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LC-MS/MS-based non-isotopically paired labeling (NIPL) strategy for the qualification and quantification of monosaccharides

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ABSTRACT

Investigation into monosaccharides is critical for studies of oligosaccharides structure and function in biological processes. However, monosaccharides quantification is still challenge due to their isomeric structure and high hydrophilic properties. Besides, it was difficult to obtain isotopic internal standards (IS) of each monosaccharide in complex matrixes. Herein, we developed a novel strategy for the qualification and quantification of monosaccharides in urine using two structure analogs 1-(4-methylphenyl)-3-methyl-5-pyrazolone (MPMP) and1-phenyl-3-methyl-5-pyrazolone (PMP) as non-isotopically paired labeling (NIPL) reagents by liquid chromatograph-tandem mass spectrometry (LC-MS/MS). The derivatized monosaccharides by NIPL method not only had sufficient retention time differences on reversed-phase column, but also exhibited predominant product ion pairs (*m/z* 189 & *m/z* 175) in the multiple reaction monitoring (MRM) mode. In this method, PMP labeled standards were adopted as one-to-one internal standards (ISs). 12 urinary monosaccharides were successfully determined and the linear ranges expanded five orders of magnitude with limit of quantification (LOQ) varied from 0.09 ng mL^{-1} to 0.36 ng mL^{-1} as well as the accuracy higher than 98.15% and the relative standard derivation (RSD) lower than 7.92%. With assistance of multivariate analysis, the targeted monosaccharide biomarkers were firstly obtained for the diagnosis of bladder cancer. By the inexpensive NIPL reagents—MPMP/PMP, the developed strategy possessed the specific advantages of low cost, simple operation, high sensitivity and high accuracy for the qualification and quantitation of monosaccharides. As expected, this method will provide an alternative application potential for targeted metabolomics analysis.

1. Introduction

Carbohydrates are existed in the form of monosaccharides, oligosaccharides and polysaccharides in various organisms including plants, animals and microorganisms [1–3]. Together with proteins and lipids, carbohydrates occupied an important position in many biological processes such as protein folding, cell recognition and cellular immunity [4–6]. Glycosylation is consisted of a certain number of monosaccharides including galactose, glucose, mannose, fructose, fucose, N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine (GalNAc) and so on [7,8]. Minor structural differences on sugar would result in large functional diversities [9]. Additionally, the most significantly altered metabolic pathways in many diseases including colorectal cancer (CRC) are involved monosaccharides [10]. Hence, the accurate qualification and quantification of these saccharides would give benefit to further explore their biological significance.

With the development of analytical technology, mass spectrometry (MS) was used as a well-accepted tool in monosaccharides analysis with its high sensitivity and high accuracy [11–13]. However, the existence of isomeric forms and hydrophilic groups in monosaccharides greatly limit the trace level analysis for their poor ionization efficiencies on MS and difficult separation capabilities on chromatography [14]. Then, the derivatization is introduced to enable reversed-phase liquid chromatograph, gas chromatograph or capillary electrophoresis separation and mass response of oligosaccharides or glycans [15–18]. The reducing end of the sugar reacts with the labeling reagents containing amino function including 2-aminopyridine(2-AP), 8-aminonaphthalene-1,3,6-trisulfonate (ANTS), 2-aminobenzoic acid (2-AA), 2-Pyridylfuran,

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2-aminobenzamide (2-AB) and 3-amino-9-ethylcarbazole (AEC) were most frequently used [19–24]. However, the major drawback to the reductive amination reaction is that its derivatization operation and post-processing steps are cumbersome, especially not suitable for acid sugar [25]. Unlikely, 1-Phenyl-3-methyl-5-pyrazolone (PMP) reported by Honda et al. was available for neutral and acid sugar derivatization with high yields and single products [26]. Meanwhile, other PMP reagent analogs including 1-aryl-3-methyl-5-pyrazonlone (AMP), 1-(4-methoxy-phenyl)-3-methyl-5- pyrazolone (PMPMP), 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid (PMPA), 1-(2naphthyl)-3-methyl-5-pyrazolone (NMP) and 1-(4-isopropyl) phenyl-3-methyl-5-pyrazolone (PPMP) were successively developed as derivatization reagents for saccharides characterization [27–31]. These works highlighted the accuracy of saccharides qualification.

For the targeted quantitation of analytes in complex biological samples, multiple reaction monitoring (MRM) performed on a triple quadrupole instrument is deemed as a unique tool by mass filtering of both parent and product ions [32,33]. To overcome the quantitative errors caused by complex matrices of biological samples, the addition of internal standards can be effectively reduced the ion suppression, thereby greatly improving quantitative analysis [34–36]. Traditionally, isotopically labeled analogs (SIL) were the first choices of quantitative internal standards for their favorable assay performance [37–39]. Han et al. developed MRM-based method to quantify neutral mono- and disaccharides employing ^{13}C-labeled internal standards in negative ion mode [40]. d0/d5-PMP stable isotopic labeling strategy was utilized for relative quantitative analysis of oligosaccharides by LC-MS [41]. However, the SIL strategy still has problems as fellows: (1) isotopic interference and mutual ionization suppression between analytes and its internal standards; (2) complicated synthesis procedures and high cost [42].

