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Abstract

N-linked glycosylation is a process mediated by glycosyltransferases to transfer sugars from glycosyl donors to proteins or lipids. Currently, biopharmaceutical products widely produced by culturing mammalian cells such as Chinese hamster ovary (CHO) cells are prevalently glycosylated, and for some biologics the N-linked glycan can be a critical quality attribute of the drugs. The impacts of cell culture on the glycan precursors - nucleotide sugars - are important for understanding intracellular glycosylation process. Robust separation of some nucleotide sugar isomers such as UDP-glucose and UDP-galactose remain a challenge in current analytical methods because of their structural similarity. Based on ion-pair reverse phase (IP-RP) chromatography, a strategy was developed in this study to resolve the separation of major nucleotide sugars including challenging isomers. The strategy applies core-shell columns and connects multiple columns in tandem to obtain sufficient theoretical plates for extending separation power and ultimately improve the resolution for the nucleotide sugars detected from cell extracts. The key parameters in the IP-RP method, including temperature, mobile phase and flow rates, have been systematically evaluated in this work and theoretical mechanisms of the chromatographic behavior was proposed.

Keywords	Nucleotide sugars; UDP-sugars; CHO; ion-pair reverse chromatography; core-shell; columns in tandem
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Highlights:

1. Separation of nucleotide sugar isomers.
2. An approach of using core-shell columns and multiple columns in tandem
3. Identification of key parameters affecting ion-pair reverse phase chromatography
4. A mechanism of the ion-pair reverse chromatography

A High Resolution Measurement of Nucleotide Sugars by Using Ion-Pair Reverse Chromatography and Tandem Columns

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Abstract

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Key words:

Nucleotide sugars, UDP-sugars, CHO, ion-pair reverse chromatography, core-shell, columns in tandem

1. Introduction

N-linked glycosylation is a cellular process with a function to transfer sugars from glycosyl donors to proteins during protein synthesis [1, 2]. This process is mediated by glycosyltransferases and occurs prevalently in mammalian cells. Nowadays, Chinese hamster ovary (CHO) cells are widely used for producing biologics and can glycosylate most recombinant proteins produced by the cells. The N-linked glycan is a necessitated and critical quality attribute of many therapeutic proteins such as monoclonal antibodies (mAbs) because it can affect proteins from a wide range of drug characteristics including stability, efficacy and safety [3].

Nucleotide sugars are a group of glycosyl donors. The abundance of this group of metabolites is a factor in the progress of glycosylation, and necessary to be understood for the culture impacts on the N-linked glycan formation on the cell produced products [4-7]. However, there has only been a small number of mechanistic investigations of the glycosylation donor's effects [1, 4, 8]; and nucleotide sugar metabolism studies are still required to gain more insights for a better understanding of the culture impacts on the N-linked glycosylation.

The common nucleotide sugars metabolized by CHO cells include guanosine diphosphate mannose (GDP-Man), guanosine diphosphate fucose (GDP-Fuc), uridine diphosphate galactose (UDP-Gal), uridine diphosphate glucose (UDP-Glc), uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), uridine diphosphate-N-acetylgalactosamine (UDP-GalNAc) and cytidine monophosphate-sialic acid (CMP-SA). A common strategy to separate these nucleotide sugars are via chromatographic technology. There are several approaches used in literature, including ion-pair reverse phase chromatography [9, 10], anion-exchange [11], liquid chromatography-mass spectrometry (LC-MS) [4, 12-15] and capillary electrophoresis [16]. The similarity between some nucleotide sugar isomers, especially UDP-sugars is an intrinsic challenge. For instance, the pair of

UDP-hex (UDP-gal and UDP-glc) differ only by the orientation of a hydroxyl (-OH) group. The similarity is also present with UDP-hexNAc (UDP-GlcNAc and UDP-GalNAc). In a chromatographic method using UV as a measurement tool, additional challenges are given by the presence of non-nucleotide sugar compounds in cell extracts such as nucleotides and amino acids, which can co-elute and absorb at the same UV wavelength. Therefore, a highly selective chromatographic method is required to provide sufficient separation between nucleotide sugar isomers and the relatively less abundant nucleotide sugars from other compounds in cell extracts.

Ion-pair reverse phase (IP-RP) chromatography has been a successful method in literature and obtained many applications [9, 17]. The principle is based on a modification to the conventional reverse phase stationary phase by introducing an ion-pair reagent (commonly tetrabutylammonium bisulfate). The first application of IP-RP chromatography to nucleotide sugar analysis was made by Thomas et al. (1991) [17] and a number of studies have applied this method [18, 19]. However, complete data for nucleotide sugar isomers were not always available because the separation was inadequate at the initial methods. Nakajima's study (2010) revised the method with an adoption of a single high carbon-load column, which had shown a significant improvement to the separation among most nucleotide sugars [20]. However, the separation between UDP sugar isomers was still limited and can be subject to laboratories using different columns and systems.

