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Original Article

Determination of oligosaccharides and monosaccharides in Hakka rice wine by precolumn derivation high-performance liquid chromatography

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ABSTRACT

This article presents a precolumn derivatization procedure with 1-phenyl-3-methyl-5-pyrazolone (PMP) reagent to detect oligosaccharides and monosaccharides in Hakka rice wine. The subsequent separation of the derivatized glucose–PMP also was performed using a mobile phase consisting of the molar ratio of acetonitrile to ammonium acetate buffer (0.1M) of 22:78 at a flow rate of 1.0 mL/min with the column temperature of 35°C, and the pH of ammonium acetate buffer at 5.5. The optimum derivation conditions were as follows: reaction temperature, 70°C; reaction time, 30 minutes; molar ratio of PMP to glucose, 10:1 (v/v); molar ratio of sodium hydroxide to glucose, 3:1 (v/v). The recovery rates were between 93.13% and 102.08% with relative standard deviation of 0.96–2.48%. The established method provides sufficient sensitivity with values of limit of detection of 0.09–0.26 mg/L and limit of quantification of 0.27–0.87 mg/L for determination of oligosaccharides and monosaccharides.

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1. Introduction

Carbohydrates play vital roles in the control of many key biological processes by acting as reciprocating compounds with proteins in molecular recognition events [1,2]. Hakka rice wine is brewed using glutinous rice and red kojic. It has low alcohol content, high nutritional value, and is healthy [3]. It is one of the most important kinds of Chinese rice wine [4]. Carbohydrate is the main component of Hakka rice wine, which is also responsible for its sweet taste [5–7]. The main component of carbohydrate is glucose, followed by

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oligosaccharides [4,5,8]. The type and content of carbohydrates in the wine play an important role in improving not only the taste and flavor of the wine but also its nutritional value. Therefore, determining the composition of sugars in rice wine is important for assessing its quality and monitoring the production process. Determination of the sugars in a variety of wines had been described in the literature [9,10]. Presence of several carbohydrates in Shaoxing (China) rice wine has been reported previously using high-performance liquid chromatography (HPLC) refractive index [11,12]. To our knowledge, however, there is no report on the direct and simultaneous qualitative and quantitative determination of carbohydrates in the Hakka rice wine. Accurate determination of the type and content of carbohydrate in Hakka rice wine is of significant importance to carry out further research on its functional components.

Analysis of monosaccharides is commonly performed using gas chromatography (GC) [13,14], liquid chromatography [15,16], or capillary electrophoresis [17]. GC/mass spectrometry with an electron-impact ion source has been the traditional method of choice for the quantification of neutral monosaccharides. However, sample preparation for GC is an extensive process, involving the derivation of sugars with specialty reagents to render them more volatile [18]. In addition, the sensitive chromatographic operating parameters for GC are not well suited for routine analysis. HPLC is commonly used to determine the composition of carbohydrates in various samples [19–21]. Monosaccharides or oligosaccharides cannot be detected directly by absorption due to the lack of chromophores in their molecular structure, which also limits the modes of detection by HPLC. For example, optical detectors do not meet the demands of modern analysis, and refractive index detector has low sensitivity, whereas using the evaporative light scattering detector incurs much cost [22]. Therefore, the derivatization of monosaccharides and oligosaccharides is indispensable to achieve highly sensitive detection.

1-Phenyl-3-methyl-5-pyrazolone (PMP) is one of the popular reagents that react with reducing carbohydrates under mild conditions, requiring no acid catalyst and causing no desialylation and isomerization. The derivative method make the sample with UV or fluorescent groups, and improve the detection sensitivity by ultraviolet and fluorescence detector for detection [23,24]. This paper is specifically concerned with analysis of monosaccharide composition and uronic acid using a PMP precolumn derivative [5,25,26]. However, the aforementioned derivative method has not been applied for qualitative and quantitative analyses of oligosaccharides in Hakka rice wine. Therefore, we improved and optimized the well-established PMP derivative method and its liquid-phase separation mode in terms of detection limit and separation capacity to make it applicable for the analysis of the Hakka rice wine.