An alternative strategy to solve these problems is the structural analogs [43,44]. For example, N-dimethyl-amino-1-naphthalene-1-sulfonyl chloride (Dns-Cl) and N-diethyl-amino-1-naphthalene-1-sulfonyl chloride (Dens-Cl) were used to measure the metabolites of tryptophan pathway in serum. In this method, both of Dns-Cl and Dens-Cl were structural similar and the Dens labeled standards were regarded as internal standards to overcome matrix effects [45]. Besides, chiral metabolomics fingerprinting was successfully achieved by using a pair of enantiomers as derivatization reagents [46].

Herein, a novel LC-MS/MS absolute quantification methodology for monosaccharides is established, based on structural similarity of 1-(4-methylphenyl)-3-methyl-5-pyrazolone (MPMP) and 1-phenyl-3-methyl-5- pyrazolone (PMP). The non-isotopically-paired labeling (NIPL) approach that could quantitate isomeric monosaccharides with excellent reliability and practicability. In this method, PMP labeled standards were adopted as one-to-one IS to compensate the influence from complex matrix effects. After systemic optimizations including chromatographic separation condition and MRM parameters by LC-MS, 12 isomeric monosaccharides were successfully identified and quantified in urine samples of human suffering from bladder cancer, which was one of the most common urinary malignancy worldwide [47]. In combination of statistical analysis, the targeted monosaccharide biomarkers of bladder cancers were profiled in visual way. To our acknowledge, the MPMP/PMP-oriented paired derivatization method was first reported, which allows key monosaccharides closely related to bladder cancer to be measured by LC-MRM. The developed method was expected to widely applied to accurate qualification and quantification of isomeric monosaccharides with high sensitivity and high accuracy. Meanwhile, PMP-derivatized monosaccharides using as non-isotope internal standards has the advantage of high stability and low cost.

2. Material and methods

2.1. Chemicals and reagents

1-phenyl-3-methyl-5-pyrazolone (PMP), 1-(4-methylphenyl)-3-methyl-5-pyrazolone (MPMP) were obtained from Aladdin company (Shanghai, China). Monosaccharides such as glucose, galactose, mannose, arabinose, ribose, xylose, rhamnose, fucose, N-acetylglucosamine (GlcNAc), glucuronic acid (GluA), Galcturonic acid (GalA) and N-acetylmannosamine (ManNAc) were purchased with the purification more than 99% (Sigma- Aldrich, Shanghai, China). Formic acid, ammonium hydroxide, ammonium acetate, methanol and acetonitrile in HPLC grade were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Derivatization optimization

A mixed standard solution containing 12 monosaccharides with the concentration of 1 mg mL^{-1} was utilized as the typical example. The mixtures were reacted with different concentrations of MPMP (10, 20, 30, 40, 65, 80, 100 and 200 mg mL^{-1}) under different time points (0.5, 1, 2, 3, 4, 5, 6 h) at different temperatures (50, 60, 70, 80, 90 °C) individually. Then the resulting solutions were centrifuged (20000 g, 4 °C, 10 min) before LC-MS/MS determination.

2.3. Urine samples collection and pretreatment

Volunteers contained 10 patients suffered from bladder cancer and 10 healthy controls were recruited from the first affiliated hospital of Zhengzhou University. After collection, urine samples were centrifuged at 8000 rpm for 10 min three times, the supernatants were combined and stored at −80 °C.

2.4. Preparation of PMP labeled non-isotopic internal standards (NILISs)

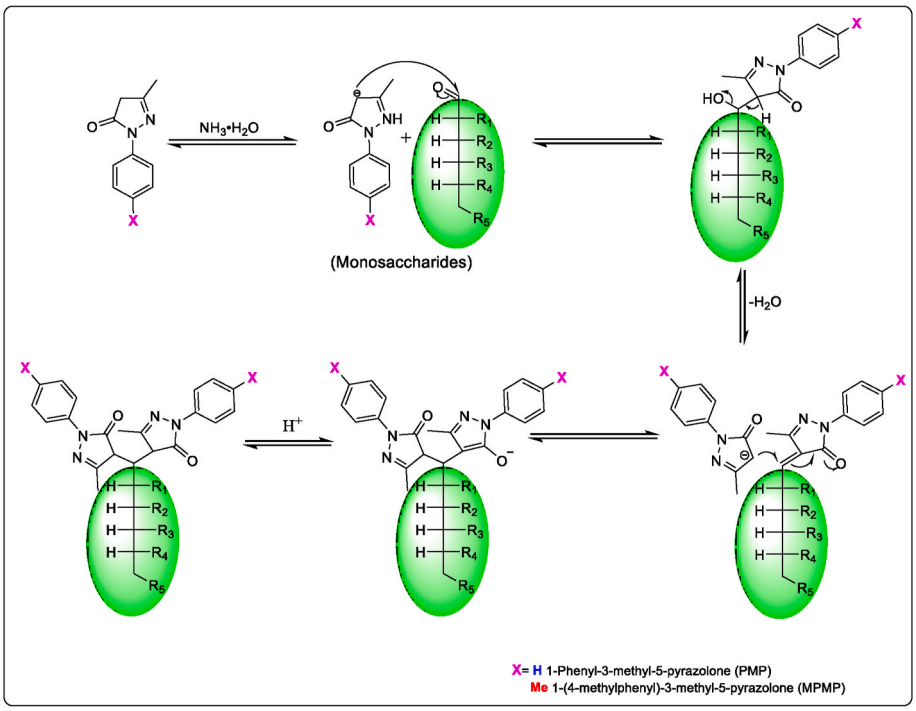
Standard solution was added to 100 mg mL^{-1} PMP in methanol containing 100 μL ammonium hydroxide. Then the mixtures were incubated under 90 °C for 60 min. After cooling to room temperature, the reaction solution was centrifuged at 8000 rpm for 5 min. Then, the supernatant was diluted by initial mobile phase before LC-MRM analysis.

