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Optimization of HPLC Detection of PMP Derivatives of Carbohydrates

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OPTIMIZATION OF HPLC DETECTION OF PMP DERIVATIVES OF CARBOHYDRATES

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ABSTRACT

Detection of carbohydrates has always been a big challenge in the world, which is still attracting numerous researchers to develop different methods to overcome various difficulties. Reducing sugars, a special group of carbohydrates containing a reducing end, have provided a possibility to combine one or more chromophores to facilitate the carbohydrate detection in spite of the lack of chromophoric group in original carbohydrates. After such kind of chemical derivatizations, the sugar derivatives can be analyzed by high performance liquid chromatography (HPLC) with ultraviolet detector (UV) and diode array detector (DAD), which have been the most common methods for the carbohydrate detection.

In order to optimize the sugar detection via the HPLC-UV and/or DAD, this study applied the chemical derivatization to add an extra luminophore into carbohydrates molecules, for which 1-phenyl-3-methyl-5-pyrazolone (PMP) was used in this experiment. The optimal conditions for derivatizations of glucose and glucosamine with PMP were obtained through the response surface methodology (RSM) experimental design, which suggested the optimal conditions, under a fixed value at pH 13 of the buffer solution, for the glucose-PMP and glucosamine-PMP derivatizations at 71 °C for 134 minutes and 73 °C for 96 minutes, respectively. The delicate difference among the optimal conditions might result from the difference of the inner-structure and inner environmental pH values of the carbohydrates. Nevertheless, this method has been proven to be a feasible and practical method with high sensitivity to determine the most monosaccharides except fructose, and disaccharides such as lactose and maltose, as well as oligosaccharides which contain the

reducing end. In addition to the effect of inner pH environment, multiple sugar rings and optical isomerism of carbohydrates might also play important roles in the yield of sugar-PMP derivatives.

Furthermore, this research involved the study of the detective power in terms of the detective sensitivity, accuracy and linearity of two common detectors, i.e., DAD and evaporative light scattering detector (ELSD), on the sugar-PMP derivatives, and the efficiency in terms of the separation capability of two common HPLC columns, i.e., C18 column and amide column. Because of different principles of DAD and ELSD in chemical detection, both popular detectors have different sensitivities and selectivities for carbohydrates. DAD is able to analyze the sugar-PMP derivatives, while ELSD is good at detecting both the PMP free sugars, sugar PMP derivatives and other sugar derivatives such as sugar alcohols, etc. Moreover, the results have demonstrated that the amide column could efficiently separate the PMP free carbohydrates rather than the sugar-PMP derivatives, and on the contrary, the C_{18} column was able to separate the sugar-PMP derivatives rather than the sugar themselves.

DEDICATION

From the bottom of my heart, I greatly appreciate my family with this work for their loves and encouragements to me to pursue my dreams. Thanks for my grandparents and parents, because of your constant supports and patience, I could improve myself continuously and at last achieve this goal. Particularly, I would appreciate my parents, Jianwu Wang and Gang Lian. All your loves, supports and encouragements cultivate self-confidence and high competitiveness on me and provide me with a wonderful education.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Carbohydrates, one of the most ubiquitous biomolecules in the world, for example, in all organism bodies and even in lake sediments (Whittaker & Vallentyne, 1957), are mainly composed by carbon (C), oxygen (O), and hydrogen (H). Normally, because of the atom ratio between oxygen and hydrogen, these substances have one common structure formula which shows as $C_x(H_2O)_y$.

The most well known function of carbohydrates is described as an energy provider to all organism bodies, for instance, through the glycolysis which provides numerous ATP in bodies. Additionally, carbohydrates play important roles in health regulation of organisms. For example, high-carbohydrate diet helps improve the insulin sensitivity (Bessesen, 2001; Daly, Vale, Walker, Alberti, & Mathers, 1997). Fukagawa et al (Fukagawa, Anderson, Hageman, Young, & Minaker, 1990) investigated the influence of high-carbohydrate and high-fiber (HCF) diets on peripheral insulin sensitivity in healthy young adults versus healthy old adults. The researchers found that the HCF diets had significantly improved the peripheral tissues' sensitivity and physiologic concentration of insulin in both healthy young and old adults. In addition, carbohydrates have been found to be associated with some risk factors of health, which indicate a series of diseases or some certain unhealthy conditions resulted from wealth in the society, including obesity, cardiovascular disease

and diabetes (Drewnowski, Kurth, Holden-Wiltse, & Saari, 1992; Ezzati et al., 2005; Meyer et al., 2000; Sacks & Katan, 2002).

Moreover, carbohydrates are also regarded as necessary bio-synthesis materials in live organisms. Briefly speaking, they usually combine with lipids or proteins after chemical reactions or biological process, yielding the glycoproteins and glycolipids, respectively. P-glycoproteins, for example, are able to block the uptake of xenobiotics and enhance their metabolisms in the bile and urine (Ambudkar, Kimchi-Sarfaty, Sauna, & Gottesman, 2003; Breedveld et al., 2005), while glycolipids are usually embedded into the biomembranes acting as molecular receptors and cell-surface markers (Yamakawa & Nagai, 1978).

Besides those biofunctions, carbohydrates play other important roles in food processings. Glucose, or carbohydrates composed only by glucose, such as sucrose and starch, under the Sn-Mon catalyst, are able to be converted into 5-hydroxymethylfurfural (HMF) which is a crucial intermediate in the fine chemical industry (Wang et al., 2012). Furthermore, according to previous papers (Matero, Mattsson, & Svensson, 1998; von Rybinski & Hill, 1998), a new nonionic surfactants called alkyl polyglycosides can be made from carbohydrates as raw materials. These surfactants have functions on either eliminating cloud point or destroying the stability of liquid crystalline phases in the water-contained system, though it depends on the length of alkyl group chain. Moreover, Gupta et al (Gupta & Kumar, 2007) reported that polylactide consisting of lactic acid resulted from carbohydrate fermentation had multiple potential applications in orthopedic sutures or drug delivery. Moreover, carbohydrates as a renewable source have potential as energy providers in transportation sector (Román-Leshkov, Barrett, Liu, & Dumesic, 2007).

Huge consumption and a wide range of applications of carbohydrates have prompted human beings to make its efforts on more scientific researches for deep understanding of carbohydrates, which is still a big challenge for researchers.

Reducing sugar analysis

Reducing sugars that contain an aldehyde group or carbonyl group on their molecules can act as reducing agents. In spite of many ways for analyzing reducing sugars, there is not a "perfect" method existing. Due to lack of chromophores which show electronic transition under ultraviolet or visible light, reducing sugars can not be detected by ultraviolet (UV) detector or diode-array detector (DAD) which are the two most commonly used analytical detectors connected with high performance liquid chromatograph (HPLC). This has limited the detection of reducing sugars. Although multiple detective methods for reducing sugar have been developed in recent years, none of these methods is considered "perfect" to possess all of these characteristics: more convenience, less cost, higher effectiveness, better reproducibility, higher accuracy and universality.

As one of the most famous analytical methods for chemical structure analysis, nuclear magnetic resonance (NMR) has been used on reducing sugar determination. Zhu et al (Zhu, Zajicek, & Serianni, 2001) investigated configurations, tautomeric equilibria and hydrates of eight aldohexoses in aqueous solution via ^{13}C NMR. The authors found that all of these eight sugars had aldehyde and hydrate signals in solution, though their ratios varied depending on the sugar configuration. Based on the results, authors concluded that the configuration at the second C position had an important effect on H magnitude of the first position C. Additionally, Barclay et al (Barclay, Ginic-Markovic, Johnston, Cooper, &

Petrovsky, 2012) have searched the tautomeric equilibria of D-fructose in D_2O via ^1H NMR. The authors observed the equilibrium of tautomeric composition of fructose under 5-50°C and also studied its mutarotation at 5-25°C, and suggested that temperature and acidic pH had no significant influence on the fructose's tautomeric composition, notwithstanding, the concentration change had a linear relationship with the environmental temperature among all forms which also matched the previous work (Goux, 1985). Moreover, a lot of experiments focused on the transformation from fructose into 5-hydroxymethylfurfural under a variety of conditions, like temperature change, catalyst existence or solvent difference, which were analyzed by NMR (Amarasekara, Williams, & Ebede, 2008; Bicker, Hirth, & Vogel, 2003; Chinnappan, Jadhav, Kim, & Chung, 2014; Moreau, Finiels, & Vanoye, 2006; Zhang, Das, Assary, Curtiss, & Weitz, 2016).

Another characteristic method, Fourier Transform Infrared Spectroscopy (FTIR), has been used as an alternative detective method for sugar analysis. However, it was usually equipped with chemometrics to search the reducing sugar in food, particularly, in liquid food. The most commonly used FTIR technique is the attenuated total reflection (ATR), which is one of the most important methods for direct sampling detection (Wilson & Tapp, 1999). Kemsley et al (Kemsley, Holland, Defernez, & Wilson, 1996) applied the FTIR-ATR technique for quantitative determination of sugars in raspberry purees, while Duarte et al (Duarte, Barros, Delgadillo, Almeida, & Gil, 2002) measured the concentration of reducing sugars in mango juice via the FTIR-ATR. Additionally, other FTIR-ATR applications have been reported, such as detection of reducing sugars in raw potato tubers

(Ayvaz, Santos, Moyseenko, Kleinhenz, & Rodriguez-Saona, 2015), in honey (Nayik, Dar, & Nanda, 2015), and in apricot fruit (Bureau et al., 2009).

Nevertheless, high performance liquid chromatograph (HPLC) system is the most desirable equipment for chemical determination in the world. HPLC equipped with a refractive-index (RI) detector is a traditional means to detect reducing sugars. Vaz et al (Vaz et al., 2011) investigated the chemical compositions of free sugars of four different wild edible mushrooms species. As a result, the authors indicated that trehalose was the main sugar in the edible mushrooms. The *C. comatus* sample contained the highest concentration of trehalose, which was 42.82 gram per 100 gram of dry weight (DW), while the arabinose (0.78g/100g DW) was only found in *A. mellea* sample. In addition, according to the glucose analysis by the HPLC-RI, it was found that, after germination, the starch in rice will be degraded into glucose, which means glucose increased along with the rice germination process (Kim et al., 2012; Moongngarm, 2011). In addition, HPLC-RI has been applied for determining sugars in the adulterated milks by dairy industry (Sharma, Rajput, Dogra, & Tomar, 2009), detecting sugar composition in milk and cheese, and even predicting sugar formulae of milk (Chávez-Servín, Castellote, & López-Sabater, 2004; Zeppa, Conterno, & Gerbi, 2001)

However, RI detector has its insurmountable drawbacks. Particularly, it is not compatible with the gradient elution more favorable for separation of reducing sugars (Cunha & Oliveira, 2006). Therefore, evaporative lighting scattering detection (ELSD) has attracted more and more attention in sugar analysis. The mechanism of ELSD is shown on **Figure 1.1** (Megoulas & Koupparis, 2005), which involves the following consecutive steps:

(1) nebulization of effluent; (2) mobile phase vaporization under high pressure and temperature; and (3) scattered light passing through the clear chamber in order to analyze the specific analyte particles. Muir et al (Muir et al., 2009) used HPLC-ELSD to search sugars and sugar alcohols in 45 vegetables and 41 fruits. The authors reported that concentrations of sorbitol and mannitol were found only in 18 vegetables, ranging from 0.09 to 2.96 gram per 100 gram of fresh weight (FW), while 7 vegetables contained raffinose and stachyose between 0.08-0.68g / 100g FW. Additionally, 19 vegetables had nystose and kestose, of which their concentrations were within 0.02-0.71g / 100g. As for fruits, the most impressive phenomenon was that five common fruits, including apple, watermelon, clingstone peach, mango and pear, had higher concentration of fructose than that of glucose. Although sorbitol can be detected in 15 fruits (0.53-5.99g/100g, FW), mannitol was only found in 2 fruits, including clingstone peach and watermelon. Another publication reported that HPLC-ELSD had high ability to detect sugar in fruit juice. For example, the sugar profile of orange juice after several weeks of storage, which mainly contains sucrose, glucose and fructose, was studied by HPLC-ELSD (Wibowo et al., 2015). The authors indicated that the percentage of sucrose hydrolysis rose from 8% to 70% along with the temperature increase from 20°C to 42°C within 8 weeks of storage. In addition, Peng et al (Pang et al., 2006) used HPLC-ELSD for sugar analysis in tobaccos, found several short-chain carbohydrates, including fructose (2 μg/ml), glucose (3 μg/ml), sucrose (2 μg/ml), maltose (4 μg/ml), xylose (2 μg/ml) and raffinose (4 μg/ml), in extracted solutions of tobacco leaves. Besides, major sugars in soybean have been analyzed by ELSD (Valliyodan, Shi, & Nguyen, 2015).

Although the ELSD has higher sensitivity and selectivity than RI, and is compatible to LC gradient elution, its efficiency and stability still can not be comparable to the UV detector or DAD.

Selection of HPLC columns for sugar analysis

Before being eluted into the HPLC detector, analytes such as sugars should pass through a LC column for chemical separation. In view of the reverse phase (RP)-HPLC, two major theories have been proposed: solvophobic and partitioning theories which are two models for chemical retention time. According to previous published papers (Knox & Parcher, 1969; Scott, 1985), due to the solvophobic effects from the mobile phase and attraction by the stationary phase on chemical particles, small chemicals would be excluded by the mobile phase and then bind on the surface of the particles of column. The principle of this phenomenon is called the solvophobic theory. As for the partitioning theory, it described that all analytes were absolutely embedded into and caught by the stationary phase, and then brought out by continuous mobile phase (Brown & Weston, 1997). Therefore, whether substances can be separated or not, the retention time of each substance depends on the chemical materials in column. In other words, column characteristics decides the retention times of analytes. In fact, there are hundreds of columns which are usually classified into five major categories, such as reversed phase (RP) HPLC column, Bio LC column, normal phase (NP) HPLC column, HILIC HPLC column, and ion exchange (IE) HPLC column. Each category includes several subcategories which are based on main functional groups of particles in column.

C_{18} column, one of the most commonly used columns in all labs, is usually regarded as a RP HPLC column. C_{18} column is loaded with 1-8 µm porous octadecyl-bonded silica gel which is a non-polar material. Based on characteristics of its particles, C_{18} column is able to separate non-polar eluents. It is good for separation of phenolic acids and flavonoids (Avula et al., 2010; Dai & Mumper, 2010; L. Peng, Song, Shi, Li, & Ye, 2008; Pyrzynska & Biesaga, 2009; Wei, Xie, Dong, & Ito, 2009). Moreover, this kind of column has a high efficiency on isolation of specific nitrosamines in tobacco. Kim et al (Kim & Shin, 2013) successfully quantified N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanabasine (NAB) and also N'-nitrosoanatabine (NAT) via LC-MS/MS coupled with C_{18} column. The authors measured the concentration of NNN in 105 electronic cigarettes from 11 shops, which ranged from 0.34 to 60.08 µg/L. Besides, the concentration of NNK was from 0.22 to 9.84 µg/L, while the concentration of NNB and NAT ranged from 0.11-11.11 µg/L and 0.09-62.19 µg/L, respectively. In addition, this particular column is also applied in other areas, for instance, for detection of disperse dyes in water sample (Carneiro, Umbuzeiro, Oliveira, & Zanoni, 2010), pesticides in environmental water (Gervais, Brosillon, Laplanche, & Helen, 2008) and polybrominated diphenyl ethers at lower level (Li et al., 2008). Due to the properties of C_{18} column, it has no ability to separate reducing sugar. However, it can be used for separation of sugar-derivatives, which will be described in more details in the next section.

Another column, specific for sugar separation, is the amide column which belongs to the HILIC column. Amide column is loaded with the silica-amino derivative materials as the stationary phase (Peng, Hou, Zhang, Shen, & Yang, 2016). One research has employed

the HPLC-RI equipped with the amide column to explore the sugar contents in the wild and commercial mushrooms (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008). The reducing sugars had the least concentrations among all carbohydrates in mushrooms, and maltose could only be detected in two types of edible mushrooms, i.e., *agaricus silvaticus* (0.44g per 100g dry powder weight) and *agaricus silvicola* (0.57g per 100g dry powder weight). Besides the sugar detection, the amide column has been used in analyses of protein- and lipid- derived oligosaccharides (Neville, Dwek, & Butters, 2008), though the authors pointed out that amide column was not as competitive as the HIAX column. Apart from the determinations of sugars and their derivatives, amide columns have been applied for amino acids and phosphoproteome separation (Albuquerque et al., 2008; Langrock, Czihal, & Hoffmann, 2006; Yoshida, 1997, 2004). Moreover, according to Malic et al (Malik & Rai, 2009), a combination of solid phase microextraction (SPME) and RP-HPLC-UV system coupled with an amide column was applied to analyze the nitroaromatic components in water.

In some specific cases, chirality, one of geometric properties in molecules and ions, is described as that the chiral atom or ion is nonsuperimposable, which means they can not be identical with their mirror image (Ouellette & Rawn, 2014b). Carbohydrates are usually associated with this characteristics, so this special property is usually applied on stationary phases. Polysaccharides, like cellulose and amylose, have been used as stationary phases of chiral column called polysaccharides-based chiral column. Polysaccharides-based chiral columns have solved some analytical problems, especially enantioseparation of underivatized amino acids (Yun, Lord, Yin, & Stringham, 2002), 20 asymmetric sulfoxides

(Cass & Batigalhia, 2003), fungicida trazolyl alcohols (Spitzer, Yashima, & Okamoto, 1999), propafenone in plasma (de Gaitani, Lanchote, & Bonato, 1998), benzazoles and benzanilides (Kubota, Sawada, Zhou, & Welch, 2010). The polysaccharides-based chiral column is also prepared for enantioselectivity of chemical derivatives: aptazepine derivatives (Cirilli et al., 2006), chiral arylpropionic acid derivatives (Matarashvili, Chankvetadze, Fanali, Farkas, & Chankvetadze, 2013), fluoxetine derivatives (Guo, Fukushima, Li, & Imai, 2002; Yu, Li, &Guo, 2006), homocamptothecin derivatives (Goossens et al., 2004), phenylcarbamate derivatives (Enomoto et al., 1996) and hydantoin derivatives (Kartozia et al., 2002). The performance of cellulose, amylose and their derivatives toward to drug separation was also evaluated (Aboul-Enein & Ali, 2002; Bonato et al., 2002; Chankvetadze, Kartozia, Yamamoto, & Okamoto, 2002;Zhang, Schaeffer, & Franco, 2005).