2. Materials and methods

2.1. Materials and reagents

The wine samples were purchased from a local Carrefour supermarket (Guangdong, China). All reagents were of analytical grade and were used as supplied. Standards isomaltose and isomaltotriose (99.9%) were purchased from Sigma (St. Louis, MO, USA). Maltose was purchased from the Identification of Chinese Pharmaceutical and Biological products (Beijing, China). Panose was purchased from Aladdin (Shanghai, China). PMP was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Acetonitrile and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Water was prepared using the Milli-Q system (Millipore, Bedford, Massachusetts, USA). All other chemicals were of reagent grade.

2.2. Derivatization procedure with PMP and the removal of the excess PMP

The derivatization procedure applied in this study was modified from that reported by Kwon and Kim [27]. The effect of various factors on the derivatization yield was investigated, including reaction temperature, reaction time, and ratios of PMP to glucose (Glu) and sodium hydroxide (NaOH) to Glu. The following test procedure was followed: dry saccharide samples (100 g) were dissolved in 100 mL of water. The sample solution (1 mL) was dissolved in a solution of 0.5M PMP in methanol. After adding 1–9 mL of 0.3M NaOH, the mixture was incubated for 10–50 minutes at 50–70°C. After cooling down to room temperature, the mixture was acidified for neutralization by adding 1 mL of an aqueous hydrochloric acid solution (0.3M). The resulting solution was extracted five times by adding 4 mL of organic solvent (for standards, diethyl ether was used; however, benzene and chloroform were also tried), followed by vigorous mixing and centrifugation at 3000*g* for 5 minutes. The organic layer was carefully removed, and the final aqueous layer was evaporated to dryness and redissolved in 1 mL of water to obtain a standardized final volume. It was filtered through a 0.45-μm membrane filter, and 5 μL of the resulting solution was injected into the analytical column.

The removal of excess PMP was an important step that ensures efficient separation by the HPLC system. Five organic reagents—chloroform, benzene, isoamyl acetate, *n*-hexane, and dibutyl ether—were chosen to purify the PMP. Excess PMP was extracted using the same volume of organic reagent under the same conditions, and the efficiency of five organic extractants for removing the excess amount of derivatizing agent (PMP surplus ratio) was compared. The recovery of the derivative product was also studied, which indicates mean recoveries of Glu–PMP (i.e., the ratio of the Glu to PMP peak area of the sample solution after extraction was compared with that of the sample solution without extraction).

2.3. High-performance liquid chromatography

An HPLC analysis was carried out on an Agilent 1100 system (Agilent Technologies Q7, Santa Clara, California, USA) to evaluate the yield of the derivatization reaction. The system consisted of a quaternary solvent delivery system, an online degasser, an auto-sampler, a column temperature controller, and an ultraviolet detector coupled with an analytical workstation. The derivatives (samples) were separated on an Agilent Eclipse XDB-C_{18} column (5 μm, 250 mm × 4.6 mm i.d.; Agilent Technologies). The mobile phase consisted of two

eluents (A and B). Eluent A was a mixture of 0.1M ammonium acetate buffer and Eluent B was acetonitrile. For degassing and sterilization, both eluents were filtered through 0.2-μm-pore hydrophilic propylene membrane filters. The mobile phase was delivered at a flow rate of 0.5–2.5 mL/min and column temperature was set at 30°C. Chromatographic separation of several derivatives and the study of the quantitative performance of the procedure were carried out under elution conditions. Several conditions of pH, flow rate, and the ratio of mobile phase were studied by comparing the resolution and retention time of PMP–sugars. The chromatograms were monitored at 245 nm and the sample injection volume was 10 μL. All experiments were carried out at least in triplicate.