2.5. derivatization of urine samples

The urine samples were diluted 1000-fold with ultrapure water. After centrifuging (8000 rpm, 4 °C, 10min) the supernatant was collected and dried under vacuum for further derivatization. Each MPMP-derivatized sample was evaporated to dryness and mixed with PMP-labeled standards (ISs) equally. After centrifugation (8000 rpm, 4 °C, 10min) and dilution, supernatant with a volume of 1 μL was injected for LC-MRM analysis.

2.6. LC-MS/MS analysis

The LC-MRM analysis was carried on ultra high performance liquid chromatography (Shimadzu, Japan) and 6500 QTRAP mass spectrometry equipped with an Ion Drive™ Turbo V source (ABSCIEX, America). Chromatography was on a LC-30AD (Shimadzu Corp, Columbia, USA) system consisting of LC-30AD pumps, SIL-30AC autosampler, and a CTO-30 A column oven. The chromatography separation was performed on a EVO C18 column (2.1 mmX100 mm, 2.6 μm; Phonex, USA) at 40 °C, using H_2O (A, ammonium hydroxide-ammonium acetate, pH = 8.72) and acetonitrile (B, 0.1% formic acid) as mobile phase. Programmed gradient adopted for separation was as follows: 0 min, 5% B; 0–10 min



Scheme 1. Derivatization of monosaccharides by MPMP or PMP through Michael 1,4-addition reaction under the catalyst of ammonia.