Detection of reducing sugar derivatives by HPLC-UV

DAD and UV detectors are two ubiquitous detectors in analytical chemistry because they have higher efficiency and selectivity than RI and ELSD, though the latter two detectors are also useful for reducing sugar analysis. DAD and UV have very similar chromatographic principles, nonetheless, DAD, the principle of which is shown in **Figure 1.2**, is less sensitive than UV because DAD usually loses more light energy when more than one variable wavelengths are working at the same time (Brown & Weston, 1997). Based on their mechanisms, both detectors have been practiced in many areas (listed in **Table 1.1**), including: (1) food analysis, (2) biological detection, (3) drug determination and (4) environment control. In food analysis, phenolic acids, vitamins, carotenoids and

flavonoids from vegetables or fruits are usually performed under UV detector or DAD (**Table 1.1)**. UV and DAD are also employed in meloxicam analysis. Bae et al (Bae, Kim, Jang, & Lee, 2007) reported that the maximum peak of meloxicam was shown under 355 nm and its recovery ranged between 77.2%-86.7%.

HPLC-UV and HPLC-DAD are not able to measure sugar directly, whereas they have higher sensitivity and resolution on sugar derivatives, thus, several methods for sugar derivatives have been developed. Schiff base (shown on **Scheme 1.1**), one of intermediates of Millard reaction, plays an important role on reducing sugar determination by HPLC-UV-DAD. The labeling reaction begins at the carbonyl groups of reducing sugars where they become a weak target for lone pair of amino groups of protein or other amines to attack and at last produces the imine derivatives. Numerous reagents containing amino groups have been applied to label reducing sugar. Hase et al (Hase, Hara, & Matsushima, 1979) first reported 2-aminopytidine as a reagent to yield the Schiff base of reducing sugar, which can be found under UV at 235 nm. Furthermore, a variety of aminonaphthalene sulfonic acid isomers were found suitable for carbohydrates analysis since they have the abilities on the following two aspects: charge of the saccharides and UV absorbance (Lamari, Kuhn, & Karamanos, 2003). For instance, 8-aminonaphythalene-1,3,6-trisulphonic acid (ANTS) has been used in mono-, di- and oligosaccharides derivatizations, of which the products exhibited the maximum peaks under 220 nm (Chiesa & Horváth, 1993). Additionally, many other reagents for yielding the Schiff base have been reported, such as 4-aminobenzoic butyl ester (Dahlman, Jacobs, Liljenberg, & Olsson, 2000; Mo, Takao, Sakamoto, & Shimonishi, 1998), 6-amino-quinoline (Greenaway, Okafo, Camilleri,

& Dhanak, 1994) and 1-aminopyrene-3,6,8-trisulfonate with its derivatives (Laroy, Contreras, & Callewaert, 2006; Mechref & Novotny, 2009).

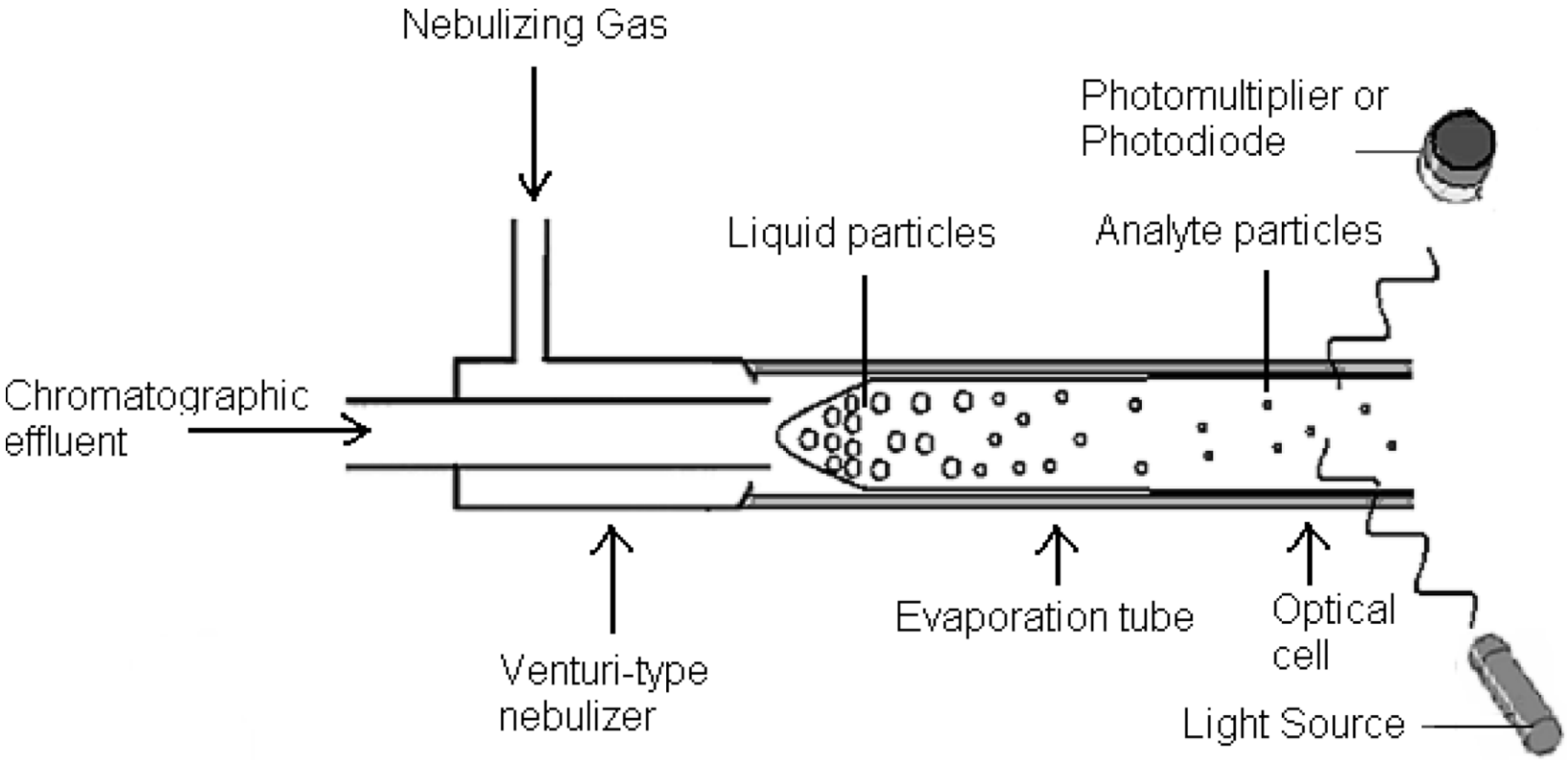
Besides the Schiff base, 1-phenyl-3-methyl-5-pyrazolone (PMP), which was at first applied for derivatizing reducing sugar (Honda et al., 1989), can also react with reducing sugars without any other side reactions such as desialylation or desulfation (shown on **Scheme 1.2**). Reducing sugar-PMP derivatives has a strong absorbance under UV light at 245 nm. Derivatization of monosaccharides in fucoidans has been investigated (Zhang, Zhang, Wang, Shi, & Zhang, 2009). Wan et al (Wan, Yang, Song, Liu, & Liu, 2013) studied the disaccharide isomers via an ion electrospray ionization multi-stage tandem mass spectrometry (ESI-MS). Derivatizations for mono-, maltooligo- and oligosaccharides with PMP were performed under matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Pitt & Gorman, 1997). Polysaccharides from *Dunaliella salina* has been hydrolyzed into several monosaccharides, which were converted into the PMP derivatives and analyzed by HPLC-UV and LC-MS (Dai et al., 2010). Furthermore, derivatives from 4-(-3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid (PMPA) that was used to derivatize di-, tri- and oligosaccharides were detected by UV and DAD (Arias, Castells, Malacalza, Lupano, & Castells, 2003; Tapie, Malhiac, Hucher, & Grisel, 2008).

Objective of this project

This research aims to study the production of the PMP derivatives of the selected reducing sugars under different chemical conditions in light of the temperature and reaction time, of which the products are detected by HPLC-UV-ELSD. Glucose, one of the representative monosaccharides, was selected to explore its optimal reaction condition for

derivatization, which was compared with optimal condition for glucosamine that shows alkalinity itself. In regards to the effects of carbon numbers, sugar ring and optical isomerism on the yields of PMP-sugar derivatives, multiple neutral sugars, including monosacchairdes like mannose, galactose, xylose and ribose, disacchairdes like maltose and lactose, were also derivatized and analyzed.

Since the sugar separation and detection are subject to columns and detectors, respectively, two HPLC detectors, i.e., DAD and ELSD, have been compared for detective sensitivity and selectivity. At the same time, two different separation columns, i.e., C18 and amide column, have been adopted to separate sugars before and after the derivatization with PMP, in order to get more insight of the sugar derivatizaiton.


Nebulizing Gas
Photomultiplier or
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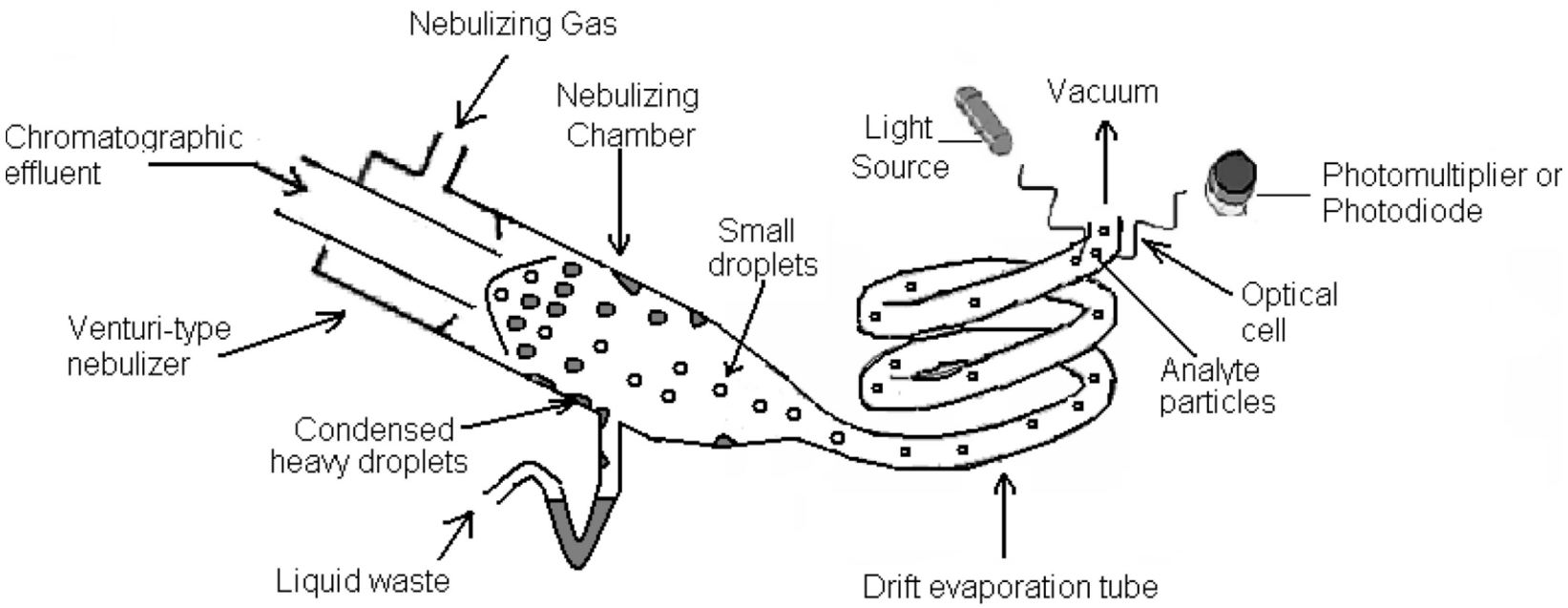
Venturi-type
nebulizer
Evaporation tube
Optical
cell
Light Source

(a)



(b)

Figure 1.1 Two types of ELSD and their mechanisms [Adapted from Twenty Years of Evaporative Light Scattering, N. C. Megoulas et al, copyright of Taylor and Francis LLC (Megoulas & Koupparis, 2005)]

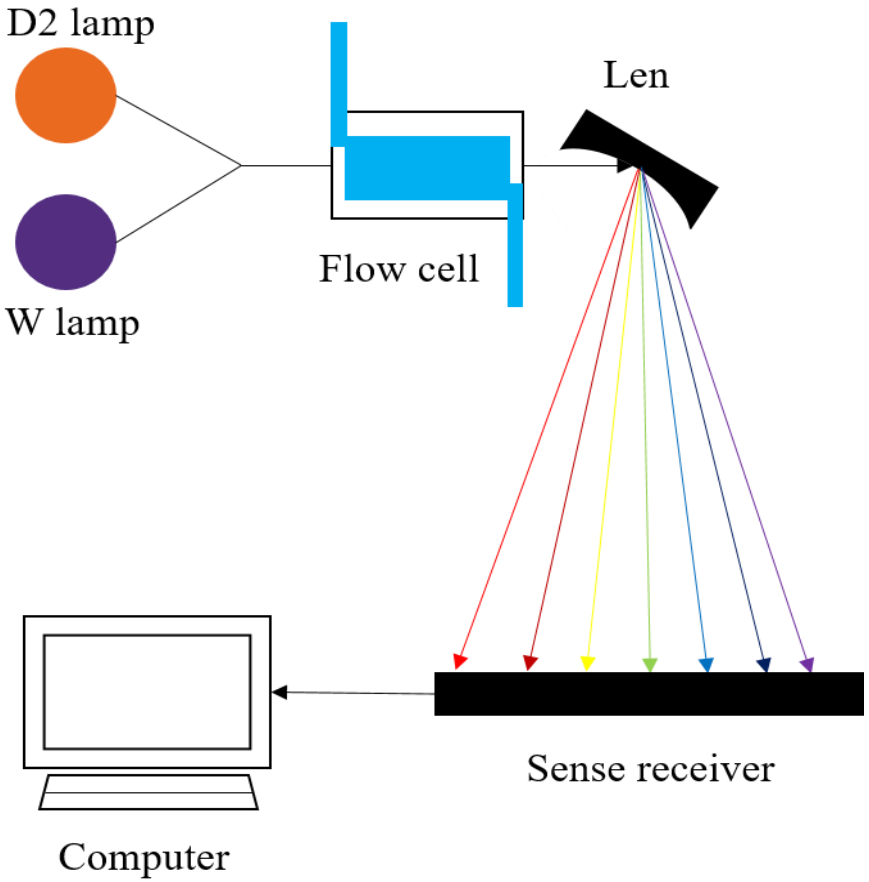
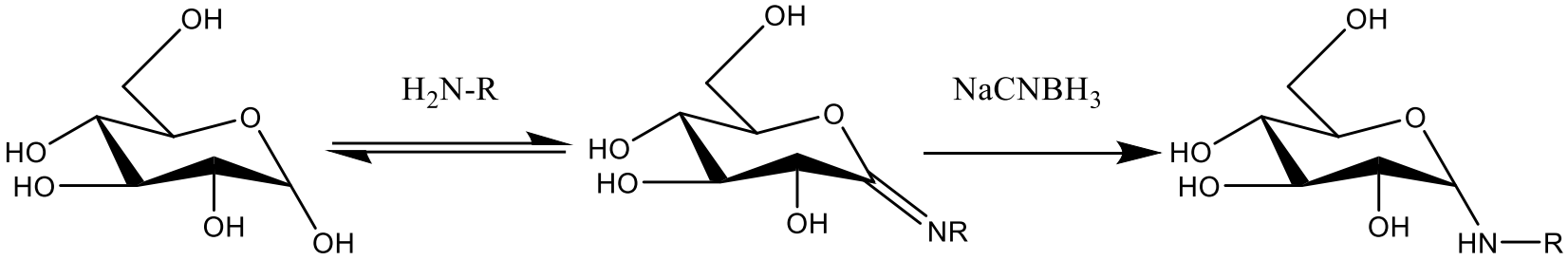