2.4. *Preparation of calibration curve and treatment of wine samples*

Five standard samples (100 mg) were accurately weighed and dissolved in water to obtain the primary stock standard solution (1.0 mg/mL). Stock standard solutions (1.0 mL) were transferred into a series of 10-mL volumetric flasks to cover the 0.2–6.0 μg/mL concentration range in a solution of 0.5M PMP in methanol. The calibration curve was constructed by plotting the peak area ratio against the final concentration of the sample. Alternatively, the corresponding regression equation was derived.

The wine sample was mixed with 70% ethanol in the ratio of 1:9, and shaken uniformly. The mixture was allowed to stand for a while until a white precipitate was produced. The supernatant was treated in accordance with the derivatization procedure.

2.5. *Sensitivity*

Limit of detection (LOD) is the lowest concentration of the wine that can be detected, but not necessarily quantitated under the stated experimental conditions. The LOD is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1 and is confirmed by analyzing a number of samples near this value using the following equation:

$$\text{Signal-to-noise ratio} = H/h \quad (1)$$

where H is the height of the spectrum corresponding to the sample, and h is the absolute value of the largest noise fluctuation from the baseline of the spectrum of a blank solution.

The limit of quantification (LOQ) is defined as the lowest concentration of the analyte that can be determined with acceptable precision and accuracy. It is quoted as the concentration yielding a signal-to-noise ratio of 10:1 and is confirmed by analyzing a number of samples near this value.

2.6. *Precision, repeatability, and recovery*

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation, or coefficient of variation of a series of measurements. The method was determined by the replicate analyses ($n = 7$) of the samples as well as the standard solutions at all calibration concentrations. The linearity of each calibration curve was confirmed by plotting the peak area ratio of the sugar to the internal standard against the sugar concentration. The unknown sample concentrations were calculated from the least-squares regression analysis of the standard curves. The standard curve samples were prepared simultaneously with the wine samples as described in the previous section.

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed "intra-assay precision." The repeatability was evaluated through the replicate analysis of different concentrations of samples, in either pure or dosage forms. Recovery method was determined by the replicate analyses ($n = 5$) of the samples at four spiked levels of 0.2 mg/L, 0.4 mg/L, 2.0 mg/L, and 4.0 mg/L.

2.7. *Statistical analysis*

All statistical designs consisted of one-way repeated measures analysis of variance, calculated using SPSS (IBM Company, New York City, New York, USA) at the 95% confidence level. Statistical differences were determined by the least significant difference test and multiple comparison procedure.

3. Results and discussion

3.1. *Optimization of the conditions for PMP precolumn derivatization*

Using glucose as a representative carbohydrate, the influence of various factors on the derivatization efficiency was optimized by changing one parameter at a time while keeping other parameters constant. The effect of reaction temperature is shown in Fig. 1A. It was found that the derivatization efficiency almost linearly increased below 70°C; however, a decrease was noted above this point due to partial decomposition of the reaction product at this condition. Based on the maximum peak area of the derivative, the optimal reaction temperature was found to be 70°C (Fig. 1A). These results are in accordance with those reported previously [26,28]. Fig. 1C illustrates that slightly higher yields were observed at PMP to glucose ratio of 10:1. As shown in a previous report [22], 1 mol reducing carbohydrate reacts with 2 mol PMP derivative to produce a better efficiency. However, in general, excess amount of derivatization reagent is used to ensure that complete reaction efficiency is achieved. The volume of NaOH was also evaluated. When the ratio of NaOH to glucose was changed, a convex curve having the maximal point at a ratio of 5:1 was obtained (Fig. 1D). The decrease of the peak area above this point is due to side reactions. The ratio of NaOH to glucose of 5:1 was chosen for further studies. Using the 5:1 ratio of NaOH to glucose and the 10:1 ratio of PMP to glucose at 70°C, the peak area of the derivative product increased as the reaction time increased until 30 minutes, but after 30 minutes, the peak area declined (Fig. 1B). However, a longer reaction