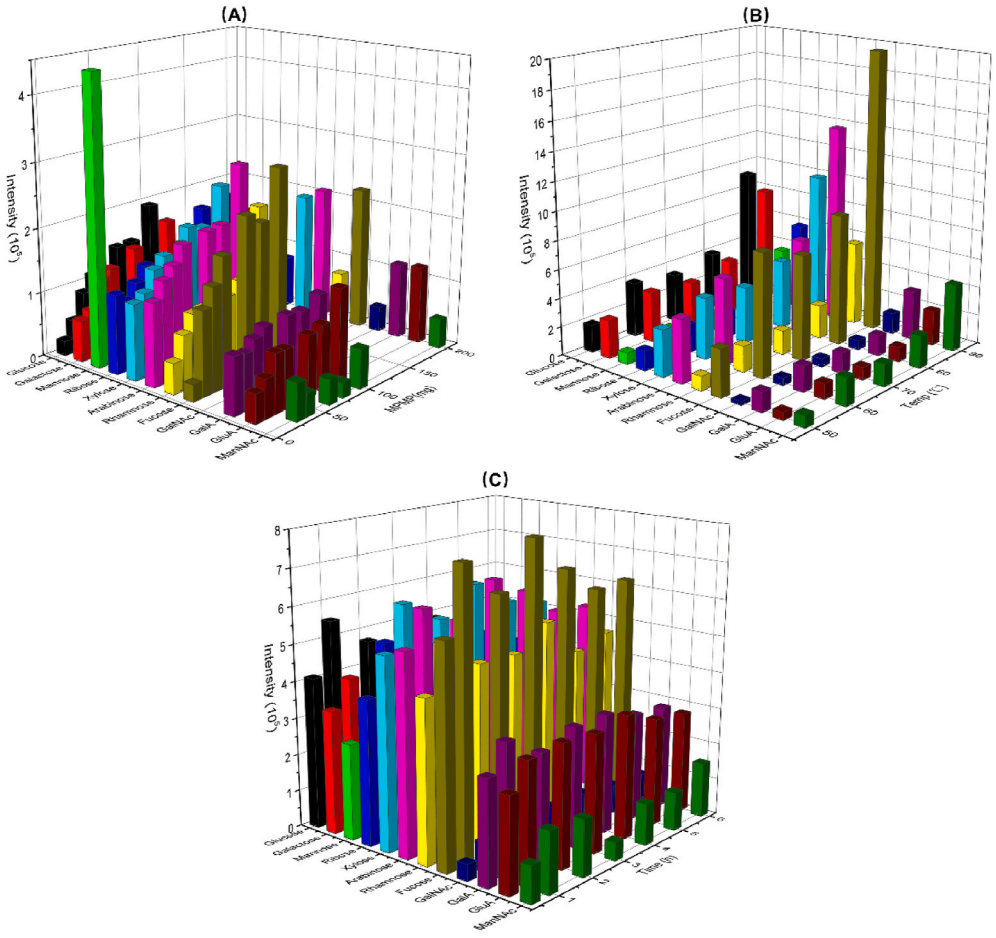
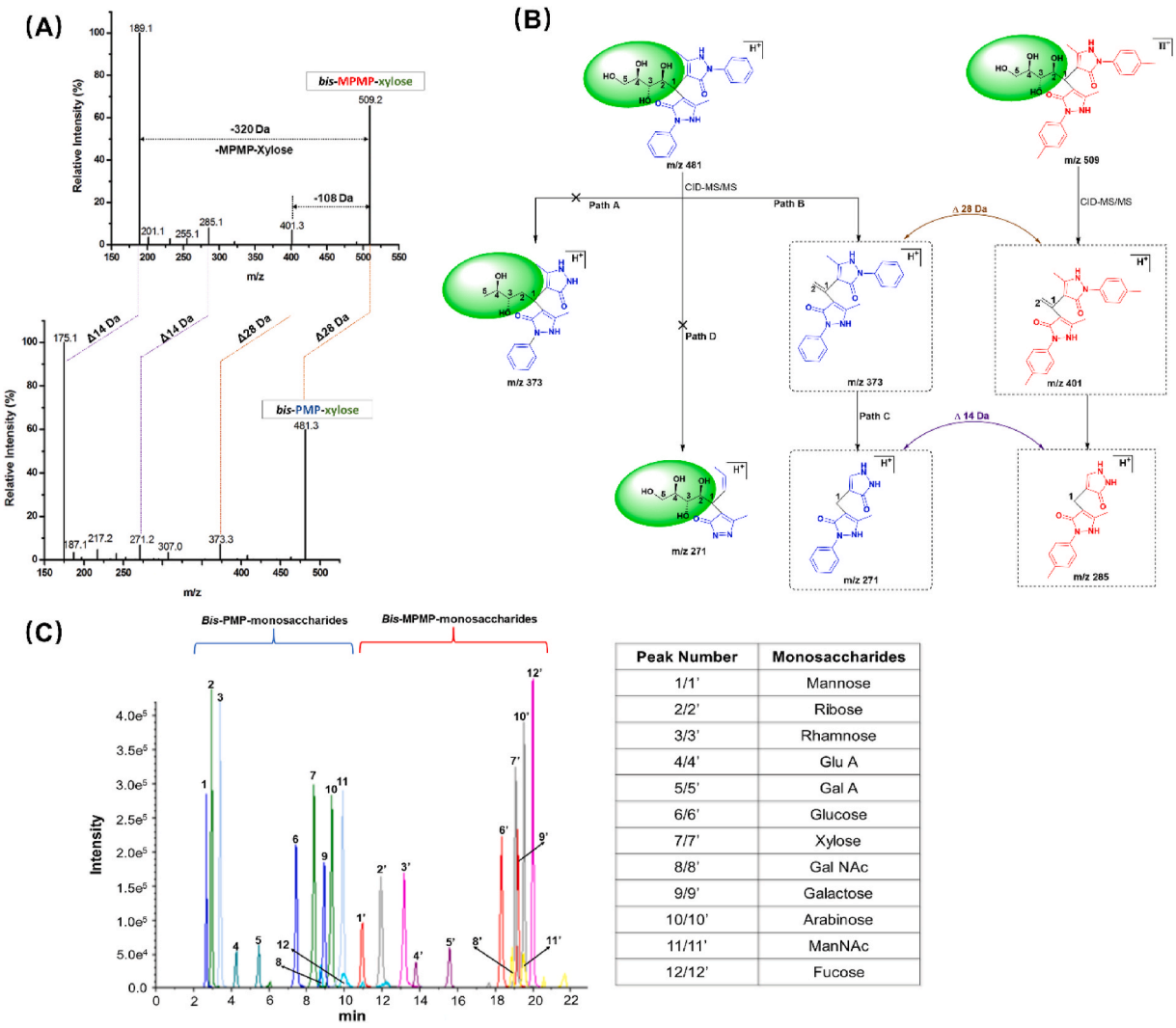


Fig. 1. Optimization of MPMP derivatization efficiency for 12 isomeric monosaccharides under different reaction conditions: (A) effect of MPMP concentration; (B) effect of temperature, and (C) effect of reaction time.



Peak Number	Monosaccharides
1/1'	Mannose
2/2'	Ribose
3/3'	Rhamnose
4/4'	Glu A
5/5'	Gal A
6/6'	Glucose
7/7'	Xylose
8/8'	Gal NAc
9/9'	Galactose
10/10'	Arabinose
11/11'	ManNAc
12/12'	Fucose

Fig. 2. (A) ESI-MS/MS spectrum of bis-MPMP-xylose and bis-PMP-xylose; (B) fragmentation pathway calculation by the comparison of *bis*-MPMP-xylose and *bis*-PMP-xylose; (C) XIC spectrum of *bis*-MPMP-Monosaccharides and *bis*-PMP-Monosaccharides using LC-MRM. Twelve monosaccharides were listed at the right table.