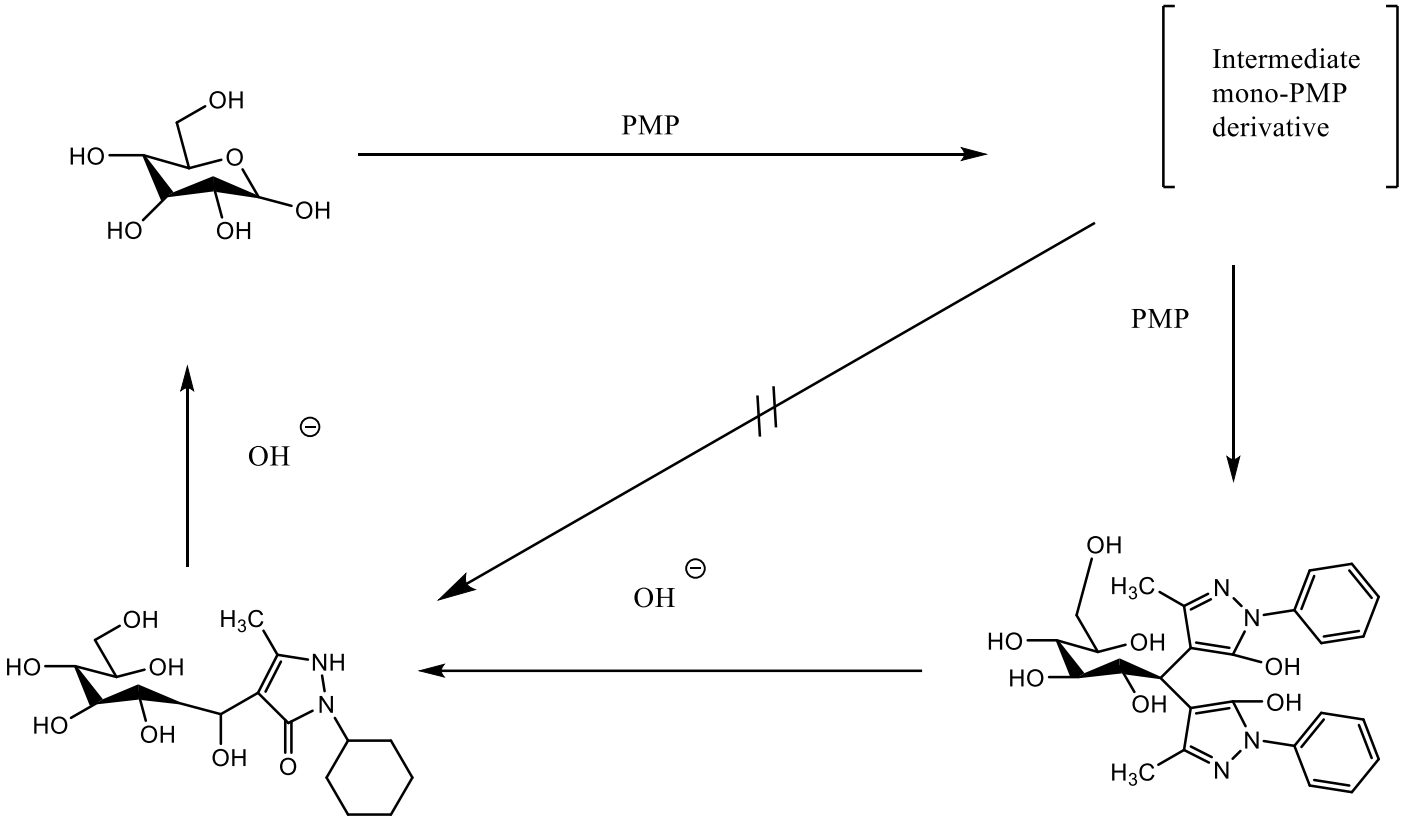
Figure 1.2 The principle of UV detector

Table 1.1 HPLC UV and DAD applications

Analytes	Matrix	Separation	Detection	LOD	Reference
Food analysis					
Flavonoids	Rooibos Tea	No mention	DAD (200-400 nm)	50 ng/ml	(Bramati, Aquilano, & Pietta, 2003)
Vitamin C, carotenoids and phenol	Papaya	C30 reverse phase column	DAD at 430 450, 470 nm	No mention	(Leal, Figueira, Tornisielo, & Regitano, 2012)
Phenolic acid	Wild mushroom	C18 column	DAD at 280 nm	No mention	(Barros, Dueñas, Ferreira, Baptista, & Santos-Buelga, 2009)
Phenolic acid	Sunflower	C18 column with C18 secure column	DAD at 280 and 320 nm	No mention	(Weisz, Kammerer, & Carle, 2009)
Phytoalexin resveratrol (3,5,4'-Trihydroxystilbene)	Pistachios and peanuts	Hypersil - ODS column	DAD at 308 nm	No mention	(Tokuşoğlu, Ünal, & Yemiş, 2005)
Biology detection					
Cystenine (Cys) and its related aminothios	Plasma, urine and cerebrospinal fluid	C8 column with ODS2 column	UV at 240 nm	0.2 pmol per injection	(Amarnath, Amarnath, Amarnath, Valentine, & Valentine, 2003; Bald, Kaniowska, Chwatko, & Glowacki, 2000)
Ascorbic acid and free malondialdehyde	Serum	C18 column	UV at 250 nm	$1.3*10^{-8}$ and $1.02*10^{-8}$ mol/L	(Karatepe, 2004)
Drug determination					
Meloxicam	Plasma	C18 column	UV at 355 nm	No mention	(Bae et al., 2007)
Metformin with derivatives	Plasma	ODS-2 column	DAD at 250 nm	10 ppb	(Tache, David, Farca, & Medvedovici, 2001)
Environment control					
Sulfonamide	Environmental water	C18 column	UV at 270 nm	0.024-0.033 µg/L	(Sun et al., 2009)
Quinolone and fluoroquinolone	Soil	C18 column	UV at 260 nm and 280nm	0.04 -0.08 µg/g	(Leal et al., 2012; Turiel, Martín-Esteban, & Tadeo, 2006)



Scheme 1.1 Schiff base route



Scheme 1.2 Reducing sugar-PMP full route

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CHAPTER TWO

OPTIMIZATION OF REACTIONS BETWEEN REDUCING SUGARS AND PMP

Abstract:

Reducing sugars, especially aldoses, have strong reactivity with 1-phenyl-3-methyl-5-pyrazolone (PMP) to result in the sugar-PMP derivatives, which can be more accurately analyzed by high performance liquid chromatography (HPLC) with diode array detector (DAD). This chemical derivation was optimized by response surface methodology (RSM) in order to achieve the best yield. Glucose and glucosamine were incubated in a water bath to react with PMP under the RSM design within a temperature range of between 60°C to 80°C, and a time range of 60 to 150 minutes. The chemical derivatives were determined by the HPLC-DAD at 248 nm. Subsequently, other monosaccharides, including pentoses (xylose and ribose), hexoses (galactose, fructose, and mannose), disaccharides (lactose, maltose, and sucrose), as well as some sugar alcohols (sorbitol, mannitol and xylitol), were investigated under the optimized condition for glucose. As a result, the optimal condition of the glucose-PMP reaction was obtained at 71°C for 133 minutes, while the optimized condition of glucosamine was obtained at 73°C for 93 minutes. However, fructose, sucrose, sorbitol, xylitol and mannitol had no chemical derivatizations, which could not be detected by the HPLC-DAD. Adding luminophore group of PMP on reducing sugars could significantly improve the sugar analyses in light of increased sensitivity and accuracy. In addition, this study demonstrated that different chemical structures of sugars and their derivatives could significantly influence the rate and yield of the PMP derivatization.

Introduction:

Carbohydrates play an irreplaceable role in biological activities where they are regarded as the basic energy substances, nutraceuticals, or source for proteins and lipids. They also serve as natural adhesives, fine chemicals and certain acid stabilizers in food technology (Becker et al., 2013). However, carbohydrate analysis still remain a big challenge in the world, though several direct analytical methods, such as uncommon NMR (Vliegenthart, Dorland, & Halbeek, 1983) and Fourier Infrared spectrometer (Tong et al., 2013), or less accurate HPLC-RI (Barreira, Pereira, Oliveira, & Ferreira, 2010; Zielinski et al., 2014) and HPLC-ELSD (Ma, Sun, Chen, Zhang, & Zhu, 2014; Shanmugavelan et al., 2013), are available. In addition, other analytical methods, such as capillary electrophoresis (CE) and gas chromatography mass spectrum (GC-MS) (Becker et al., 2013; Račaitytė, Kiessig, & Kálmán, 2005), have been developed.

In regard to the chemical structures of carbohydrates that lack of luminophore groups, carbohydrates can not to be detected by UV and fluorescence detectors that are both widely used in academic and industrial labs. In order to explore more analytical techniques for the carbohydrates' analysis, particularly to meet the huge demand in industry, the derivatives of carbohydrates that have higher detective sensitivity have attracted more attentions in recent years. Among the carbohydrate derivatives, the 1-phenyl-3-methyl-5-pyrazolone (PMP) derivative that was first proposed by Honda et al (Honda et al., 1989) is considered to be one of the most sensitive substances, which has a strong absorbance under UV light at 245 nm. In addition, the author's group has drawn the mechanism of PMP methods (See **Scheme 2.1**). Although there are many studies reporting the PMP labeling for analysis of

reducing sugars, such as those extracted from shark fins and Hakka rice wine (Bai et al., 2015; Xian et al., 2015), a few research papers have reported the optimization of the reaction conditions (Rühmann, Schmid, & Sieber, 2014), let alone comparison of optimal conditions for different sugars. Therefore, this research focuses on studies of efficiency and yields of the chemical derivatization of different reducing sugar reacted with PMP.

In the case of the derivatization process, several factors, such as the reaction time, reaction temperature and solvent pH, could remarkably influence the yields of reducing sugar-PMP derivatives. However, if just a single variable of the aforementioned factors is analyzed, cross-impact of the variables would usually be neglected. On the other hand, a complete experimental design to explore the relationships of the exploratory variables is time consuming. In this context, the response surface methodology (RSM) has been suggested to find the most valuable point, or called optimization to simplify the experimental design and, simultaneously, maximize the production, or minimize the cost, side reactions, etc,. RSM has been applied in many fields, such as extraction process, enzymatic clarification, degradation and many other chemical reactions (Dong, Pan, Zou, He & Wang, 2015; Lee, Yusof, Hamid, & Baharin, 2006; Ren, He, Wang & Cheng, 2016). In regard of the RSM, central composite design (CCD) is often used to support the optimization of no more than two-level factorial or fraction factorial design, which are usually coded as -1 and 1, containing three important parts: (1) integrated factorial or fraction design; (2) axial points (also called star points) with α distance between experimental points and center; (3) a central point (Bezerra, Santelli, Oliveira, Villar & Escaleira, 2008).

Additionally, this study initially used two different aldehyde hexoses, i.e, glucose and glucosamine, as the models for the RSM optimization of sugar-PMP derivatizations. Although these two monosaccharides consist of different parts in their second carbon of their ring structures, their reactions with PMP still follow the same chemical principle (shown in **Scheme 2.1**). Moreover, this study has investigated other aldehyde hexoses, pentoses, ketoses, alditols, disaccharides and oligosaccharides, and compared their efficiencies of reaction with PMP.

Experimental Design

Chemicals and reagents

The following chemical standards were purchased from Sigma-Aldrich (America), including D (+)-glucose, D-(-)-ribose, D-(+)-xylose, D (+)-glucosamine, D-(+)-lactose, D-(-)-fructose, D-(+)-mannose, D-(+)-maltose monohydrate, D-sorbitol, D-mannitol, α-, β- and γ-cyclodextrin, 1-phenyl-3-methyl-5pyrazolone (PMP) and ammonium acetate. Galactose was purchased from Fisher Scientific (New Jersey). Hydrochloric acid, 37% (analysis), was obtained from ACROS ORGANICS (part of Thermo Fisher Scientific). HPLC-grade chloroform with 0.75% ethanol, as well as HPLC-grade acetonitrile, acetic acid (glacial) and sodium hydroxide, were provided by Fisher scientific.

Pretreatment of sugar standard

Approximate 5 mg of glucose was weighed using a XS-200D analytical balance, and transferred into a glass tube, followed by an addition of 10 mL of distilled water. The glucose was dissolved in water facilitated by Fisher vortex mixer until no observable

particles were observed. The aqueous solution was then filtered with 0.45 µm Nylon filter (MACHEREY-NAGEL Co.). Other sugar standards were pretreated as glucose.

Preparation of reactants and buffer solutions

An amount of 87 mg of PMP powder was weighed accurately and transferred into a clean glass tube, which was mixed with 1 mL of methanol to prepare 0.5 M PMP-methanol solution. Meanwhile, in order to prepare a fresh 0.3 M NaOH solution, 6 grams of sodium hydroxide (NaOH) was dissolved in a glass bottle that contained 500 mL of distilled water, while 0.3M hydrochloric acid (HCl) solution was prepared from the concentrated 37% HCl solution diluted by distilled water. A buffer solution composed of ammonium acetate (7.7 g/L) was adjusted to pH 5.51 by acetic acid, which was used as the mobile phase A of the reverse-HPLC.

Preparation of reducing sugar derivatives

The preparation of reducing sugar derivatives with PMP was achieved based on the experimental modification of a previous report (Dai et al., 2010). In detail, an aliquot of 100 µL of sugar standard solution (500 ppm) was mixed with 100 µL of 0.3 M NaOH solution in a 1.5 mL micro-centrifuge tube (VW, North American Co), adjusted to pH 13 with 0.3 M NaOH. Then, this mixture, i.e., 200 µL of sugar-NaOH solution, was added with 100 µL of 0.5 M PMP-methanol solution, mixed by 1 minute via vortex at the fourth level-shaking rate, followed by incubation in the water bath at 70 °C for 120 minutes. After the reaction was completed, the sample was neutralized with 100 µL of 0.3 M HCl solution. Then, HPLC-grade chloroform was added into the tube as an extraction agent for clearance of PMP residues. The mixture was shaken vigorously for 1 minute before the upper layer,

i.e., the water-methanol layer, was separated. This extraction procedure was repeated three times. Finally, the supernatant was filtered through a 0.45 μm filter into a HPLC auto-sampler vial (12×32 mm, Sigma-Aldrich Co) for chemical determination.

Experimental design

There are some variables in regard to the chemical derivatization, including solution temperature, pH and reaction time. According to a previous report (Strydom, 1994), high pH value (pH=13) is a necessary for the sugar-PMP derivatization as proposed by Honda et al (Honda et al., 1989). In this context, the reaction time and temperature of RSM were selected as the primary factors in the first step of RSM design, for which a two-factor central composite design (CCD) coded with three levels (i.e., -1, 0, 1) was performed in order to fulfil an accurate analysis. Two independent variables were depicted as time (X_1) and temperature (X_2), respectively, which were also coded from -1 to 1. The experimental design of RSM for glucose and glucosamine are shown in **Table 2.1** and **2.2**, where the experimental response (ER) means the area of each peak shown in reverse HPLC-DAD. The function of Y was used for predicting the optimal condition, expressed as the following equation (1)

$$Y = \sum b_i X_i + \sum b_j X_j + \sum b_{ii} X_i^2 + \sum b_{jj} X_j^2 + \sum b_{ij} X_i X_j + b_0 \qquad (1)$$

where the Y represents the predicted response or dependent variable. The aforementioned coefficients in the equation indicate the effects of linear (b_i and b_j), quadratic (b_{ii} and b_{jj}) and interaction (b_{ij}) and the constant coefficient (b_0). X_i and X_j are the independent variables.

Reverse HPLC-UV Condition

The HPLC-DAD system consists of a CBM-20A controller, a DGU-20A degasser, a LC-20AT pump, a SIL-20A HT auto-sampler, a FRC-10 collector, a SPD-M10A UV detector and a CTO-20A column oven, which was purchased from Shimadzu Corporation (Shimadzu Co, North America). Reducing sugar-PMP derivatives were separated by an Eclipse Plus C18 column (4.6 * 250 mm, 5 μm particle, Agilent, North America), of which the mobile phase was constituted by ammonium acetate buffer (A) and acetonitrile (B). The solvent B runs from 20% to 30% during the first 45 minutes and decreases to 20% in the subsequent 10 minutes. Other settings were as following: flow rate at 0.3 mL/min, column oven at 30°C.

Relative response

Since the reducing sugars, such as pentose, hexose, di- and oligosaccharides, have similar physio-chemical characteristics but different molecular weights, their relative responses (RR) in comparison with that of glucose were determined, based on the reactant molarity, for the sugar derivatives that were all prepared in 500 ppm (μg/mL) of sugars. The RR herein is defined as the ratio of HPLC peak area of each sugar derivative and the glucose derivative under the same optimal condition of glucose-PMP derivatization, which was presented as following equation.

$$RR = \frac{AYsample}{AYglucose} X \frac{MWsample}{MWglucose} \qquad (2)$$

where the RR is the relative response. AY represents the average of chromatographic area of each peak shown on the HPLC-DAD. MW is the abbreviation of molecular weight of each sugar

Statistical analysis

All measurements were conducted in triplicate to obtain the mean ± standard deviation (SD). Data of the sugar-PMP experimental design (shown in **Table 2.1** and **2.2**), the yields of sugar derivatives (Y number see equation 1) and analysis of variance (ANOVA) were calculated via JMP Pro 12.2.0 (SAS Institute Inc). $P<0.05$ indicates the statistically significant difference.

Data and discussion

Optimization of glucose-PMP derivatization

The yield of glucose-PMP derivative could be influenced by the reaction time (X_1) and temperature (X_2) of the derivatization, which was performed under the response surface methodology (RSM). In addition, four star points and two replicates at the central point were employed to construct a quadratic model of the reaction, which is shown in **Table 2.1**. As a consequence, the yield (or experimental response, Y) of glucose-PMP was obtained within a range from $1.51*10^7$ to $3.74*10^7$ (**Table 2.1**), which fits to a second-order polynomial equation after excluding the interaction effect, expressed as following:

$$Y = 37.21*10^6+3.56*10^6X_1+3.00*10^6X_2-6.08*10^6X_1^2-10.74*10^6X_2^2 \quad (3)$$

where Y means the dependent variable of the yield of glucose-PMP derivative, while X_1 and X_2 refer to the variables for the reaction time and reaction temperature, respectively (see **Table 2.1** and **2.3**).

Analysis of variance (ANOVA) that was fulfilled by the Fisher's statistical test is shown in **Table 2.4.** Based on the ANOVA of the yields of the glucose-PMP derivatives obtained from the RSM model in 2 factors with 3-levels (see **Table 2.1**), its F-value that is expressed as the ratio of the mean square of regression and error is 80.16 (**Table 2.4**),

resulting in its P-value at 0.0004 that is less the 0.05, which means that at least one of factors have impacted significant influence on the yields of glucose derivatives (Muralidhar, Chirumamila, Marchant, & Nigam, 2001).

Based on the differences of the predicted and actual values of the glucose-PMP derivatization from the RSM model, the statistical analysis showed that the experimental Pro>F=0.2063 for the lack of fit is larger than 0.05, implying that there is no significant lack of model fit, or the current model (or equation 3) can provide an expected prediction to match the true value of glucose-PMP derivatives (see **Table 2.4**). In other words, a significant lack of fit assumes that there may be some other variances or factors unaccounted for the hypothesis of the RSM model and the induced equation, which will result in the rejection of the hypothesis or the model to find an alternative one (Bashir, Aziz, Yusoff, & Adlan, 2010; Muhamad, Abdullah, Mohamad, Rahman, & Kadhum, 2013).