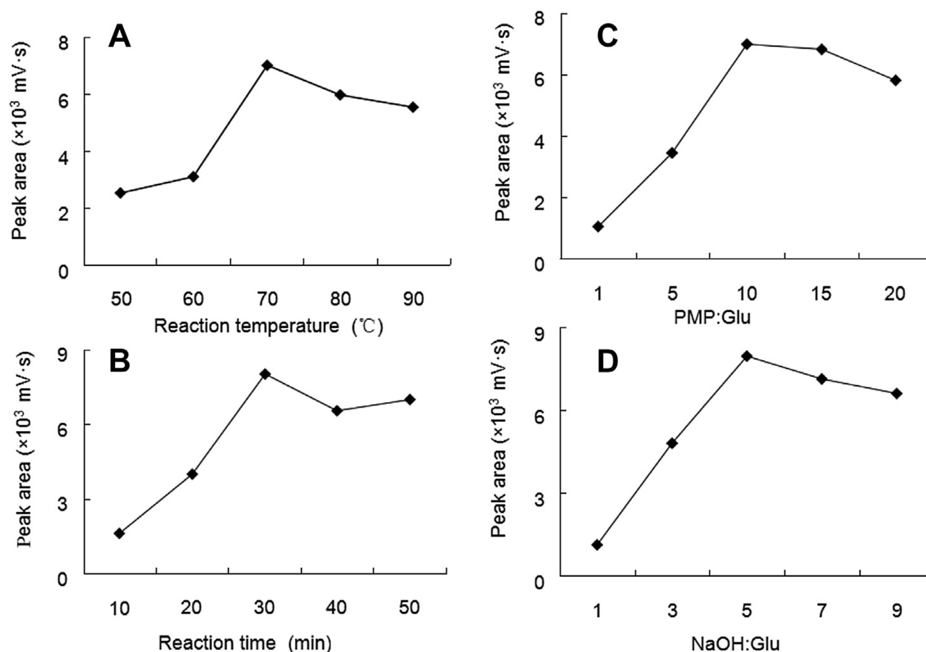

Fig. 1 – Effect of (A) reaction temperature, (B) reaction time, (C) volume of 1-phenyl-3-methyl-5-pyrazolone (PMP), and (D) volume of NaOH on the peak area of glucose–PMP. Glu = glucose, NaOH = sodium hydroxide.

time has been used for this purpose in other studies [29,30]. Honda and colleagues [23] first introduced PMP as a derivatization reagent for reducing sugars in 1989. Based on their results, a recommended procedure for PMP precolumn derivatization was established as described under the "Materials and methods" section.

3.2. *Selection of organic solvent for cleanup of reaction mixture*

Although the PMP precolumn derivatization method is useful, it has certain shortcomings. The large amounts of reagent for derivatization and removal of the excess PMP before performing HPLC of the derivatives were indispensable. Table 1 compares the efficiencies of cleanup by extraction with several organic solvents, together with recoveries of Glu–PMP. The use of dibutyl ether and chloroform produced the best result in both removal of the excess PMP and recovery of the PMP derivative. However, extracting with chloroform made the separation of two phases more obvious and the stratification more conducive. After three extractions with chloroform, the PMP remaining rate reached 1.5%. However, multiple extraction would lead to the loss of sugar derivatives, and therefore, the recovery of Glu–PMP too low. Using chloroform and dibutyl ether could guarantee Glu–PMP recovery rates of 98.2% and 98.6%, respectively.

Table 1 – Comparison of the efficiencies of extraction of PMP by different organic solvents.

Reagent	PMP remaining rate (%)	Recoveries of Glu–PMP (%)
Chloroform	1.5	98.2
Benzene	4.2	71.8
Isoamyl acetate	3.3	80.3
n-Hexane	63.1	67.4
Dibutyl ether	2.0	98.6

Glu = glucose; PMP = 1-phenyl-3-methyl-5-pyrazolone.