90% B. The column was re-equilibrated with initial mobile phase conditions for 5 min. Samples were analyzed in positive mode. The optimized MS instrument parameters were set as follows: ion source temperature (TEM) was 550 °C, and ion spray voltage was set as 5500 V. Gas 1, Gas 2 and Curtain gas were 30, 15, 20 psi respectively. Collision gas was set to medium, and Q1/Q3 were set to unit resolution. Collision energies and declustering potentials were optimized by direct infusion of MPMP/PMP-derivatized monosaccharide standards with the concentration of 100 ng mL^{-1} in Analyst v1.7. The MRM transitions and parameters of analytes were listed in Table S1.

2.7. Method validation

Standard stock solutions of monosaccharides were prepared in H_2O to obtain concentrations of 1 mg mL^{-1}. Nine-level mixed standard working solutions at the concentration of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 ng mL^{-1} were served as calibrators. They were prepared with ultrapure water through serial dilution of stock solutions for method validation. Solutions of MPMP, and PMP were all prepared in methanol with concentrations of 1 g mL^{-1} respectively. They were diluted with methanol to the desired level before use. All stock and working solutions were prepared fresh.

The calibrations were conducted over the analysis of derived 9-level monosaccharide standards with a wide concentration range (0.01–100 ng mL^{-1}). The linear regression of calibration curves was established with weighting factor of $1 / x^2$. The limit of quantification (LOQ) and limit of detection (LOD) were equivalent to a concentration where 10 and 3 times of the signal-to-noise ratio, respectively. Precision was evaluated within a run (n = 5) for intra-assay and five consecutive days for inter-assay (n = 5). In general, RSDs were represented for the precision, and no more than 15% were required. The accuracy was carried out at three different concentrations in five replicates. The value of the accuracy was assessed by the recoveries of labeled monosaccharides within the range of 85–115%.

2.8. Data analysis

The LC-MRM raw data was treated by Analyst (Version 1.7) and Multi Quant (Version 2.1) respectively. The data was expressed as the mean ± S.D. Both principal component analysis (PCA) and orthogonal partial least squares (OPLS-DA) analysis were performed on SIMCA-p (Version 14.0).

3. Results and discussion

3.1. Method development and optimization

The derivatization mechanism of MPMP/PMP and monosaccharides

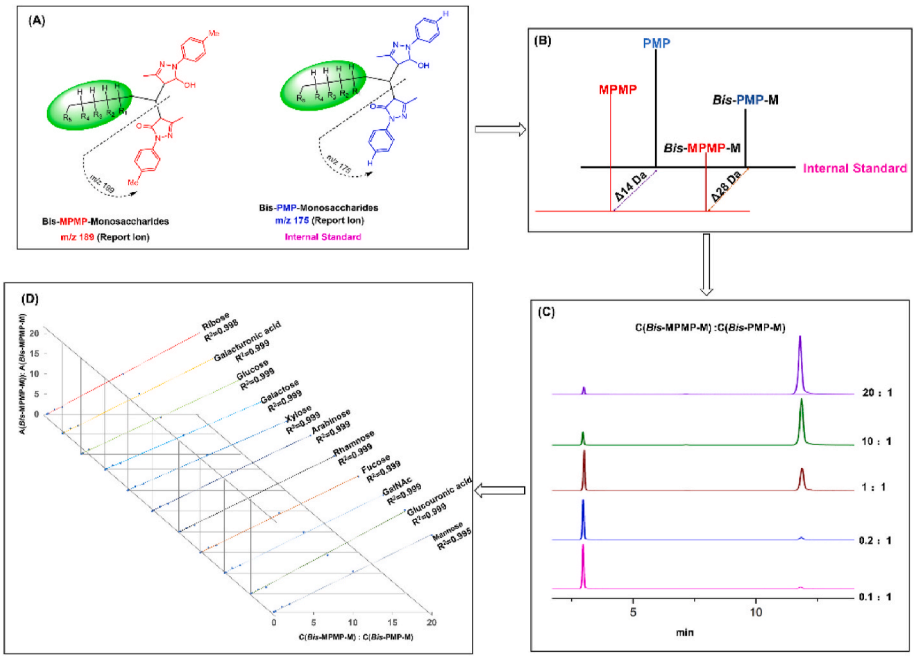


Fig. 3. (A) The structure of monosaccharides derivatized by MPMP and PMP. (B) The description of NIPL strategy for the quantitation of monosaccharides by LC-MRM. (C) Relative quantification of MPMP/PMP derivatized monosaccharide standards under various ratios (0.1:1, 0.2:1, 1:1, 10:1, 20:1). (D) The linearity plots of their expected values on the x-axis and their measured ratios on the y-axis.