P-values (see **Table 2.3**) were used to estimate the significance of each coefficient of the dependent variables at a certain level α and also shows the influence of a single factor or interacted impact of several factors (Wang et al., 2014). When the α level is selected as 0.05, the coefficient is significant when its P-value is less than 0.05. However, there is an agreement in statistics that, if the P-value is more than 0.1, it should be eliminated (Aghaie, Pazouki, Hosseini, Ranjbar, & Ghavipanjeh, 2009; Körbahti & Rauf, 2008). Herein, the coefficients of the second-order polynomial equation (shown in **Table 2.3**) profile the effects of reaction time and reaction temperature, of which the linear effects (X_1 and X_2) and quadratic effects (X_1^2 and X_2^2) are significant since their P-values are less than 0.05.

However, the interaction effect (X_1X_2) is not significant due to its P-value at 0.5825, more than 0.05.

Moreover, t-test is another frequently used model in statistics, which usually tests if each coefficient of effect equals to zero, namely, whether this effect is significant to influence the yields of glucose derivatives. In regards of the t-values (**Table 2.3**), all the coefficients, except that of the interaction effect of reaction time and temperature, have significant influence on the yields of the glucose-PMP derivative (Li & Fu, 2005).

In addition, to check whether the predicted values fit the actual values (**Figure 2.1**), their correlation coefficient (R-square) was also analyzed. Based on a previous report, a good fit model usually requires a minimum R^2 at 0.8 (Joglekar, May, Graf, & Saguy, 1987). In this context, the value of R^2 between the predicted values and the actual values was 0.9901 (**Table 2.3**), meaning it is a good model of fit within the range of experimental values.

Analyses of glucosamine-PMP derivatives

Like the study of the glucose-PMP derivatives, the second-order polynomial model, as well as its linear, interaction and quadratic effects, for predicting the yields of the glucosamine-PMP derivatives, are listed in **Table 2.5**, and shown as following:

$$Y = 38.1*10^6+1.49*10^6X_1+1.65*10^6X_2+3.58*10^6X_1X_2-6.20*10^6X_1^2-4.58*10^6X_2^2 \quad (4)$$

where Y represents the predicted yields of the glucosamine-PMP derivatives, X_1 and X_2 represent the independent variables of the reaction time and reaction temperature, respectively. In addition, the R-square of the equation (4) is 0.9963 (see **Table 2.5)**, suggesting that the predicted values highly fit the actual data (shown in **Figure 2.2)**.

ANOVA data is listed in **Table 2.6**. A low probability of F-value (Pro>F<0.0001), which is less than the setting α value at 0.05, means the rejection of the null hypothesis which assumes that all factors have no significant influences on the yields of glucosamine-PMP derivatives. From the same **Table 2.6**, the significance of lack of fit is 0.0907, which is larger than 0.05, indicating the current model for the prediction of the yields of glucosamine-PMP derivatives is sufficiently enough.

As described previously, P-value can be used to estimate the significance of each factors in the experimental design. Since all the P-values in **Table 2.5** are less than 0.05, all the factors have significant influences on the glucosamine-PMP derivatization. In detail, all effects, including the linear effects (X_1 and X_2), quadratic effects (X_1^2 and X_2^2) and interaction effect (X_1X_2), have significantly influenced the yields of the glucosamine-PMP derivatives, which is slightly different from the effects on the optimization of glucose-PMP derivatization, for which the interaction effect is not significant. This slight difference might result from the following factors: (1) experimental error during the process of the chemical derivatization; and (2) different structures of the studied monosaccharides that resulted in different chemical reactivities.

Comparison of glucose- and glucosamine-PMP derivatizations

In light of the RSM for optimization of the sugar-PMP derivatizations, two profiled 3D response surfaces have clearly exhibited the trends of interactions of two independent variables, which are shown in **Figure 2.3** and **Figure 2.4** that are representative of the glucose-PMP and glucosamine-PMP derivatives, respectively. Elliptical response plot are accomplished when there is an ideal relationship between the two factors, including the

reaction time and temperature, for the yields of derivatives. The main function of constructing the response surface is to explore the optimal condition of an experiment, namely, reaction time and reaction temperature in this experiment, in order to maximize the response (Tanyildizi, Özer, & Elibol, 2005). Compared with the curvature of curves of the reaction time in **Figure 2.3**, temperature curves produce steeper curvature, which supports our observation that the reaction temperature has influenced the yields of glucose derivative more significantly (see **Figure 2.3**), while the reaction time is a more important factor for glucosamine derivative yields (see **Figure 2.4**).

In addition, the contour map below the 3D response surface serves as another function for analyzing the variables, i.e., the reaction time and temperature, at the same time, while fixing all other factors (pH, for instance) at a constant level (for example, coded value at zero), which are beneficial for the understanding of both main and interaction effects of these two given factors (Adinarayana, Ellaiah, Srinivasulu, Devi, & Adinarayana, 2003; Lee, Yusof, Hamid, & Baharin, 2006). Two contour maps are perfectly elliptical (see **Figure 2.3** and **Figure 2.4**), which suggest that the interaction of both time and temperature existed in accordance with the response surface. Based on these results, it was concluded that the optimal condition for the glucose-PMP derivatization is under 70-73°C for 120-140 minutes, while the optimal condition for glucosamine-PMP derivatization is between 70-73°C for 90-96 minutes. Based on the 3D response surface with the elliptical contour of **Figure 2.3**, the maximum yield of the glucose-PMP derivative is produced when the reaction condition is under 71°C for 134 minutes, while the optimal condition of the glucosamine-PMP derivative is under 73°C for 96 minutes (see **Figure 2.4)**. Although the

average of the recorded highest responses of glucose-PMP derivative is $3.74*10^7$ (n=3) obtained under the condition at 70°C for 120 minutes, its predicted maximum value is 3.4% greater than the actual value at the same condition. However, it is suggested to use the condition at 70°C and 120 minutes as the optimal condition for the glucose-PMP derivatization because the longer reaction time and higher temperature will cost much more. Similarly, the best condition for the glucosamine-PMP derivatization is suggested at 70°C for 90 minutes because the predicted value at the optimized condition at 73°C for 96 minutes is only 2.3% higher than the actual yield at $3.81*10^7$.

Compared with the glucose-PMP derivatization, the glucosamine-PMP derivatization costs less energy, needs shorter reaction time, but has higher synthetic yields, which might be due to the different structures of glucose and glucosamine. There are two possible assumptions: (1) breaking the bond energy of the aldehyde group of glucosamine is lower than that of glucose, which was due to the higher oxidation state of the first carbon of glucosamine; (2) the amino group on the second carbon functions as a catalyst due to its alkalinity in aqueous solution, though in nature it is a common formation for the glucosamine with one molecule hydrochloride having an acidic pH (Santhosh & Mathew, 2008), thus accelerating the reaction completion.

Different reducing sugar-PMP derivatives

As shown in **Table 2.7**, several sugars and sugar alcohols were observed with no chemical response (or detective signals), which signifies that they did not react with the PMP reagent. For example, cyclodextrins that have a torus-like macro ring shape have three types known as Schardinger's α-, β- and γ- cyclodextrin, which do not contain the

reducing end, thus failed to be oxidized by PMP (Szejtli, 2013). Moreover, sugar alcohol, such as sorbitol and mannitol, are not able to react with PMP due to the lack of an aldehyde group that can not form alcohol-PMP derivatives.

Though fructose is a very reactive reducing sugar, it can not produce the fructose-PMP derivative either because of the following two reasons. On one hand, its carbonyl group can not be reduced enough to react with PMP. In other word, its carbonyl group is more stable than the aldehyde group in reducing sugars (Ouellette & Rawn, 2014a). On the other hand, reversible and non-reversible transfiguration usually occur simultaneously when monosaccharides suffer the heated alkaline condition. There are four possible pathways for monosaccharide transformation under an alkaline condition: (1). ionization; (2). mutarotation; (3). enolization and (4). degradation (Bamford, Bamford, & Collins, 1950; Brands & van Boekel, 2001). In the second step, in spite of "the complex system of fructose" which actually contains higher amount of pyranose and furanose after a certain time, fructose is still mainly composed of about 66% of β-pyranose (Angyal & Bethell, 1976; Sinnott, 2007), which is stable enough to reject the reaction with PMP. Enolization, also known as the "Lobry de Bruyn -Alberda van Ekenstein transformation", begins in equilibrium by the same 1,2- and 2,3-enediol anion species during the constant pH environment (Eggleston & Vercellotti, 2000). Furthermore, enediol intermediate of fructose also undertakes irreversible degeneration action in the methanol, which at last converts into a few racemic methyl lactates without catalyst in the alkaline-methanol solution at lower temperature 80°C (Holm, Saravanamurugan, & Taarning, 2010; Taarning et al., 2009). Compared with the aforementioned aldoses, the other part of fructose would

be degraded due to more than 33% furanose in the equilibrium converted into glucose or mannose (Bharose & Verma, 2016; MacLaurin & Green, 1969). However, due to improper condition for fructose transformation and stability of its carbonyl group, few fructose molecules and even no fructose molecule can be isomerized to glucose or mannose thus reacting with the PMP. As a consequence, no fructose-PMP derivative was observed in the chemical reaction and/or detected by HPLC-UV.

The relative responses (RRs) of mannose and galactose are larger than that of glucose that was set at a base value of 1, while the RRs of xylose and ribose are less than 1 (**Table 2.7**). In an agreement to previous reports (Bamford et al., 1950; Brands & van Boekel, 2001), monosaccharides usually experience four same stages in an alkaline solution, though various pathways for different monosaccharides could happen. In this study, under the same optimal condition as that for glucose, the ribose-PMP derivative has a lower RR at 0.730. One hypothesis is that higher energy or longer reaction time has raised higher probability to destroy the ribose-PMP derivatives. Ribose is the furanose which is less stable than pyranose, for example, glucose. Based on this explanation, the ribose derivatives only need less energy or shorter reaction time than those needed by the glucose derivatives to reach the maximum yields. However, the optimal condition for the glucose derivatization at 70°C for 120 minutes might not be the optimal experimental condition for the ribose derivatization, or even expedite the degradation of the ribose derivatives. As a result, its response of HPLC-DAD was smaller. In comparison, xylose is considered "simple" in the mutarotation stage, but its PMP derivative has a lower response, which needs more investigation. Although glucose, mannose and galactose are also "simple" in

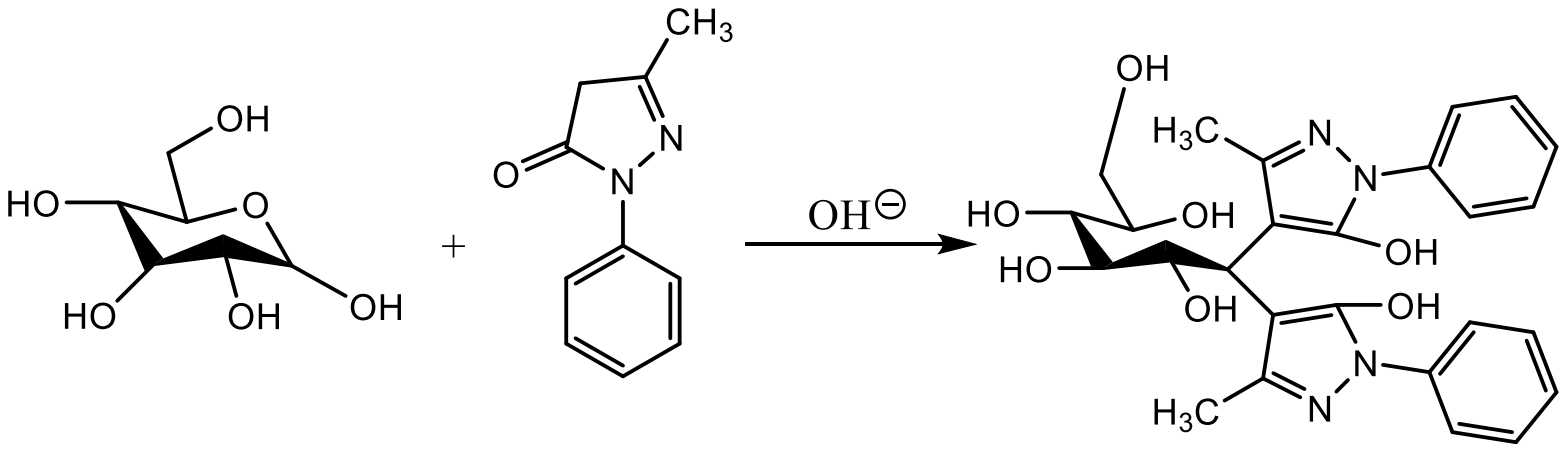
mutarotation stage, they are difficult to be enolized than fructose (Gao, Kobayashi, & Adachi, 2015), thus reacting with PMP easier. Additionally, the disaccharides, such as maltose and lactose, have lower RR than mono-aldose. This phenomenon was attributed to the additional unit of monosaccharide conjugated to another monosaccharide, which has a negative stereo impact on their chemical reactivity.

Conclusion

Application of PMP derivatization of sugars have provided many analytical benefits. The PMP method has shown to be highly reproducible, more sensitive and better accurate for sugar analysis in light of the conjugation of two molecular chromophoric groups on sugars (see **Scheme 2.1**), which allows the sugar derivatives to be detected by UV and DAD detectors. The RSM method can offer a 3D visual graph highlighting the effect of the interactions of factors on the yields of sugar-PMP derivatives with high reliability. The RSM optimization of glucose-PMP and glucosamine-PMP revealed that their optimal conditions were at the 70°C for 120 minutes and at 70°C for 90 minutes, respectively. From these two RSM optimizations, other reducing sugars were derivatized to investigate the effect of chemical structure on the effectiveness of the derivation reaction. Monosaccharides, especially hexose, have higher chemical reactivity, as well as detective responses than the studied pentoses (such as ribose and xylose) and disaccharides (such as maltose and lactose). However, there was no sign that fructose could react with the PMP effectively under the studied condition.

In summary, this research has not only been useful for finding the optimal condition of reducing sugar derivatization with PMP, but also beneficial for possible determination and

investigation of the chemical reactivity of different sugars. In order to figure out the effect of molecular inner environmental pH on the PMP derivatization, glucuronic acid and galacturonic acid have been suggested for further investigation. Meanwhile, more work for reducing sugar analysis should be conducted, such as kinetics of sugar-PMP process.



Scheme 2.1 Principle of glucose-PMP derivatization

Table 2.1 Experimental design of glucose-PMP derivatization

Run	Actual and coded levels of variables		ER ($*10^7$)*
	Time (mins) (X_1)	Temp (°C) (X_2)	(Y)
1	90 (-1)	60 (-1)	1.51
2	90 (-1)	80 (1)	1.93
3	150 (1)	60 (-1)	2.07
4	150 (1)	80 (1)	2.63
5	90 (-1)	70 (0)	2.69
6	150 (1)	70 (0)	3.55
7	120 (0)	60 (-1)	2.24
8	120 (0)	80 (1)	3.06
9	120 (0)	70 (0)	3.74
10	120 (0)	70 (0)	3.68

*Experimental response: the results are representative for yields

Table 2.2 Experimental design of glucosamine-PMP derivatization

Run	Actual and coded levels of variables		ER(*10^7)*
	Time (mins) (X_1)	Temp (°C) (X_2)	(Y)
1	60 (-1)	60 (-1)	2.73
2	60 (-1)	80 (1)	2.38
3	120 (1)	60 (-1)	2.36
4	120 (1)	80 (1)	3.43
5	60 (-1)	70 (0)	3.08
6	120 (1)	70 (0)	3.29
7	90 (0)	60 (-1)	3.22
8	90 (0)	80 (1)	3.48
9	90 (0)	70 (0)	3.81
10	90 (0)	70 (0)	3.79

*Experiment response: the results are shown as yields

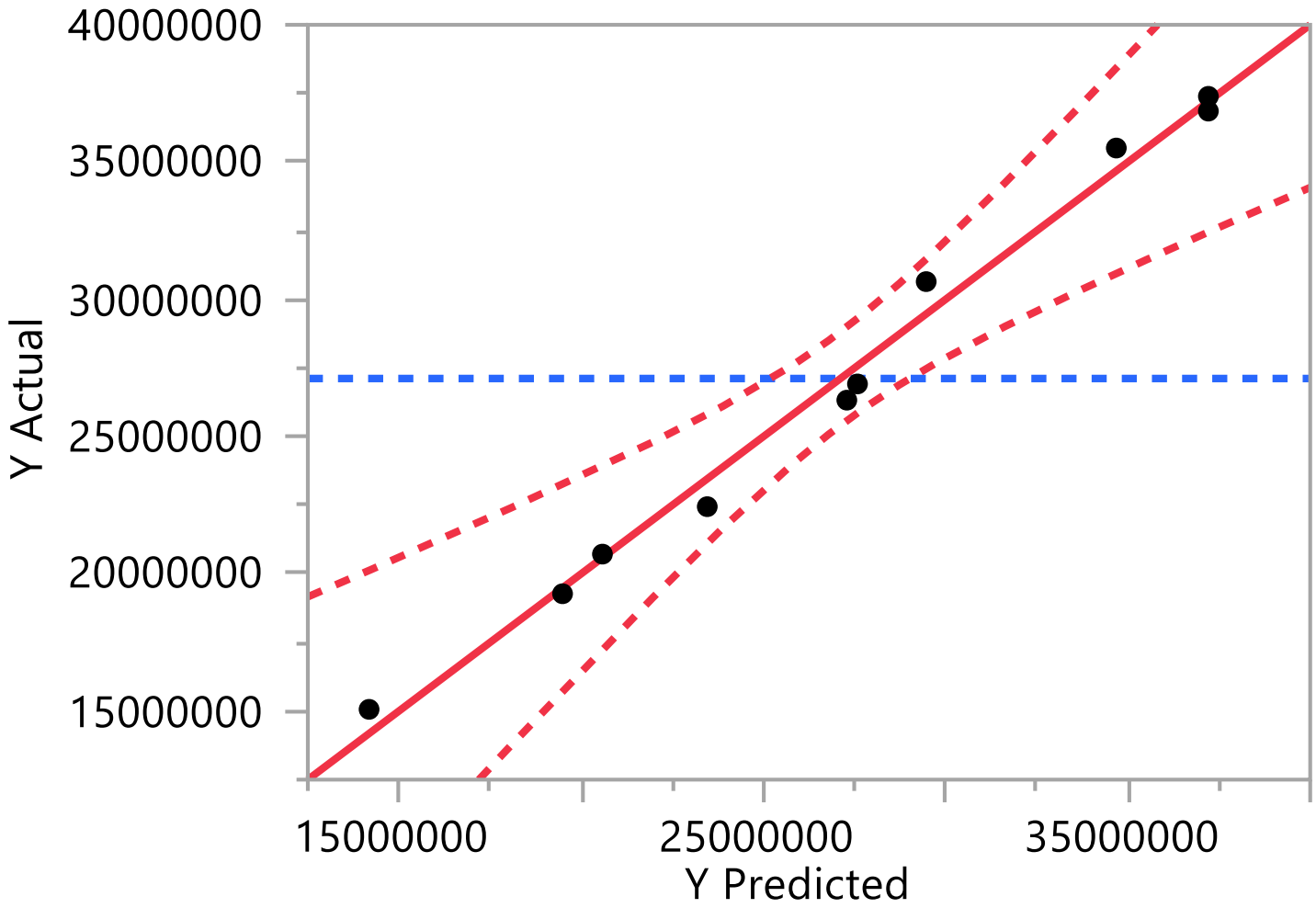


Figure 2.1 Predicted yield vs actual yield of the glucose-PMP derivative

Table 2.3 Second order response contents and the regression analysis of glucose derivatives yield