3.3. *Optimization of chromatographic separation of PMP derivatives*

The proportion of mobile phase used in liquid chromatography plays an important role in gradient elution. Different proportions of mobile phase determine different polarities and different solubilities of the test substance in the stationary and mobile phases, which have a direct impact on both retention time and separation efficiency. Separation of PMP derivatives of selected samples was investigated using various stationary and mobile phases. The stationary phase of Agilent Eclipse XDB-C_{18} was the most suitable for this purpose. The retention behaviors of these PMP derivatives were examined using mixtures of 0.1M ammonium acetate buffer with various pH values and different proportions of acetonitrile. Fig. 2A and 2B show the effect of acetonitrile concentration on retention and resolution of PMP derivatives for the acetate buffer. With the increase in the proportion of acetonitrile, a decrease was noted in the retention time. Under this condition, the resolution also decreased. Satisfactory separation without peak tailing and maximum peak area was obtained with a mobile phase consisting of acetonitrile ranging from 18% to 21% (v/v) at ambient temperature. However, the separation of isomaltose and panose was not perfect. Considering the comprehensive requirements of suitable resolution and short elution time, 22% acetonitrile was chosen as the suitable mobile phase for the analysis.

To examine whether a lower mobile phase pH offers better separation, the influence of pH on the resolution and retention time of the formed complex sugar was investigated over

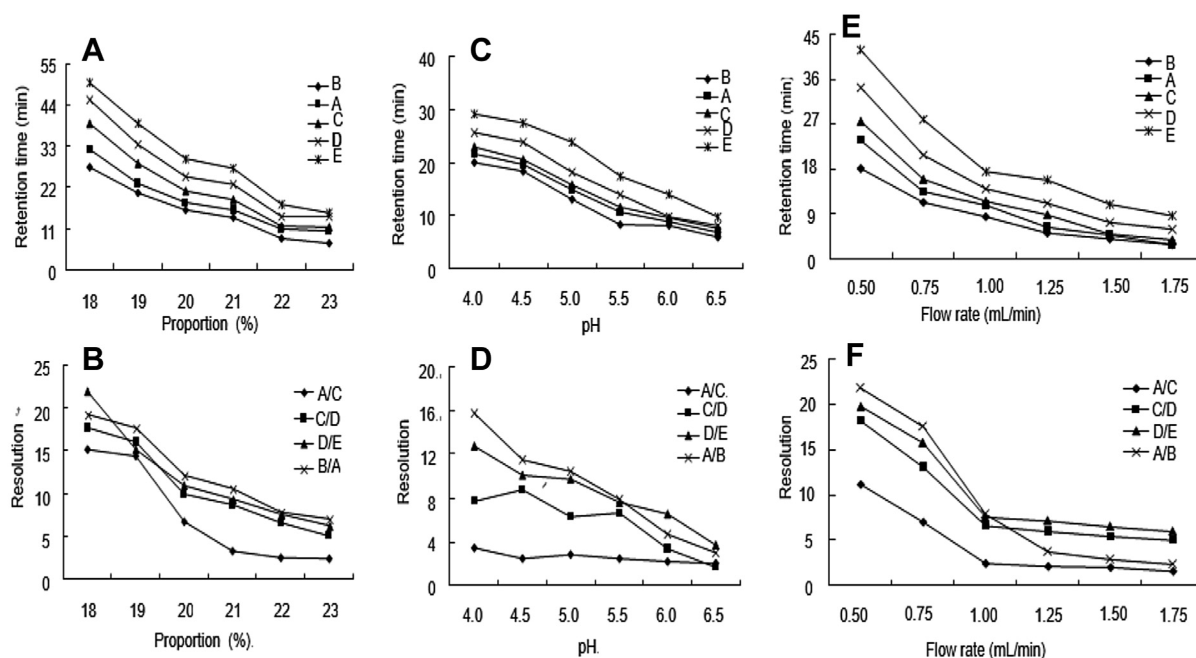

Fig. 2 – Effect of acetonitrile concentration on (A) retention time and (B) resolution of sugar–1-phenyl-3-methyl-5-pyrazolone (sugar–PMP) derivation. Effect of pH of acetate buffer on (C) retention time and (D) resolution of sugar–PMP derivation. Effect of flow rate of mobile phase on (E) retention time and (F) resolution of sugar–PMP derivation. A = isomaltose; B = isomaltotriose; C = panose; D = maltose; E = glucose.