in base medium was shown in Scheme 1. This reaction was proceeded slowly between monosaccharide with aldo-form and MPMP/PMP by a reversible 1,4-Michael addition. Taking MPMP for instance, different MPMP concentration, temperature and reaction time were continuously investigated to achieve the optimum conditions based on signal enhancement. Each monosaccharide was derivatized with MPMP with the label concentration from 10 to 200 mg mL^{-1}. The optimal concentration for MPMP was 100 mg mL^{-1}, as derivative product signal improved with the increase of MPMP concentration from 10 to 100 mg mL^{-1} and reduced at 200 mg mL^{-1} (Fig. 1A). The influence of reaction temperature and derivatization time were exhibited in Fig. 1B and C. The results indicated 90 °C for 60 min provided the best mass spectrometric response for individual bis-MPMP derivatives. Taken together, we determined that the optimal conditions for MPMP-labeling should be performed under 90 °C for 60 min with 100 mg mL^{-1} MPMP.

To study the fragment patterns and identify the fragment ions for MRM monitoring, twelve monosaccharides derivatized by MPMP or PMP were used for ESI-MS/MS analysis. Taking xylose for example, The characteristic fragment ion namely $[PMP + H]^+$ (*m/z* 175) was observed for bis-PMP-xylose. Similarly, the most abundant product ion of $[MPMP + H]^+$ (*m/z* 189) was observed for *bis*-MPMP-xylose.Fragments including *m/z* 401, *m/z* 285 and *m/z* 201 were observed for *bis*-MPMP-xylose and *m/z* 373, *m/z* 271, *m/z* 187 for *bis*-PMP-xylose (Fig. 2A). The fragment mechanism or pathway were calculated as Fig. 2B. For the fragment ion *m/z* 373 of *bis*-PMP-xylose, it might involve two paths as fellow: (A) loss of two molecular of H_2O on sugar ring and one molecular benzene ring on PMP; (B) the elimination of a molecule of water on C2, occupied with further cleavage of C2/C3 bond. According to the difference of 28 Da (-$2CH_2$) between two ions *m/z* 401 and *m/z* 373, it suggested that the path B was the reasonable fragmentation approach. Besides, for the fragment ion of *m/z* 271 from *bis*-PMP-xylose, two possible cleavage paths were concluded:(C) the dissociation of benzene ring of PMP, accompanied with further cleavage of the other PMP; (D) the cleavage on one PMP residue and the cleavage of C1/C2 bond. However, the mass difference between *m/z* 285 and *m/z* 271 were 14 Da (-CH_2) indicated the loss of one MPMP or PMP residue, followed by fragmentation on the labeling reagent, indicating the path D was favored. For CID-MS/MS spectra of other eleven monosaccharides labeled by PMP and MPMP (Fig. S1 and Fig. S2), the dissociation principles could be illustrated by the mass shift (14 Da or 28 Da), including C–C bonds cleavage of sugar, the elimination of water and so on. This method was a breakthrough to the traditional isotopic labeling approach ($^{13}C/^{18}O$) to determine the cleavage pathway by CID-MS/MS [48].

Additionally, predominant fragment ions contaning pronated MPMP moiety (*m/z* 189) for *bis*-MPMP-monosaccharides and pronated PMP moiety (*m/z* 175) for *bis*-PMP-monosaccharides were generated by tandem mass spectrometry. They were selected as quantitative MRM transition monitoring, and the monitored MRM parameters were detailed in Table S1. After MPMP and PMP labeling, the separation of isomeric monosaccharide derivatives was performed on an C18 column. To further improve the chromatographic performances and response signals of target analytes, the compositions of the mobile phase and chromatographic column were optimized. EVO C18 column were adopted for its excellent tolerance in wide pH ranging from 1 to 12. With ammonium hydroxide-ammonium acetate (pH = 8.72) and acetonitrile containing 0.1% formic acid as mobile phase, twelve monosaccharides derivatized with MPMP and PMP were well separated within 21min under optimized gradient elution procedure (Fig. 2C). The PMP-labeled twelve monosaccharides were eluted in 10 min and the MPMP derivatives were separated in the next 10 min due to the lower hydrophobicity of MPMP. Significantly, the elution sequence of individual *bis*-MPMP-monosaccharides is consistent with *bis*-PMP-monosaccharides, respectively.

3.2. Method validation

It's believed that a stable isotopically labeled (SIL) analogue is the perfect internal standard in LC-MS/MS analysis due to its specific physicochemical property toward target molecule [49]. However, SIL reagents are always commercially unavailable or very expensive, and it's impractical to explore IS for each monosaccharide. In this paper, we developed a non-isotopically paired labeling approach, in which the monosaccharides were derivatized with MPMP, and the monosaccharide standards were labeling by PMP to produce bis-PMP-derivatives that

Table 1
The retention time, calibration, linear ranges, limits of detection, limits of quantification, and precision for the determination of 12 monosaccharides (n = 5) by non-isotopically paired labeling strategy using LC-MRM.