	Term	Coefficient*	Glucose ($*10^6$)	Standard error ($*10^5$)	T-value	P-value**	
	Intercept	b_0	37.21	6.99	53.23	<0.0001	Significant
Effects of linear	X_1	b_1	3.56	4.77	7.44	0.0017	Significant
	X_2	b_2	3.00	4.77	6.29	0.0033	Significant
Effects of interaction	X_1X_2	b_{12}	0.35	5.85	0.60	0.5825	
Effects of quadratic	X_1^2	b_{11}	-6.08	7.66	-7.94	0.0014	Significant
	X_2^2	b_{22}	-10.74	7.66	-14.03	0.0001	Significant
	R-square	0.9901					
	Adjusted R-square	0.9778					

*$Y=b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_1X_1+b_{22}X_2X_2+b_0$

**Significant at 0.05

Table 2.4 ANOVA of glucose derivatization

Source*	DF	Sum of squares (*10^{13})	Mean square (*10^{13})	F Ratio	P-value
Model	5	54.82	10.96	80.16	0.0004
Residual	4	0.55	0.14		
Lack of fit	3	0.53	0.17	12.25	0.2063
Pure error	1	0.01	0.01		
Total	13	55.91			

*Significant at 0.05

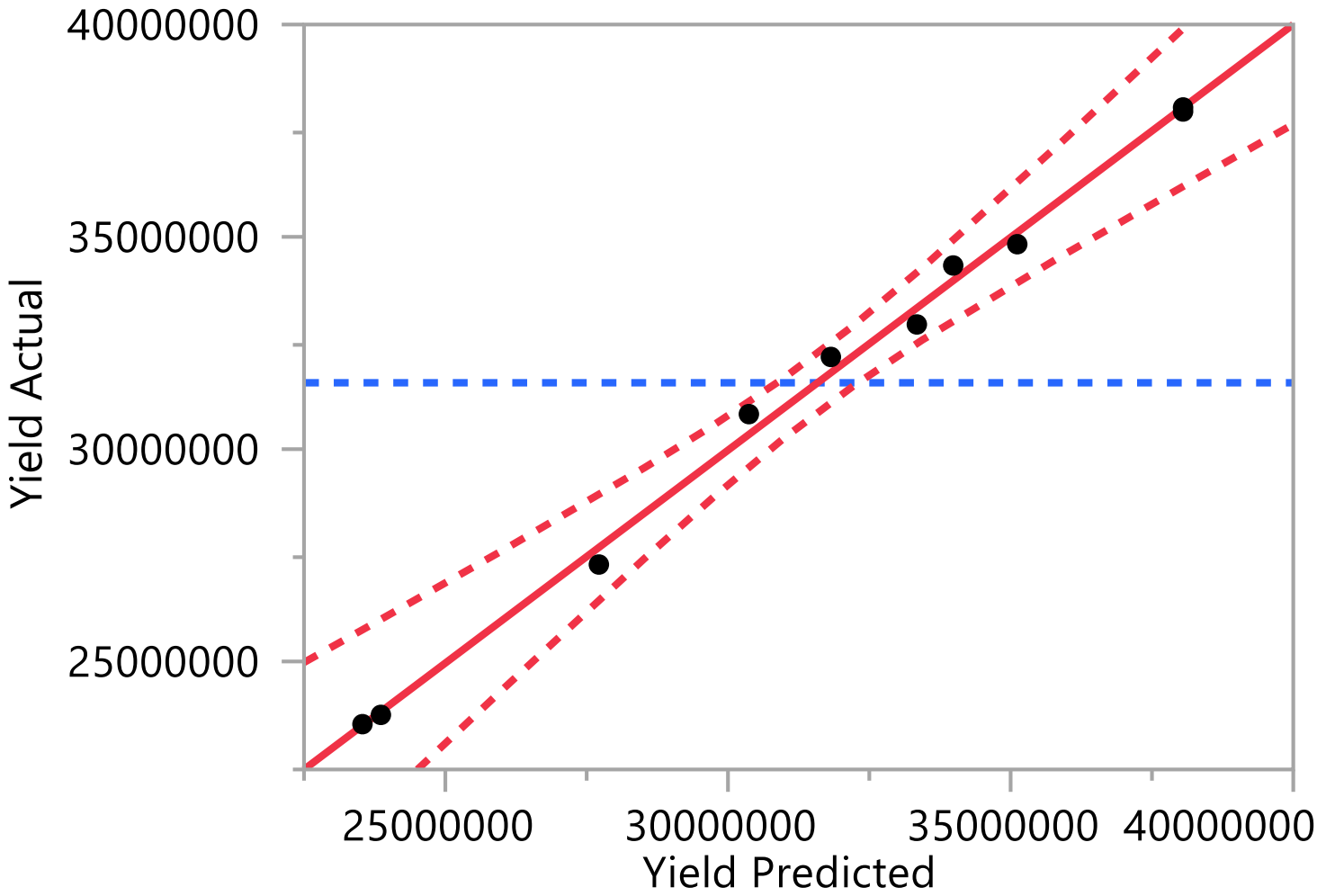


Figure 2.2 Predicted yield vs actual yield of the glucosamine-PMP derivative

Table 2.5 Second order response contents and the regression analysis for glucosamine derivatives yield

	Term	Coefficient*	Estimated ($*10^6$)	Standard Error ($*10^5$)	T-value	P-value**	
	Intercept	b_0	38.10	2.83	134.56	<0.0001	Significant
Effects of linear	X_1	b_1	1.49	1.93	7.69	0.0015	Significant
	X_2	b_2	1.65	1.93	8.53	0.0010	Significant
Effects of interaction	X_1X_2	b_{12}	3.58	2.37	15.12	0.0001	Significant
Effects of quadratic	X_1^2	b_{11}	-6.20	3.10	-20.00	<0.0001	Significant
	X_2^2	b_{22}	-4.58	3.10	-14.79	0.0001	Significant
R-square	R-square	0.9963					
Adjusted R-square	Adjusted R-square	0.9918					

*$Y=b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_1X_1+b_{22}X_2X_2+b_0$

**Significant at 0.05

Table 2.6 ANOVA of glucosamine derivatization

Source*	DF	Sum of squares (*10^{13})	Mean square (*10^{13})	F Ratio	P-value
Model	5	24.60	4.20	219.68	<0.0001
Residual	4	0.09	0.02		
Lack of fit	3	0.08	0.02	65.26	0.0907
Pure error	1	0.0005	0.0005		
C. Total	13	24.78			

*Significant at 0.05

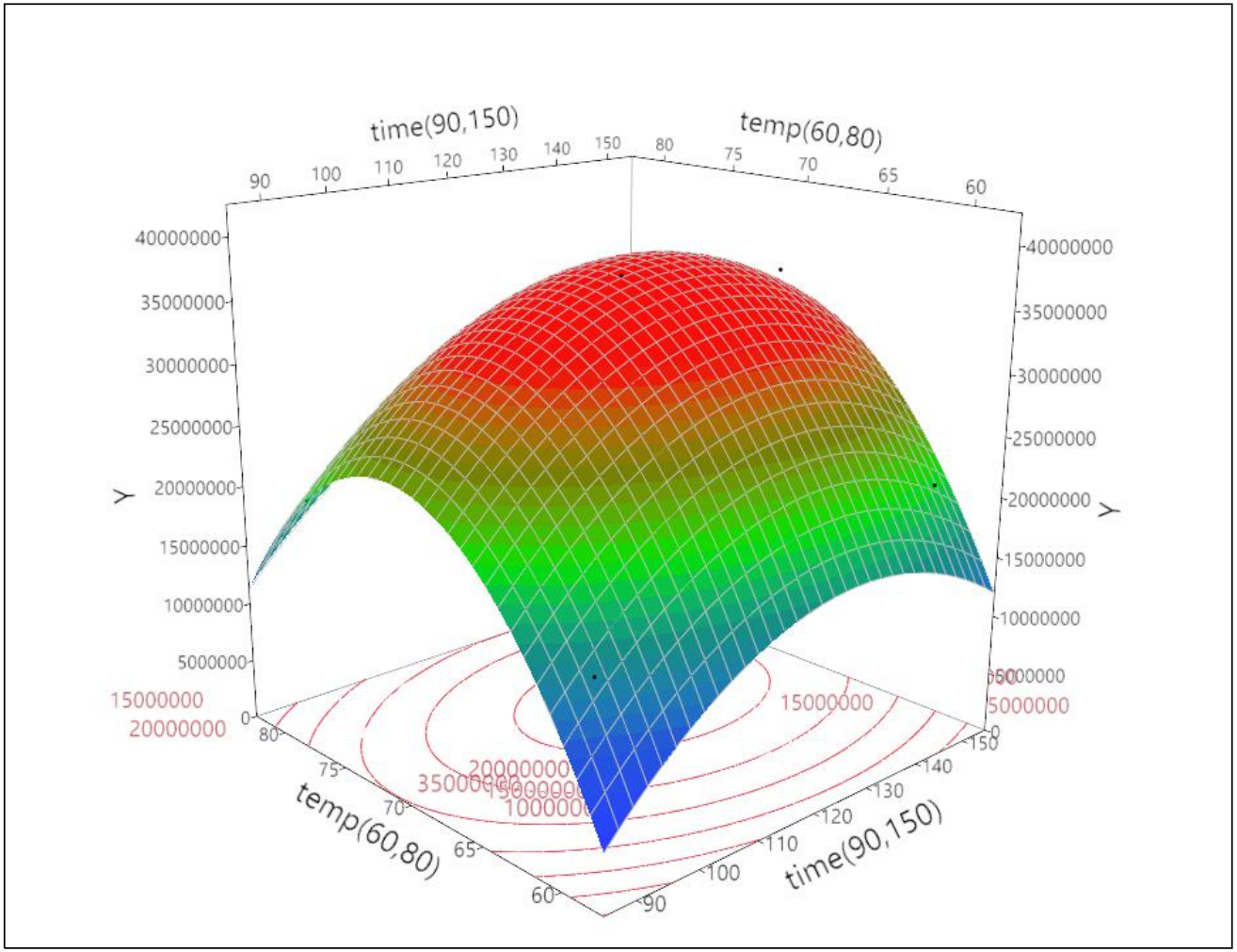


Figure 2.3 RSM plot of the glucose-PMP derivatization

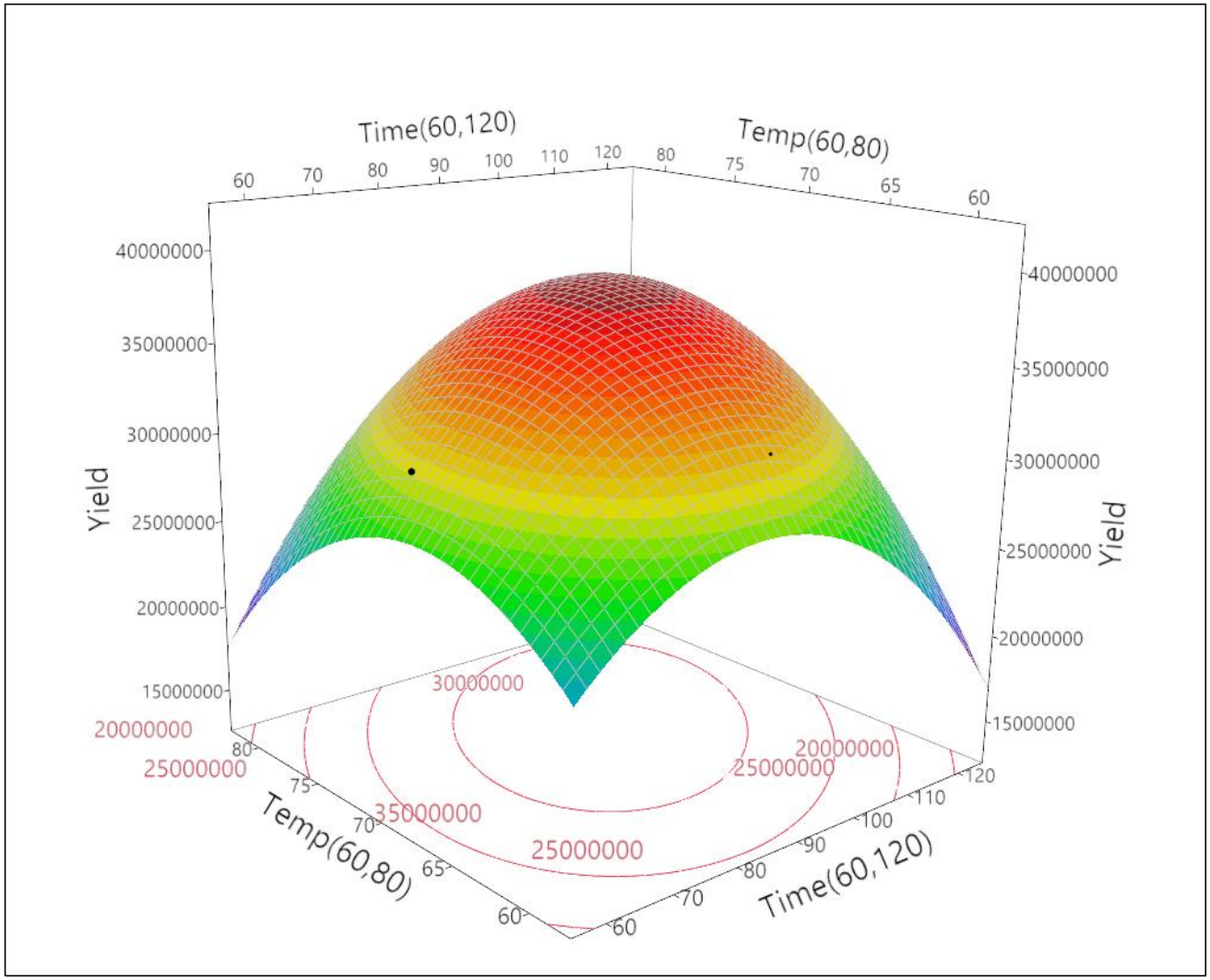


Figure 2.4 RSM plot of the glucosamine-PMP derivatization

Table 2.7 Comparison of responses of different reducing sugar-PMP derivatives

Units# of sugar	Sugars' name	MW[a]	PPM[b] (μg/μL)	M (mmol/mL)[c] $*10^{-3}$	AY[d]$*10^{7}$	RR[e]
Monosaccharide-Aldose	Glucose	180	500	2.78	3.739±0.396	1
	Galactose	180	500	2.78	4.865±0.507	1.301
	Mannose	180	500	2.78	4.145±0.414	1.109
	Glucosamine	179	500	2.79	3.295±2.051	0.876
Monosaccharide-Ketose	Fructose	180	500	2.78	0	0
Monosaccharide-Pentose	Ribose	150	500	3.33	3.277±0.484	0.730
	Xylose	150	500	3.33	2.861±0.198	0.638
Disaccharide	Maltose	342	500	1.46	1.710±0.294	0.869
	Lactose	342	500	1.46	1.799+0.118	0.914
	Sucrose	342	500	1.46	0	0
Sugar alcohol	Sorbitol	182	500	2.75	0	0
	Mannitol	182	500	2.75	0	0
Cyclodextrin	α	973	500	0.514	0	0
	β	1135	500	0.441	0	0
	ϒ	1297	500	0.386	0	0

Note:

[a]:Molecule weight; [b]:Parts per million; [c]:Molarity; [d]:Average yield-represented by the average of the area of peak show on HPLC-DAD chromatograph;

[e]: Relative response=$\frac{AYsample}{AYglucose} X \frac{MWsample}{MWglucose}$

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CHAPTER THREE

DETERMINATION OF REDUCING SUGAR-PMP DERIVATIVES VIA HPLC WITH DIFFERENT COLUMNS AND DETECTORS

Abstract

Qualification and quantification of carbohydrates remain a big challenge in the world. Fortunately, reducing sugars, especially aldoses in their open-ring structures, have strong reducibility with 1-phenyl-3-methyl-5-pyrazolone (PMP), resulting in the sugar-PMP derivatives that can be detected by reverse phase high performance liquid chromatography (RP-HPLC) with UV detector at 248 nm. With aid of two HPLC columns, i.e., Eclipse Plus C_{18} and X-bridge amide columns, and two HPLC detectors, diode array detector (DAD) and evaporative light scattering detector (ELSD), this study compared the separation and detection of some sugars, including monosaccharides such as glucose, mannose, galactose, glucosamine, fructose, ribose and xylose, disaccharides such as sucrose, lactose and maltose, and oligosaccharides like oligochitosan, with their possible PMP derivatives, as well as some other sugar derivatives including sugar alcohols and cyclodextrins, which were separated by the aforementioned two columns, and simultaneously detected by the HPLC-DAD-ELSD system. The results showed that the C_{18} column had a strong capacity to separate the aforementioned reducing sugar-PMP derivatives rather than the sugar themselves. On the contrary, the amide column could effectively separate the original sugars instead of the sugar-PMP derivatives. This phenomena demonstrated that the polarity of reducing sugars decreased after their PMP derivatization. In addition, both DAD

and ELSD were able to monitor the reducing sugar-PMP derivatives, but the DAD had a higher sensitivity than the ELSD. Moreover, eight linear regressions for the detection of aforementioned mono- and disaccharides were constructed with satisfactory R^2. Additionally, adding PMP into chitosan degraded oligomer molecules could significantly improve the oligochitosan analysis.