the pH range of 4–6 using the ammonium acetate buffer. As shown in Fig. 2C and 2D, increasing the pH of ammonium acetate buffer (pH 4.0–5.5) resulted in a subsequent decrease in both elution time and resolution. When pH exceeded 5.5, its downward trend remained constant. Because of the increase in the pH of the mobile phase, anion affinity of the residual silanols on the surface of the C_{18} column stationary phase, and the weak alkalinity of the sugar derivative are improved. pH 6.0 showed poorer resolution for maltose, panose, and isomaltose. From these results, the mixture of 0.1M ammonium acetate buffer (pH 5) and acetonitrile concentration (22% v/v) provided the best separation of these monosaccharide derivatives.

The effects of different flow rates of mobile phase on resolution and retention time were also tested. The results showed that the retention time declined and the resolution decreased with the increase in the flow velocity of the mobile phase. In accordance with the retention times and the symmetry of the peaks, flow rate of 1 mL/min was found to be most suitable. The chromatograms of the five sugar derivatives are shown in Fig. 3. All components have reached the baseline separation.

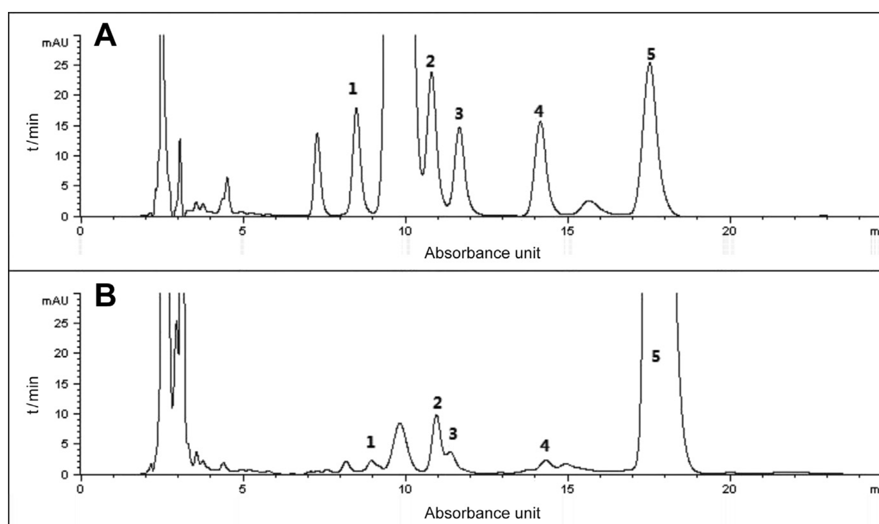

Fig. 3 – (A) Chromatograms of sugar standards derivation; (B) chromatograms of sugar derivation of wine sample. 1 = isomaltotriose; 2 = isomaltose; 3 = panose; 4 = maltose; 5 = glucose.

Table 2 – Calibration curve parameters, correlation coefficient, recoveries, LOD, and LOQ for all sugar–PMP samples.

Sample	Standard curve	r	Recovery (%)	RSD1 (%)	RSD2 (%)	RSD3 (%)	LOD (mg/L)	LOQ (mg/L)
1	$y = 925.60x + 13.69$	0.9999	96.21	2.33	2.55	1.87	0.09	0.27
2	$y = 304.91x - 0.66$	0.9992	95.43	1.12	1.22	2.34	0.15	0.45
3	$y = 675.99x + 5.46$	0.9997	102.08	2.48	1.57	2.20	0.11	0.33
4	$y = 739.39x + 18.74$	0.9997	93.13	2.15	2.14	2.85	0.21	0.63
5	$y = 135.07x + 397.50$	0.9999	99.87	0.96	0.67	1.96	0.26	0.78

Sample numbers used indicate the following: 1 = isomaltose; 2 = isomaltotriose; 3 = panose; 4 = maltose; 5 = glucose.
LOD = limit of detection; LOQ = limit of quantification; PMP = 1-phenyl-3-methyl-5-pyrazolone; r = correlation coefficient; RSD1 represents the standard deviation of recovery; RSD2 represents the result of injection precision ($n = 7$); RSD3 represents the results of repeatability ($n = 3$).