No.	Name	Retention Time (min)	calibration curve	coefficient (R^2)	Liner Range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Accuracy (%)	RSD (%)	
									Intra-day	Inter-day
1	Glucose	18.32	y = 0.8129x+0.0124	0.9999	0.01–100	0.0256	0.09	99.83	4	4.66
2	Galactose	19.21	y = 0.773x+0.0105	0.9998	0.01–100	0.0173	0.06	98.98	2.6	5.8
3	Mannose	10.9	y = 0.9826x-0.1993	0.9949	0.01–100	0.102	0.34	99.04	3.4	3.96
4	Ribose	11.91	y = 0.9204x-0.0991	0.9984	0.01–100	0.0261	0.09	99.17	2.6	5.73
5	Xylose	19.09	y = 0.7755x+0.0203	0.9997	0.01–100	0.0129	0.04	99.17	1.3	2.64
6	Arabinose	19.54	y = 0.8473x + 0.0594	0.9995	0.01–100	0.0105	0.04	99.17	2.7	4.81
7	Rhamnose	13.14	y = 0.8818x - 0.0529	0.9998	0.01–100	0.048	0.16	100.48	3.4	6.77
8	Fucose	20.01	y = 0.8567x + 0.0403	0.9995	0.01–100	0.00532	0.02	98.15	3.1	7.92
9	GalNAc	18.89	y = 0.8798x + 0.0966	0.9993	0.01–100	0.0619	0.21	101.67	3.6	3.51
10	GalA	15.56	y = 0.8845x + 0.0771	0.9992	0.01–100	0.0439	0.15	99.18	6.3	7.27
11	GluA	13.79	y = 1.0077x - 0.0242	0.9998	0.01–100	0.107	0.36	100.19	6.3	4.74
12	ManNAc	19.50	y = 1.5416x - 0.063	0.9999	0.01–100	0.0266	0.09	99.18	2.1	3.81

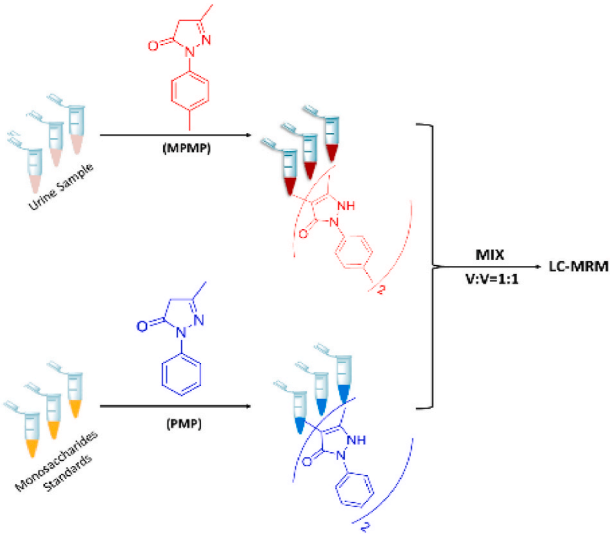


Fig. 4. Workflow of Non-isotopically paired labeling strategy for the quantification of monosaccharides in human urine.

was adopted as internal standards. The mixture of bis-MPMP-derivatives and bis-PMP-derivatives were used for LC-MS/MS analysis in MRM mode. The fragment ion of $[MPMP + H]^+$ (*m/z* 189.1) for *bis*-MPMP--Monosaccharides and product ion of $[PMP + H]^+$ (*m/z* 175.1) for *bis*-PMP-Monosaccharides were adopted as report ion pair for monosaccharides quantification (Fig. 3A and B).

In order to investigate the feasibility of non-isotopically labeling paired method for quantitative analysis, the linear dynamic range was verified by changing he ratios of *bis*-MPMP-monosaccharides and bis-PMP-monosaccharides respectively. Monosaccharides labeled by MPMP and PMP were mixed in different ratios ranging from 0.1:1 to 20:1 and examined by LC-MRM (Fig. 3C). The experimental concentration ratios were plotted against corresponding expected concentration ratios. As shown in Fig. 3D, NIPL method was linear across the 0.1 to 20 with R^2 value higher above 0.995, suggesting that good relative quantification capability could be obtained by NIPL strategy using LC-MRM. Moreover, the exhibited slopes were between 0.81 and 1.54, implying that NIPL method was available for monosaccharides quantification as isotopic ratio by MS analysis [50]. However, absolute quantification of specific monosaccharides could provide more exact and reliable information for direct confirmation of concentration changes and dynamics. Based on NIPL-LC-MRM method, the absolute concentration of monosaccharides could be obtained by spiking PMP labeled standards with known concentration into the MPMP derivatized samples. As shown in Table 1, *bis*-MPMP-monosaccharides were linear