Introduction

Carbohydrates are the most ubiquitous biomolecules in nature, which occur within all living organisms, and even in certain marine or underground sediments. One unambiguous agreement in the scientific society is that carbohydrates play vital roles in biological systems. For example, the most well-known role of carbohydrates is to supply energy. However, carbohydrate detection still remains a big challenge due to their complex structures. So far, numerous analytical methods for sugar analysis have been proposed, including RP-HPLC with refractive index (RI) detector (Barreira, Pereira, Oliveira, & Ferreira, 2010), RP-HPLC with evaporated light scattering detector (ELSD) (Shanmugavelan et al., 2013), pulsed amperometric detector (PAD) coupled with high-performance anion exchange chromatography (HPAEC) (Hou, Chen, Shi, Zhang, & Wang, 2008; Ouchemoukh, Schweitzer, Bey, Djoudad-Kadji, & Louaileche, 2010), ^{1}H nuclear magnetic resonance spectroscopy (NMR) (Hohmann et al., 2015) and Fourier-transform infrared spectroscopy (FTIR) (Wolkers, Oliver, Tablin, & Crowe, 2004). Though these methods have very convincing results in regards of their detections, the expensive prices of some of the aforementioned instruments and their maintenance costs have restricted their practical applications in academy and industry.

Without chromophoric groups in their structures, carbohydrates are difficult to be detected by some common sensitive detectors, such as UV, DAD and fluorescence detectors. Recently, chemical derivatization of sugars has been comprehensively advocated in order to improve the sugar determination. Among all the sugar derivative agents, 1-phenyl-3-methyl-5-pyrazolone (PMP) was the first to be applied for sugar analysis (Honda

et al., 1989), which was sensitive under the UV detection at 248 nm. Later, the mechanism of sugar-PMP synthesis was further studied (Saba, Shen, Jamieson, & Perreault, 1999) (see **Scheme 3.1**). In general, many previous studies have reported the PMP derivatization as a successful method for sugar detection, for instance, the sugars extracted from maple and crop rhizospheres, and the reducing sugar involved in mechanism of biological reactions (McRae & Monreal, 2011; Nakamura et al., 2011; Taga & Kodama, 2012; van Straaten et al., 2013). Nevertheless, few studies have compared the efficiency of different HPLC columns and detectors on the sugar-PMP detection, let alone the exploration of chemical properties of sugar-PMP derivatives.

The function of HPLC column is to separate a variety of analytes. For example, C_{18} column, which is composed of silica-based reversed-phase materials, is the most popular reverse phase chromatography (REC) column since it can provide highly efficient separation power with desirable mechanical strength (Majors, 2009). Therefore, C_{18} column is suitable and often adopted for isolation of weak and/or nonpolar organic compounds, for example, capsaicin and its derivatives, anthocyanins, phenolic compounds and other compounds (Daood et al., 2014; Gómez-Caravaca, Verardo, Berardinelli, Marconi, & Caboni, 2014; Törrönen, Hellström, Mattila, & Kilpi, 2017). In contrast to the C18 column, the separation mode of an amide column belongs to the hydrophilic interaction chromatography (HILIC) since the column is fully filled by silica-amino derivative materials as the stationary phase that was firstly proposed about 30 years ago (Alpert, 1990). Amide column has strong efficiency on separation of polar compounds,

like xanthine, hypoxanthine, sugar and phenolic acid (Kotoni, Ciogli, Villani, Bell, & Gasparrini, 2014; Laiakis et al., 2014; Restivo, Degano, Ribechini, & Colombini, 2014).

This study focuses on the comparison of two different columns, named C_{18} and amide columns, for their efficiency on separation of sugars and sugar-PMP derivatives, both of which were under the same HPLC condition coupled with diode array detector (DAD) and ELSD system for detection of the eluted sugars and their PMP derivatives. In addition, the current research is to investigate the detective parameters, such as precision and relative standard deviation (RSD), of the detection for the aforementioned sugars and their PMP derivatives.

Experiment

Chemicals and reagents

The following sugar standards and chemicals were obtained from Sigma-Aldrich (USA): D-(+)-mannose, D (+)-glucose D-(+)-xylose, D (+)-glucosamine, D-(-)-ribose, D-(+)-lactose, D-(+)-maltose monohydrate, 1-phenyl-3-methyl-5-pyrazolone (PMP) and ammonium acetate. Galactose was purchased from Fisher Scientific (New Jersey, USA). Chitosan oligomer mixture with degree of polymerization ranged from 2 to 10 was bought from Dalian Glycobio Co, People's Republic of China. 37% Hydrochloric acid (analytical grade), and 99% formic acid (analytical grade) were purchased from ACROS ORGANICS (part of Thermo Fisher Scientific, USA). All other chemicals and reagents, including 3 mL of C_{18} solid-phase extraction columns (SPE cartridge) and chloroform with 0.75% ethanol (HPLC-grade), as well as HPLC-grade acetonitrile, acetic acid (glacial), methanol (HPLC-grade) and sodium hydroxide were provided by the Thermo Fisher scientific (USA).

Pretreatment of sugar standards

All of the reducing sugar standards (see **Table 3.1**) were divided into two groups to prepare the mixture A including glucose, xylose, glucosamine, and maltose, and mixture B including galactose, mannose, ribose and lactose. They were weighed (see **Table 3.1**) by an XS-200D analytical balance, and respectively moved into two of 15 ml clean glass tubes. Moreover, 10 ml of distilled water was added into each tube. The two mixture solutions were shaken vigorously by a Fisher vortex mixer until the chemicals were completely dissolved in the solution to be used as the stock solution, which were immediately filtered by 0.45 μm nylon filters (MACHEREY-NAGEL Cor.). Then, the stock solution were diluted by 2, 4 and 10 times (**Table 3.1)** for HPLC analyses for construction of linear regressions.

Preparation of reactants and buffer solutions

As described in Chapter 2, 87 mg of PMP was mixed with 1 ml of methanol in a clean glass tube to make 0.5 M PMP-methanol solution. Meanwhile, 6 grams of sodium hydroxide (NaOH) was dissolved in 500 mL of distilled water to prepare 0.3 M NaOH solution, while the concentrated 37% hydrochloric acid (HCl) solution was diluted by distilled water to 0.3 M solution. Ammonium acetate (7.7 g/L) was adjusted to pH 5.51 by acetic acid to make the mobile phase A of RP-HPLC, while the mobile phase B was composed by 100% acetonitrile. In addition, 500 mL of acetonitrile (HPLC-grade) was mixed with 500 mL of distilled water with addition of 0.25 mL of 99% formic acid (analytical grade) to form another mobile phase for the C_{18} SPE elution.

The procedure of reducing sugar derivatization

As mentioned in the second chapter, the reducing sugar derivatives were prepared based on the previous report with some modifications (Dai et al., 2010). Briefly speaking, 100 µL of the sugar standard solution and 100 µL of 0.3 M NaOH solution were at first mixed in a 1.5 mL micro-centrifuge tube (VW, North American Co), which was added with 100 µL of the 0.5 M PMP-methanol solution. The solutions were mixed vigorously by a vortex mixer for about 1 minute. Then, the mixture was incubated in a water bath at 70 °C for 120 minutes. Once the incubation ended, the aqueous liquid was neutralized by adding 100 µL of the HCl solution, followed by addition of 1 mL chloroform (HPLC-grade) to extract the remaining PMP. The chloroform layer was discarded and the upper layer was kept in the micro-centrifuge tubes for further treatment. This extraction process was repeated three times.

Solution cleanup by SPE column

The supernatant was then cleaned up by the SPE cartridge based on the previous report (Rozaklis et al., 2002). A C_{18} SPE cartridge was at first conditioned with 1 mL of acetonitrile, followed by adding 1 mL of the eluent which consisted of 500 mL/L acetonitrile containing 0.25 mL/L formic acid in water. Finally, 1 mL of distilled water was added to pass through the column before the sugar-PMP solution was added. After the sugar-PMP solution was added, the C_{18} cartridge was washed by 1 mL distilled water twice. Moreover, the column was loaded with 1 mL HPLC-grade chloroform in order to remove the remaining excess PMP reagent. Then, the absorbed sugar-PMP derivative was eluted out by the aforementioned mobile phase consisting of acetonitrile-formic acid in water, and

collected in new tubes. The collected eluent was filtered through a 0.45 μm nylon filter before the HPLC analysis.

RP-HPLC-DAD-ELSD analysis of sugar-PMP derivatives

The RP-HPLC-DAD-ELSD system was consisted of a CTO-20A column oven, a SPD-M10A DAD detector, a ELSD-LYII detector, a FRC-10 collector, a SIL-20A auto-sampler, a LC-20AT pump, a DGU-20A degasser, a CBM-20A controller and HPLC solution software (Class-VP 7.4), which was purchased from Shimadzu Corporation (Shimadzu Co, North America). The RP-HPLC-DAD-ELSD system was also connected with one Eclipse Plus C_{18} column (4.6*250 mm, 5 μm particle, Agilent, North America) or an X-bridge amide column (4.6*250 mm, 3.5 μm particle, Waters, North America). The mobile phase was composed by ammonium acetate buffer solution (A) and acetonitrile (B). The solvent B was programmed from 20% to 30% during the first 45 minutes and then declined to 20% in another 10 minutes. Other general settings were as follows: the flow rate of the mobile phase was 0.3 mL/min, column oven was at 30 °C; temperature of drift tube and gain value of ELSD were set at 80 °C and 9, respectively; the chamber pressure was settled as 355 kPa and the pressure of the nebulizer gas (N_2) was controlled at 100 psi.

Method optimization

The sugars were prepared in three concentrations (**Table 3.1**) in order to construct calibration curves. Each concentration was measured three times. Linear regressions were constructed by the concentration vs. the corresponding peak area.

Recovery and precision of method

Recovery should be considered during the sample preparation. The average recovery of each reducing sugar-PMP derivatives was calculated by the following formula:

$$\text{Recovery \%} = \text{observed amount} / \text{true value} * 100\%$$

where observed amount was calculated from the linear regression, and true value was the actual concentration of the injection.

Precision was presented as RSD% which was calculated by an average number of triplicate of the measurements at the concentration of the second dilution. Result was defined as the following:

$$\text{RSD\%} = (\text{SD}/\text{Mean}) * 100\%$$

where SD means the standard deviation and Mean presents the mean value.

Chitosan analysis

Chitosan oligomers, which have a degree of polymerization from 2 to 10, were initially weighed by 5 mg to prepare a 500 ppm chitosan oligomer solution. All of its measurements adopted the same procedures as those mentioned above, and were repeated three times. However, the current preliminary study was lack of pure mono-, di- and oligochitosan standards, so the qualification of these oligochitosan will be further studied.

Data and discussion

Identification of multiple sugars via HPLC-DAD-ELSD

According to **Figure 3.1** and **3.2**, the reducing sugar-PMP derivatives of monosaccharides and disaccharides can be effectively separated by the C_{18} column and detected unambiguously by DAD. The separation chromatogram demonstrated that different sugar structures caused retention time of derivatives in the C_{18} column varying

from each other. In addition, a separation chromatogram of a combined mixture of mixture A and mixture B is shown in **Figure 3.3**, which exhibits seven peaks instead of eight peaks because of the overlap of two peaks of two sugars, i.e., lactose and maltose, which could not be separated under the setting condition of the RP-HPLC. In regards of maltose and lactose, which are composed by glucose and galactose via α-(1-4) glycosidic bond to connect another glucose, respectively. These two disaccharides have similar structures, particularly the same reducing end of glucose to form the sugar-PMP derivatives. Such kind of structural similarity has made them difficult to be separated herein, and needs further investigation.

Results of ELSD detection are shown in **Figure 3.4**, **3.5** and **3.6**, which present the separation of eight reducing sugar-PMP derivatives. Two detectors connected in tandem resulted in slightly different retention times of the same sugar-PMP derivative, which was ascribed to the required time for effluent to travel through connected tubes. For example, the retention time of glucosamine was between 31.2−33.0 minutes detected by the DAD, and between 32.0−33.5 minutes by the ELSD.

HPLC-DAD-ELSD method validation

In development of a new HPLC method, the system should be optimized for assurance of reliability of its detective characteristics, including the analytical linearity, accuracy and precision. All of the linear regressions for the eight reducing sugar-PMP derivatives exhibited an excellent linearity, based on the DAD measurements of three points in the range of concentrations (as shown in **Table 3.3**). As a result, each R^2 of the detection linearity was more than 0.9940 (close to 1), indicating that the instrument has shown a

reliable linear regression for its detection of the analyte within the range of the test concentrations (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). Although all R^2 values of the linear regressions obtained from the ELSD were also more than 0.9630, exhibiting a certain degree of linearity within the range of the test concentrations, it was reported that ELSD usually exhibited an non-linear relationship between the signal intensity and its sample concentration within a wide range of concentrations (Amaral et al., 2004; Stolyhwo, Colin, & Guiochon, 1985). Therefore, previous work has suggested to make a modification of data processing, by which each signal intensity and its corresponding sample concentration was generated through the $\log_e$ conversion and plotted so as to improve the regression linearity (Kimball, Arjo, & Johnston, 2004; Taylor, Pennell, Abriola, & Dane, 2001). As a result, the linearity of the regressions obtained from the ELSD was all higher than 0.96 within the range of 120 ppm to 570 ppm, after the data conversion (shown in **Table 3.4**).

In addition, the detection precision and recovery of the sugar-PMP were compared between the HPLC-DAD and HPLC-ELSD, which is summarized in **Table 3.5**. It is observed that there is a great fluctuation of the ELSD precision, which is reflected by its RSD% data ranging from 2.0%-23.0%. This phenomenon was attributed to the response of ELSD that might be subject to be positively affected by the diameter of the effluent particles (Vervoort, Daemen, & Török, 2008). In contrast, the RSD% values of DAD for the eight reducing sugar derivatives were lower than those of ELSD, which are in a range of 3.3%-8.2%. Although the ELSD seems to have better analytical precisions than DAD for the derivatives of four sugars including mannose (5.8 vs 7.1), glucosamine (2.0 vs 3.4),

maltose (2.5 vs 3.8), and glucose (2.7 vs 3.3), the DAD has exhibited much better precisions in other three reducing sugar derivatives, including ribose (7.0 vs 10.4), lactose (8.2 vs 23.0), and galactose (6.4 vs 18.9). Moreover, the recoveries of DAD are in the range of 94.5%-101.8% versus the range of 100.0%-100.6% for the ELSD (**Table 3.5**).

Comparison of separation effectiveness of different columns

The Eclipse Plus C_{18} column and X-bridge amide column are two common columns for chemical separation. Theoretically, a stationary phase with smaller particles will improve the effectiveness of chemical separation in the same length column, which depends on the following equation strictly: N=L/H, where N presents the high plate number, L means the column length and H is the height equivalent to a theoretical plat (HETP) (Hanai, 2007). As a result, higher column performance and resolution would be achieved by means of decreasing particle size and size diffusion, although smaller particle size will concurrently induce the higher back pressure of the HPLC column (Motokawa et al., 2002; Nguyen, Guillarme, Rudaz, & Veuthey, 2006). Additionally, in order to separate the analytes more efficiently, the ratio between the adsorbent particle and the hydrodynamic diameter of the sample particle should be more than four, thus minimizing the possibility of block force and increasing the mass transfer kinetics (Unger, Skudas, & Schulte, 2008). There are two theories, i.e., solvophobic and partitioning theories, which are often used to explain the models for calculation of retention time. The former explains the solvophobic effect on isolation of analytes from solvent and binding to the surface of the stationary phase (Knox & Parcher, 1969; Scott, 1985), while the latter represented that all solutes

were fully embedded into the stationary phase (Brown & Weston, 1997). Nevertheless, in most cases, these two theories are often used together for explanation of retention time.

The C_{18} column has shown a higher efficiency on separating the reducing sugar-PMP derivatives than the amide column since the former was packed with 5 µm porous octadecyl-bonded silica based, non-polar materials. As shown in **Figure 3.3**, the order of the separated peaks of the sugar-PMP derivatives is shown as the follows: mannose, glucosamine, ribose, maltose and lactose, glucose, galactose, and xylose. This result was consistent to the previously reported papers, except the appearance of xylose derivative (Guan & Li, 2010; Kuang et al., 2011; Shen & Perreault, 1998). Because mobile phases A and B were both polar, the order of non-polarity of sugar derivatives can be concluded from the retention time according to the mechanism of C_{18} column separation. The order of sugar derivatives' non-polarity was following (from lower to higher): mannose derivative< glucosamine derivative< ribose derivative< glucose derivative< galactose derivative. The xylose-PMP derivative may be more influenced by its particle diameter. However, other natures of the C_{18} stationary phase and other external factors might have also affected the retention time of xylose-PMP derivative, too.

As shown in **Table 3.6**, it is found that the XBridge amide column has no ability to separate the reducing sugar-PMP derivatives, though the column has a higher selectivity on series of peptides compared to the TSK gel Amide-80 column (Kalíková, Kozlík, Gilar, & Tesařová, 2013). In fact, the XBridge amide column has a great capability for the separation of common reducing sugar themselves. Though there are still some debates of retention time prediction in HILIC mode, due to the type of loaded substances, polarity of

analytes and mobile phase, partitioning model has been accepted highly for endorsement among the HILIC modes, especially in the amide column of HPLC systems (Guo & Shah, 2016; Xiong & Liu, 2016). In the case, all analytes are kept in the stationary phase at first, and then flushed by the carrier liquid later. Nevertheless, the XBridge amide column will lose its power to separate the sugar-PMPs since the polarity of reducing sugars decreases after reacting with the PMP, although the change depends on the nature of reducing sugars. However, this should be further investigated.