3.4. *Sample precision and method repeatability*

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Applying the aforementioned liquid chromatographic method, the linear regression equation of every sugar derivative was obtained. The regression plot showed that there was a linear dependence of the peak area on the concentration of five working solutions of sugar derivative. The consecutive repeat injection analysis ($n = 7$) yielded relative standard deviations of the peak area of five sugar derivative that were <2.6%. Precision of the method was evaluated through repeatability and intermediate precision. Upon examining the repeatability of the overall operation of the PMP precolumn derivatization, the HPLC method indicated that the relative standard deviation of peak area of each PMP–sugar sample was <3.0%.

3.5. *Standard curves and recovery*

The calibration curve of Glu–PMP showed excellent linearity in the range of concentration studied. Table 2 summarizes the percentage recovery of LOD and LOQ, and the recoveries of the method were between 93.13% and 102.08%, relative standard deviation was between 0.96% and 2.48%. This result indicates that the detection efficiency for HPLC using the precolumn method was satisfactory and consistently independent.

3.6. *Analysis of oligosaccharides and monosaccharides in Hakka rice wine*

Because the method presented in this paper was successful for monosaccharide analysis, it has been extended to oligosaccharide analysis as well. Therefore, the method was applied to the determination of sugar in rice wine. Three wine samples were analyzed using a precolumn HPLC system under optimized conditions. The content of five sugars is presented in Table 3. The content of five sugars in the same wine sample showed significant differences ($p < 0.05$); additionally, the content of the same sugar in different wine samples also showed significant differences ($p < 0.05$). The content of glucose was highest in three different wine samples. The remaining four wine samples had a lower content of glucose. The difference among these three wine samples was mainly due to different processing technologies applied.

4. Conclusion

In this study, an optimized rapid precolumn derivation HPLC method has been developed and validated for the determination of oligosaccharides and monosaccharides in Hakka rice wine. The developed method is more economical than mass spectrometry, and it also has a lower LOD. The derivatization procedure can be completed successfully under mild conditions without the need for high temperatures and long derivatization times. The precision and reproducibility of the method were less than 2.6% and 3.0%, respectively. Five sugars had good linear correlation coefficients and the recoveries were between 93.13% and 102.08%. Five sugars in three Hakka rice wine samples were respectively detected using the improved method. Our results show that that the wine samples evaluated had significantly different content of five sugars. Therefore, this method can be used for daily quality testing in common laboratories, thereby enabling sample separation. In addition, the method also shows promise for use in the analysis of carbohydrates in Hakka rice wine.

Table 3 – Content of oligosaccharides and monosaccharides in Hakka rice wine (g/L).

Sample	Sugars				
	Isomaltotriose	Isomaltose	Panose	Maltose	Glucose
1	$0.20 \pm 0.008^{*}$	$0.96 \pm 0.03^{*}$	$0.16 \pm 0.002^{*}$	$0.39 \pm 0.01^{*}$	$37.05 \pm 1.46^{*}$
2	$0.23 \pm 0.01^{*}$	$0.69 \pm 0.03^{**}$	$0.13 \pm 0.004^{*}$	$1.20 \pm 0.04^{*}$	$44.05 \pm 1.12^{*}$
3	$0.31 \pm 0.009^{*}$	$0.85 \pm 0.02^{**}$	ND	$0.21 \pm 0.009^{*}$	$55.80 \pm 1.08^{**}$

Results are presented as mean ± standard deviation of triplicate replicated measurements.
*The difference was significant ($p < 0.05$).
**The difference was not significant ($p > 0.05$).
ND = not detected.

Conflicts of interest

All authors declare no conflicts of interest.

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