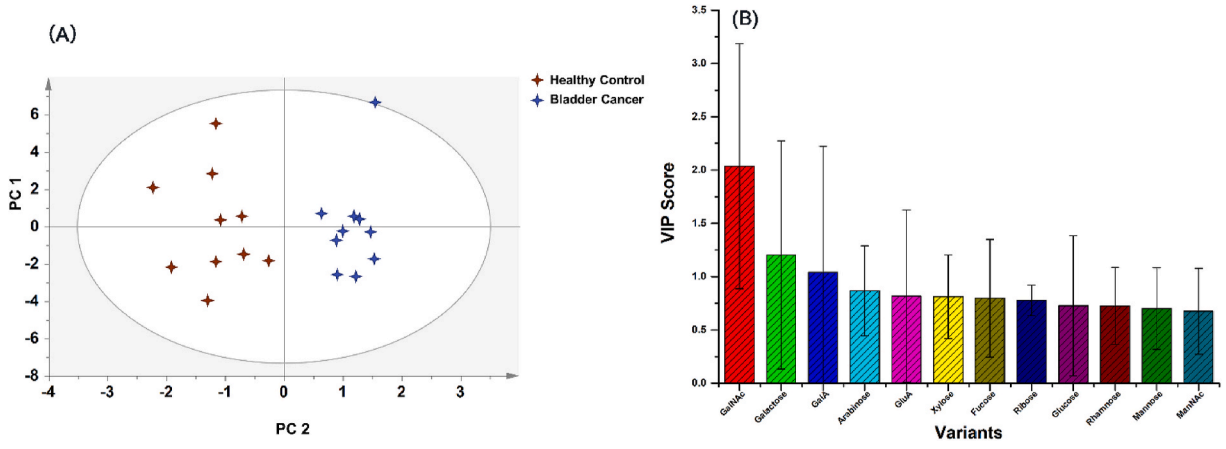


Fig. 5. (A) Score plots of OPLS-DA displayed between healthy controls (n = 10) and bladder cancer group (n = 10); (B) Relative variable important variants based on VIP scores.

across 0.01–100 ng mL^{-1} with the accuracies higher than 98.15%, indicating the excellent ability NILP strategy on absolute quantitative analysis. It was found that the MPMP-labeled monosaccharides could be quantified with LODs ranging from 0.09 to 0.36 ng mL^{-1}, and LOQs between 0.0256 and 0.107 ng mL^{-1}. The intra- and inter-assay RSDs, as determined by five replicates, were about 1.3%–6.3% and 2.64%–7.92%. Hence, the NILP method provides excellent reliability for the relative and absolute quantification of monosaccharides.

3.3. Method application

The validated NIPL strategy was applied to determine the monosaccharides in urine sample from two types of human samples, namely normal and bladder cancer group. The monosaccharides-targeted quantification was determined by spiking PMP labeled standards with known concentration into the MPMP labeled samples. Consequently, the absolute concentration of monosaccharides in urine sample was quantified by the peak area ratios of MPMP- and PMP-labeled pair of monosaccharides respectively (Fig. 4). Here, concentrations of glucose, galactose, mannose, ribose, xylose, arabinose, rhamnose, fucose, GalNAc, GalA, GluA and ManNAc were generated in the human urine sample (Table S2).

We further investigated the changes of the twelve monosaccharides of bladder cancer urine samples compared to the healthy controls by the quantitative results obtained by our developed NIPL method. The multivariate analysis was initially performed by unsupervised classification method named the principal component analysis (PCA). The first two components (PC1 and PC2) explained 76.2% of the total variance. The established PCA model (Fig. S3) had a good quality with R^2X of 85.9% but poor predictability with Q [2] of 47.8%. Consequently, OPLS-DA was applied to obtain a higher degree of group separation and a better illustration of the variables responsible for classification. As shown in Fig. 5A, the bladder cancer group were statistically distinguishable from healthy controls in OPLS-DA score plot with R^2X of 73.2%, R^2Y of 87.7% and Q [2] of 84.9%. The value of variable important variants in the OPLS-DA model indicated the corresponding changes of monosaccharides, which were responsible for the discrimination between bladder cancer group and healthy controls. In Fig. 5B, the variable importance of projection (VIP) value of GalNAc, Galactose and GalNAc more than 1.0 revealed significant difference between bladder cancer and healthy controls, indicating they could be considered as the potential biomarker for bladder cancer diagnosis.

4. Conclusion

In this work, a new commercially available MPMP which possess similar structure with PMP, was used to label monosaccharides. A LC-MRM method based on novel non-isotopically paired (NIPL) method was established and validated for the simultaneous qualification and quantification of twelve monosaccharides by serving one-to-one corresponding internal standards in urine samples with high sensitivity and high accuracy. Together with multivariate analysis, the quantitative results were selected as discriminating variables. Subsequently, correlation networks has been utilized to determine the most reliable potential biomarkers. Three monosaccharide biomarkers have been identified including GalNAc, Galactose and GalNAc. The combination of NIPL strategy and statistical analysis might provide more clues on the exploration and elucidation of pathogenesis of bladder cancer.

Credit author statement

Ya-Mei Peng: Methodology, Software, Investigation, Data Processing, Theoretical analysis, Writing - original draft. **Jian-Zhang Pan**: Conceptualization, Supervision, Writing modification, Project administration, Funding acquisition. **Qun Fang**: Conceptualization, Supervision, Writing modification, review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122336.

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