In addition, the original partially hydrolyzed chitosan oligomers could neither be separated by the C_{18} column nor the X-bridge amide column in my experiment. In contrast, the chitosan oligomer PMP derivatives could be efficiently separated from each other after passing through the C_{18} column (shown in **Table 3.6**), and also accurately detected by both the DAD and the ELSD, which is in accordance with several published reports (Han, Zeng, Lu, & Zhang, 2015; Tsigos, Zydowicz, Martinou, Domard, & Bouriotis, 1999; Xiong et al., 2009).

Comparing of DAD and ELSD

DAD, a common powerful chromatography detector, is classified as a first-order instrument which can receive a series of ordered array involving in a vector of data (Booksh & Kowalski, 1994). Though the DAD and UV detectors use the similar chromatographic principles, the former is less sensitive than the latter since the former is subject to lose more light while more variable wavelengths are working at one time, typically in a range of the wavelengths between 190-700 mm (Weston & Brown, 1997).

Table 3.7 compares the columns and detectors from another perspective. It is clear that the DAD detector can not detect the reducing sugars at all, since they are lack of UV chromophores. Though some common chromophores, such as hydroxyl (-OH), amine (-NH_2) and even aldehyde (-CHO), can be excited at low UV value (180 to 210 nm), the determination below 210 nm is much harder and inconvenient (Asher, 1988; Snyder, Kirkland, & Glajch, 1997). However, once these sugars are bound with the benzene and penta cyclic rings, they will possess the absorptivity within the near ultraviolet value, which will facilitate the DAD detection.

The principle of ELSD operation has been described in detail by Megoulas (Megoulas & Koupparis, 2005). Briefly speaking, ELSD detection mainly follows three successive steps: (1) nebulization of effluent; (2) vaporization of the mobile phase under high pressure and temperature; (3) scattered light passing through the clear chamber in order to analyze the uniform analyte particles. Because of the universality and higher accuracy of the ELSD, it is considered to be one popular detector, functioning as a complementary and alternative detector to UV detector. Based on the ELSD principle, any form of sugars could be detected by ELSD, which is supported by the result shown in **Table 3.7**, regardless of the separation. Additionally, some non-reducing sugars, sugar alcohols such as sorbitol and mannitol, and ketoses like fructose, can not be analyzed by DAD since they do not react with PMP, although they can be separated by the amide column and detected by the ELSD. Furthermore, chitosan oligomers derivatives are measured under both DAD and ELSD effectively, however, these oligomers should be further characterized.

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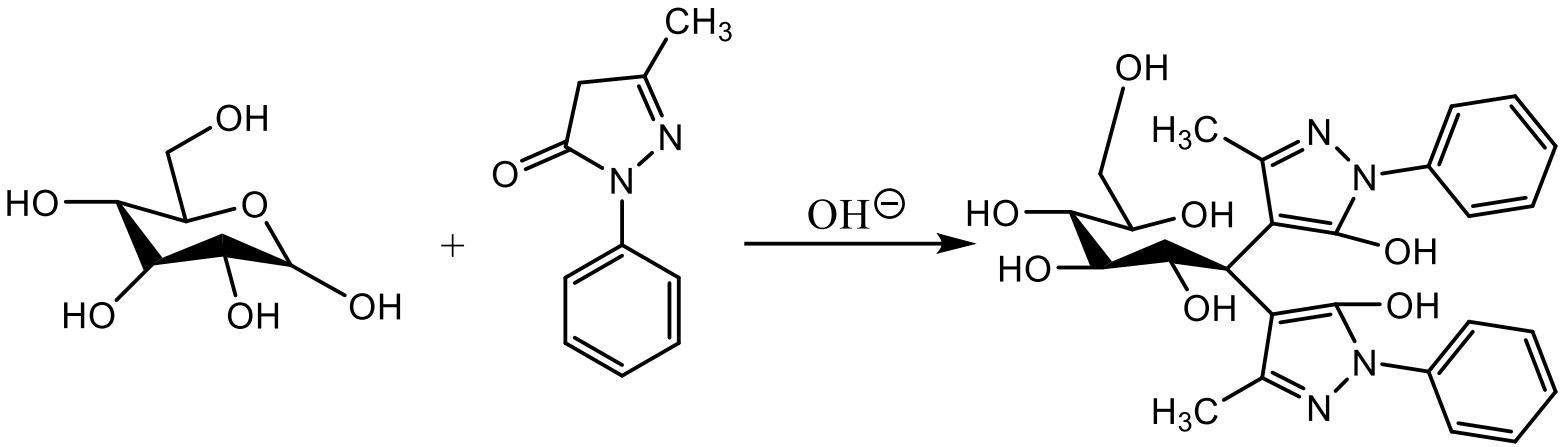
Conclusion

In this study, two detectors (i.e., DAD and ELSD) equipped with two different columns (i.e., Eclipse Plus C18 column and X-bridge amide columns) were tested for their efficiency and applicability on determination of reducing sugars and sugar-PMP derivatives. The results showed that different stationary phase materials packed in the two columns had resulted in significant different properties in terms of the anlaytical resolution and effectiveness on chemical separation. On the other hand, DAD and ELSD employ completely different principles in chemical detection which allow them to show significantly different sensitivities and selectivities of analytes. In summary, carbohydrates themselves are more likely to be separated and detected competently by an amide column equipped with ELSD, while DAD combined with C_{18} column promises to solve the determination of carbohydrates derivatives. Finally, regarding the efficient separation and confirmation of degree of polymerization of carbohydrate oligomers, such as chitosan oligomers, more research work is needed.



Scheme 3.1 Principle of glucose-PMP derivative

Table 3.1 Initial weight and each gradient dilution concentration of reducing sugar

Group	Reducing sugar name	Initial weight (mg)	Initial concentration (ppm)[a]	First dilution X 2 (ppm)	Second dilution X 4 (ppm)	Third dilution X 10 (ppm)
Mixture A	Glucose	5.7	570	285	142.5	45.6
	Xylose	5.2	520	260	130	41.6
	Glucosamine	4.9	490	245	122.5	39.2
	Maltose	4.9	490	245	122.5	39.2
Mixture B	Galactose	4.9	490	245	122.5	49
	Mannose	5.1	510	255	127.5	51
	Ribose	5.3	530	265	132.5	53
	Lactose	4.8	480	240	120	48

Note: a: parts per million

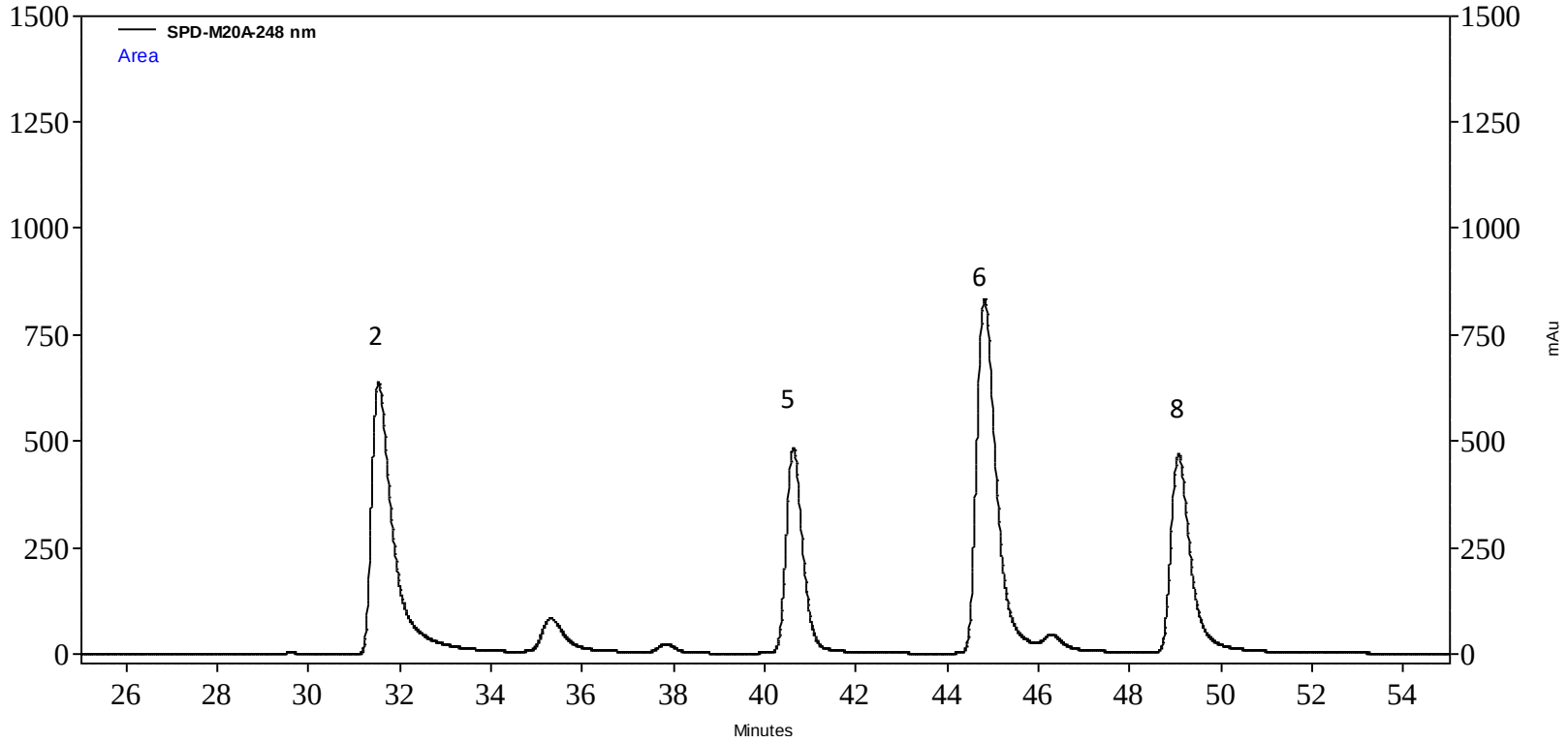


Figure 3.1 HPLC chromatogram of mixture A composed of the PMP derivatives of the following sugars with their peak numbers: 2. glucosamine, 5. maltose, 6. glucose, and 8. xylose. They are separated by the C18 column and detected by DAD.

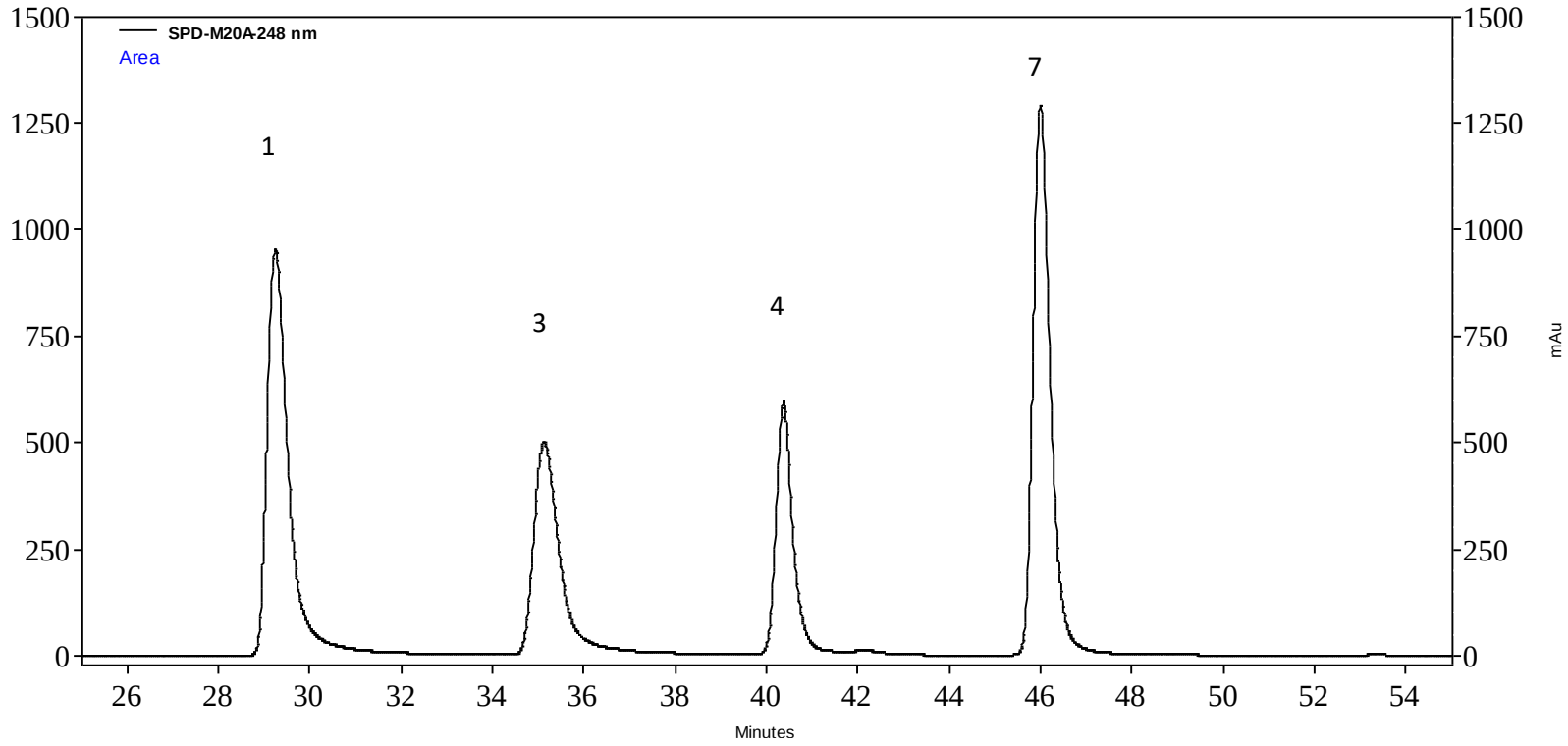


Figure 3.2 HPLC chromatogram of mixture B composed of the PMP derivatives of the following sugars with their peak numbers: 1. mannose, 3. ribose, 4. lactose, and 7. galactose. They are separated by the C18 column and detected by DAD

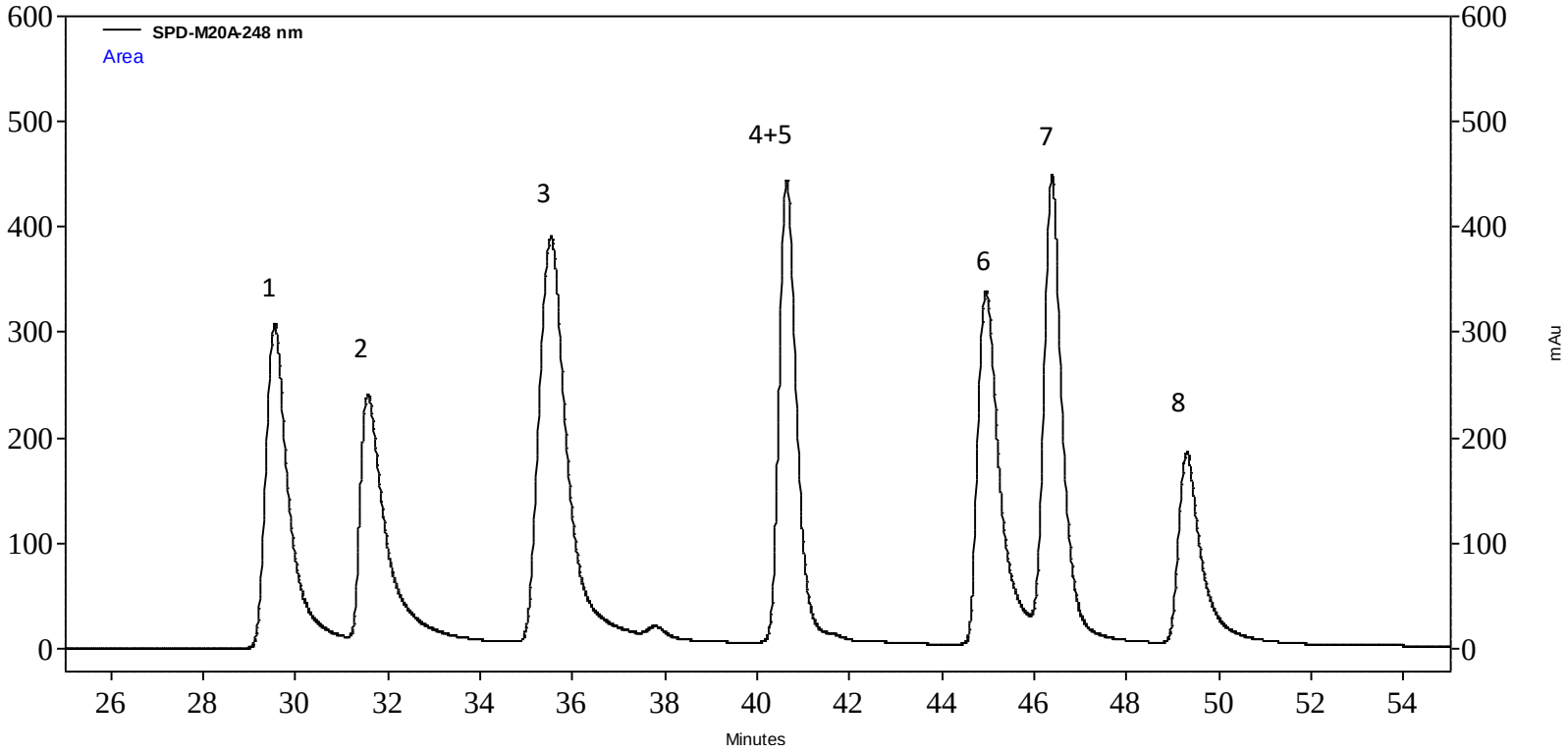


Figure 3.3 HPLC chromatogram of mixture C composed of the PMP derivatives of the following sugars with their peak numbers: 1. mannose, 2. glucosamine, 3. ribose, 4+5. lactose + maltose, 6. glucose, 7. galactose, and 8. xylose. They are separated by the C18 column and detected by DAD

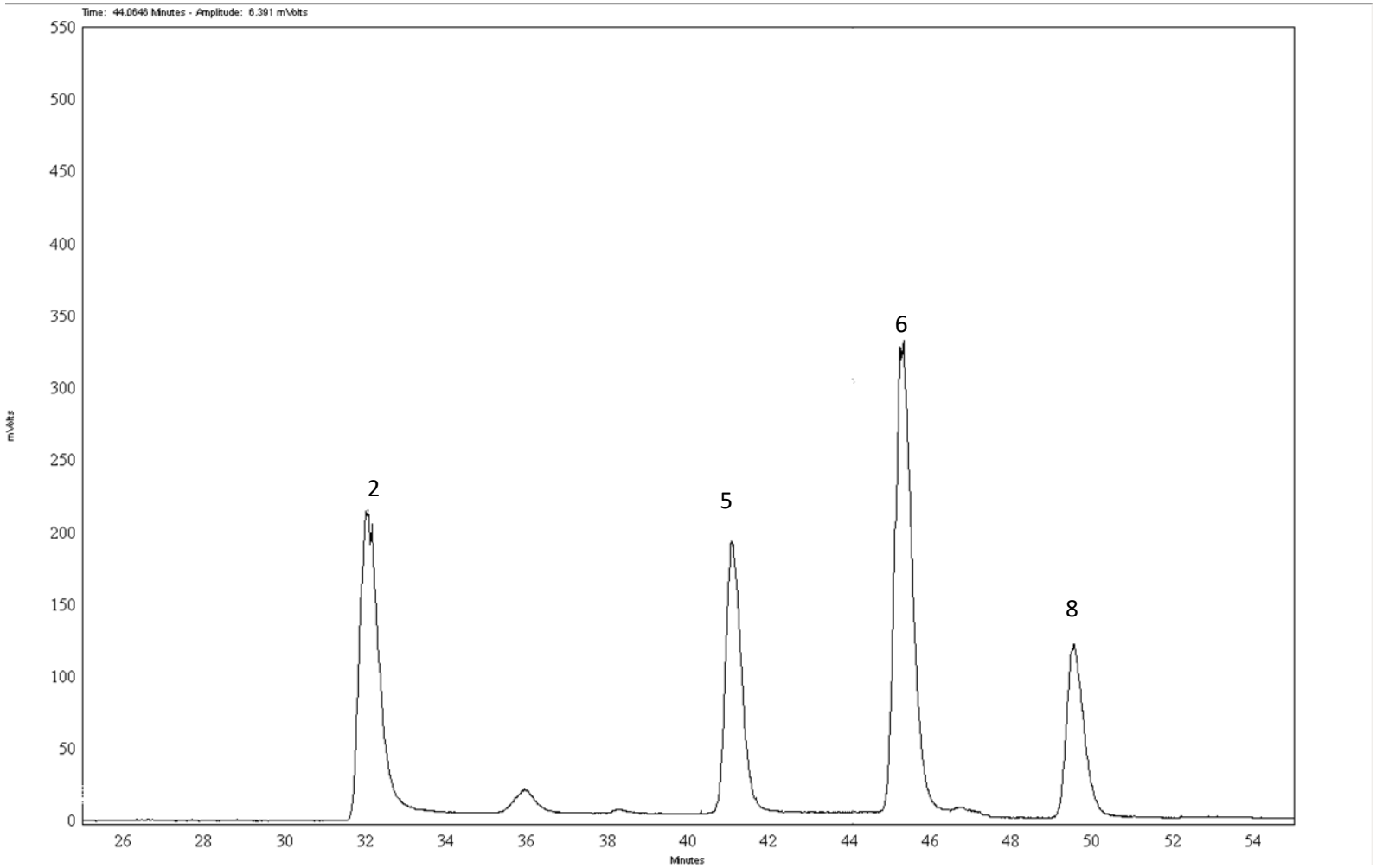


Figure 3.4 HPLC chromatogram of mixture A composed of the PMP derivatives of the following sugars with their peak numbers: 2. glucosamine, 5. maltose, 6. glucose, and 8. xylose. They are separated by the C18 column and detected by ELSD

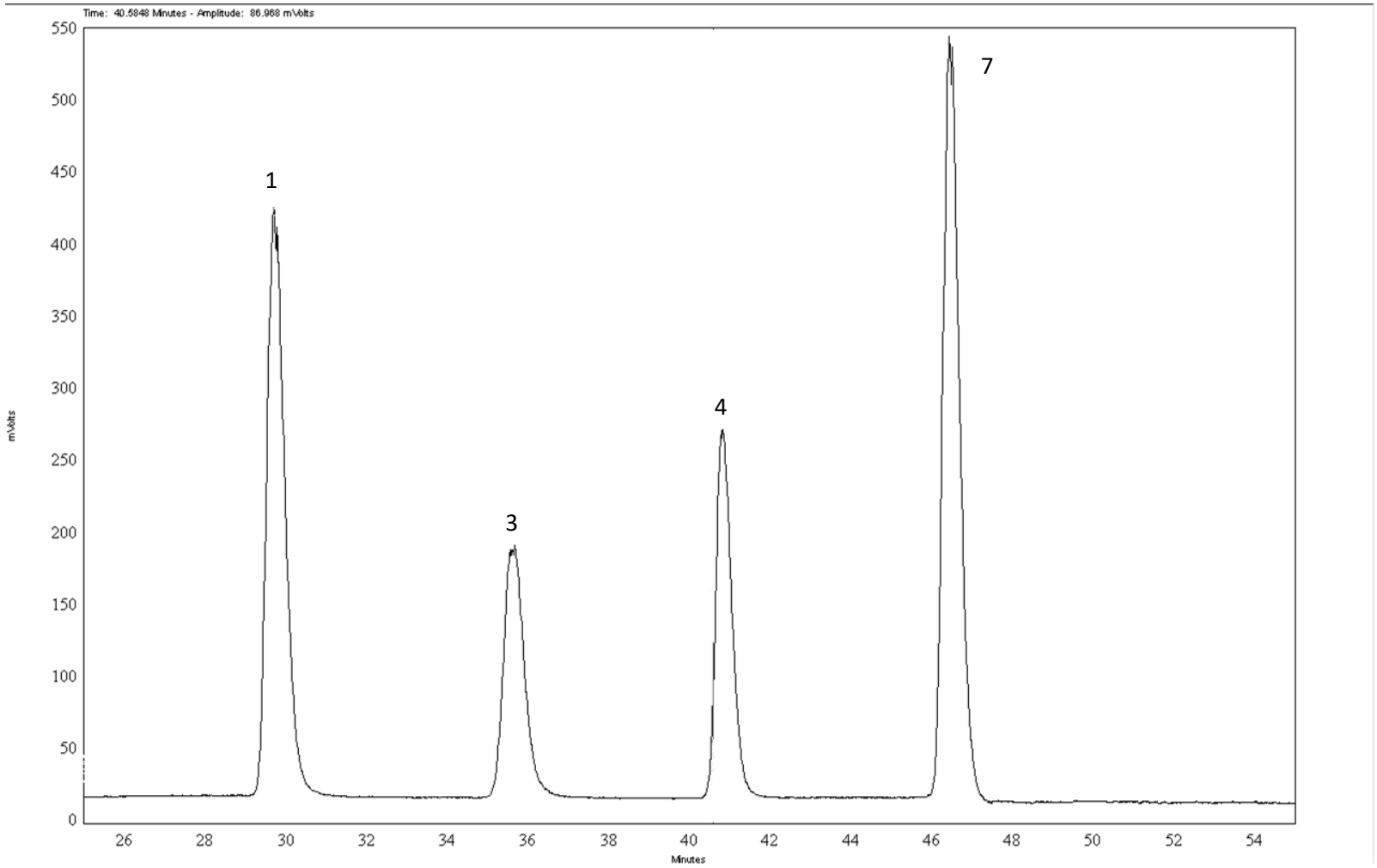


Figure 3.5 HPLC chromatogram of mixture B composed of the PMP derivatives of the following sugars with their peak numbers: 1. mannose, 3. ribose, 4. lactose, and 7. galactose. They are separated by the C18 column and detected by ELSD

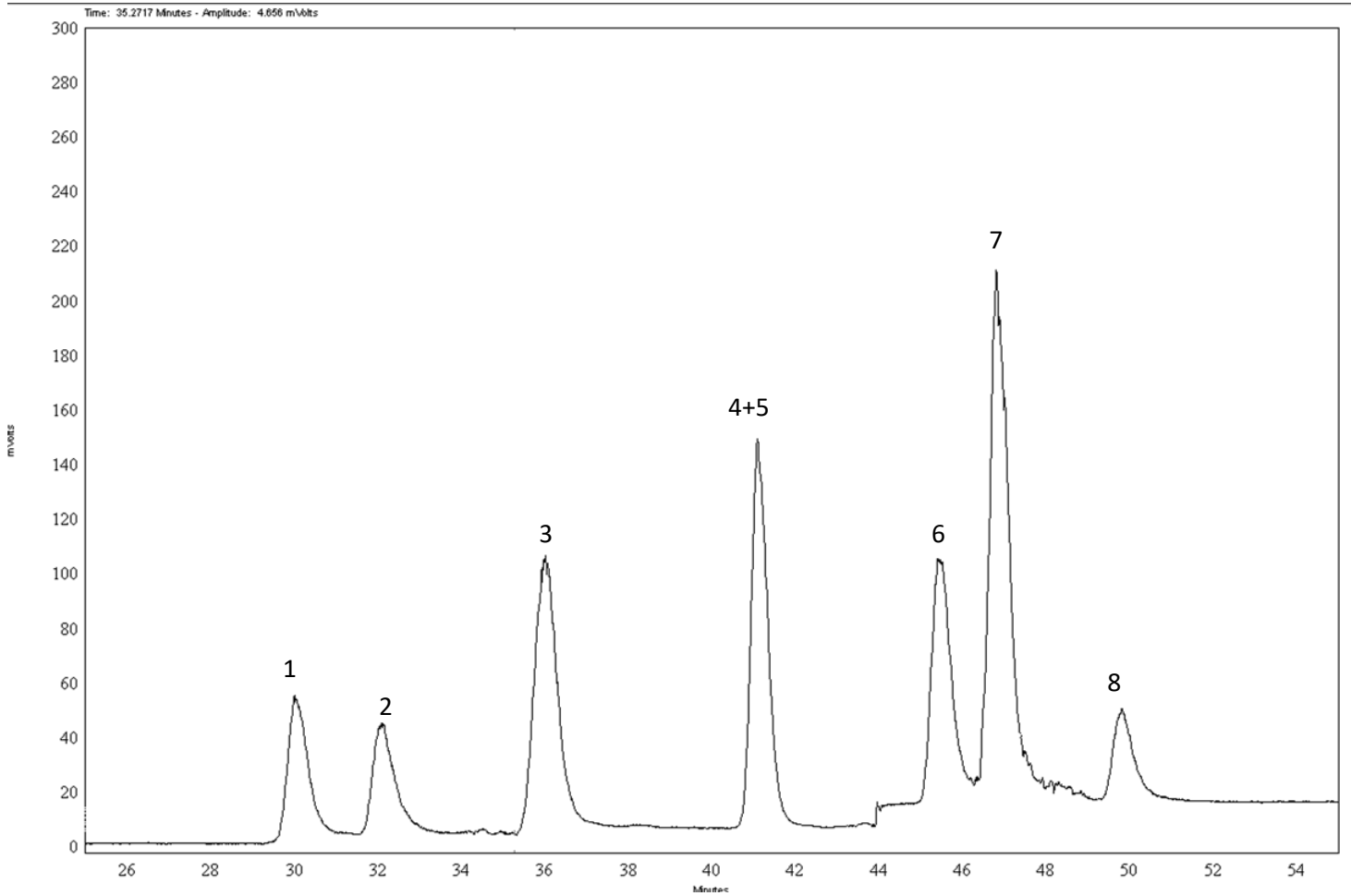


Figure 3.6 HPLC chromatogram of mixture C composed of the PMP derivatives of the following sugars with their peak numbers: 1. mannose, 2. glucosamine, 3. ribose, 4+5. lactose + maltose, 6. glucose, 7. galactose, and 8. xylose. They are separated by the C18 column and detected by ELSD

Table 3.2 Retention time of each sugar PMP-derivatives detected by HPLC-DAD

# Compound	Compounds name	DAD retention time	ELSD retention time
1	Mannose	28.8-31.0	29.5-30.5
2	Glucosamine	31.2-33.0	32-33.5
3	Ribose	34.7-36.6	35.5-36.2
4	Lactose	40-41.5	40.7-41.4
5	Maltose	40.1-41.4	40.5-41.9
6	Glucose	44.2-46.1	44.8-46.2
7	Galactose	45.9-47.5	46.3-47.1
8	Xylose	48.5-50.6	49.1-50.7

Table 3.3 Analytical linearity of HPLC-DAD of different reducing sugars-PMP derivatives detected at 248 nm

Carbohydrates	Linear regression y=ax+b		R^2	Linear range (PPM)
	a ($*10^6$)	b ($*10^6$)		
Mannose	0.1023	-2.6548	0.9957	51-225
Glucosamine	0.087	-2.0508	0.996	39.2-245
Ribose	0.0587	-1.4179	0.9997	53-265
Maltose	0.0513	-1.2263	0.9969	39.2-245
Lactose	0.0587	-1.5462	0.9941	48-240
Glucose	0.0895	-2.6413	0.9973	45.6-285
Galactose	0.1187	-2.5121	0.9968	49-245
Xylose	0.0539	-0.8045	0.9748	41.6-260

Note: a: Co-efficient of the linear regression, expressed as number $*10^6$; b: intercepts of the linear regression, expressed as number $*10^6$; y means chromatographic response area,; x represents the concentration, show as PPM which is particle per million.

Table 3.4 Analytical linearity of HPLC-ELSD of different reducing sugars-PMP derivatives

Carbohydrates	Linear regression y=ax+b		R^2	Linear range (PPM)
	a ($*10^5$)	b ($*10^5$)		
Mannose	1.9703	-6.562	0.9854	127.5-510.0
Glucosamine	1.9626	-6.4731	0.9916	122.5-490.0
Ribose	1.6324	-4.8048	0.995	132.5-530.0
Maltose	1.9013	-6.484	0.9953	122.5-490.0
Lactose	2.411	-9.6297	0.9874	120.0-480.0
Glucose	1.7867	-5.4867	0.9949	142.5-570.0
Galactose	2.4068	-8.9856	0.9927	122.5-490.0
Xylose	1.7959	-6.1654	0.9637	130.0-520.0

Note: a: Co-efficient of the linear regression, expressed as number *106; b: intercepts of the linear regression, expressed as number $*10^6$; y=In(response area), unit of which is 10^5, x=In(concentration) which was shown as particle per million (PPM).

Table 3.5 Comparison of HPLS-DAD-ELSD method

Carbohydrates[a]	%RSD[b]		Recovery (%) [c]	
	DAD	ELSD	DAD	ELSD
Mannose	7.1	5.8	101.5	100.2
Glucosamine	3.4	2.0	97.8	100.1
Ribose	7.0	10.4	99.6	100.0
Maltose	3.8	2.5	98.1	100.1
Lactose	8.2	23.0	101.8	100.8
Glucose	3.3	2.7	98.2	100.1
Galactose	6.4	18.9	101.3	100.3
Xylose	3.4	5.2	94.5	100.6

Note: [a]: detected at 248 nm; [b]: RSD %=(SD /Mean)*100; [c]: Recovery=measure value/ true value *100

Table 3.6 Detection and separation of different sugar and sugar-PMP derivatives by two detectors and two columns

Sugar	Name	C18				Amide			
		DAD		ELSD		DAD		ELSD	
		Original	PMP[a]	Original	PMP	Original	PMP	Original	PMP
Monosaccharides	Xylose	X	√	D	√	S	D	√	D
	Ribose	X	√	D	√	S	D	√	D
	Glucose	X	√	D	√	S	D	√	D
	Galactose	X	√	D	√	S	D	√	D
	Glucosamine	X	√	D	√	S	D	√	D
	Mannose	X	√	D	√	S	D	√	D
	Fructose	X	X	D	X	S	X	√	X
Sugar alcohol	Sorbitol	X	X	D	X	S	X	√	X
	Mannitol	X	X	D	X	S	X	√	X
Disaccharides	Lactose	X	√	D	√	S	D	√	D
	Maltose	X	√	D	√	S	D	√	D
	Sucrose	X	X	D	X	S	X	√	X
Oligosaccharides	α[b]	X	X	D	X	S	X	√	X
	β[c]	X	X	D	X	S	X	√	X
	γ[d]	X	X	D	X	S	X	√	X
	Chitosan[e]	X	√	D	√	X	D	D	D

Note: √: both detection and separation are good; X: neither detection nor separation is good; D: Detection is good, cannot separate; S: separation is good, cannot detect; [a]: PMP-derivatives; [b]: α-cyclodextrin; [c]: β-cyclodextrin; [d]: γ-cyclodextrin [e]: chitosan oligomer

Table 3.7 Detection and separation of different sugar and sugar-PMP derivatives by two detectors and two columns

Sugar	Name	DAD				ELSD			
		C18		Amide		C18		Amide	
		Original	PMP[a]	Original	PMP	Original	PMP	Original	PMP
Monosaccharides	Xylose	X	√	S	D	D	√	√	D
	Ribose	X	√	S	D	D	√	√	D
	Glucose	X	√	S	D	D	√	√	D
	Galactose	X	√	S	D	D	√	√	D
	Glucosamine	X	√	S	D	D	√	√	D
	Mannose	X	√	S	D	D	√	√	D
	Fructose	X	X	S	X	D	X	√	X
Sugar alcohol	Sorbitol	X	X	S	X	D	X	√	X
	Mannitol	X	X	S	X	D	X	√	X
Disaccharides	Lactose	X	√	S	D	D	√	√	D
	Maltose	X	√	S	D	D	√	√	D
	Sucrose	X	X	S	X	D	X	√	X
Oligosaccharides	α[b]	X	X	S	X	D	X	√	X
	β[c]	X	X	S	X	D	X	√	X
	γ[d]	X	X	S	X	D	X	√	X
	Chitosan[e]	X	√	X	D	D	√	D	D

Note: √: both detection and separation are good; X: neither detection nor separation is good; D: Detection is good, cannot separate; S: separation is good, cannot detect; [a]: PMP- derivatives; [b]: α-cyclodextrin; [c]: β-cyclodextrin; [d]: γ-cyclodextrin [e]: chitosan oligomer

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