Therefore, in this study, the technique of IP-RP was further developed to increase the separation for nucleotide sugars. The strategy applied was based on core-shell particle columns and connections of multiple columns in tandem, as a principle to gain more theoretical plates in the chromatographic separation. Major nucleotide sugars including UDP sugar isomers were separable by this method. Important parameters to the separation and reproducibility were identified, and a mechanism has been provided for the behavior observed in the IP-RP chromatography method.

2. Methods

2.1 Materials

The standards used in the study include tryptophan and 12 nucleotides: adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), adenosine diphosphate (ADP), cytidine diphosphate (CDP), guanosine diphosphate (GDP), uridine diphosphate (UDP), adenosine monophosphate (AMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), uridine monophosphate (UMP) and 8 nucleotide sugars including CMP-SA, UDP-Gal, UDP-Glc, GDP-Man, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc and GDP-Glucose (GDP-Glc), one that does not naturally exist in CHO cells and was used as an internal control. All these compounds were purchased from Sigma-Aldrich (St. Louis, MO). Each standard was prepared into stock solution and the aliquots were stored at -20 °C. Standard mixture was made from individual standard stock solution prior to use. Tetrabutylammonium bisulfate (HPLC grade), 1 M of both potassium phosphate dibasic solution and potassium phosphate monobasic solution, perchloric acid (PCA), potassium hydroxide and methanol (HPLC grade) were also purchased from Sigma-Aldrich (St. Louis, MO). The PCA was diluted to 0.5 M solution before use.

2.2 Cell culture and sampling

A vial of CHO-GS cells were thawed and seeded in Gibco™ FortiCHO medium (ThermoFisher Scientific, Waltham, MA). The cell culture was inoculated with 0.5 million/mL of viable cells in 125 mL shake flask with 30 ml working volume. Cell count was performed on Cedex HiRes Analyzer (Roche, Basel, Switzerland). Corresponding cell culture sample volume collection targeted around two to three million cells, and were prepared by centrifuging at 1000 rpm for 5 min. After discarding the supernatant, 1 mL of cold PBS was added to wash the pellets by re-

suspending the cell pellets. Subsequently, another centrifugation step was conducted at 1000 rpm for 5 min. The PBS was discarded and the pellets were quickly frozen in dry ice and stored at -80 °C until extraction.

2.3 Extraction of nucleotide sugars

After pellets were thawed, 200 µL of 0.5 M PCA was added to re-suspend the cell pellets. An aliquot of 0.5 µL of 20 mM GDP-Glc standard was spiked (The GDP-Glc is absent in natural extracts and thus spiked to cell extract as an internal control). The mixed solution was incubated on ice for 5 min and centrifuged at 2000 ×g for 3 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and kept on ice. Another 200 µL of 0.5 M PCA was then added to re-suspend cell pellets for a second time, followed by a spike of another 0.5 µL of 20 mM GDP-Glc standard. The mixture was incubated on ice for 2 min and centrifuged at 18000 ×g for 3 min at 4 °C. The supernatant was merged with the previous one. An aliquot of 56 µL of 2.5 M potassium hydroxide in 1.1 M dipotassium hydrogenphosphate was added and incubated on ice for 2 min to neutralize the solution. The sample was then centrifuged at 18000 ×g for 1 min to remove formation of potassium perchlorate precipitate. Thereafter, the supernatant was filtered by a 0.22 µm PVDF syringe filter into a clean Eppendorf tube. The sample was stored at 4 °C.

2.4 HPLC analysis

The separation and detection for nucleotide sugars were performed on an Agilent 1100 high performance liquid chromatography (HPLC) system paired with a diode array type of UV detector (Agilent Technologies, Santa Clara, CA). Buffer A was made of 0.1 M potassium phosphate and 8 mM tetrabutylammonium phosphate and was adjusted to pH 6.5. The buffer A was purified by 0.22 µm filter prior to use and stored at 4 °C. Buffer B contained 70% mobile A and 30% methanol. The HPLC column used was Kinetex® 2.6 µm 100 × 4.6 mm (Phenomenex, Torrance CA).

Columns were connected using short and narrow tubing. A Kinetex® C18 guard column was installed at the head of the first column. An optimized method using two columns in tandem was run at a flow rate of 0.6 mL/min with the following conditions: 0-16 min: 5% B (isocratic separation of major nucleotide sugars); 16-16.5 min: 100% B; 16.5-30 min: 100% B (removing late-eluting compounds); 30-30.5 min: 5% B; 30.5-45 min (re-conditioning): 5% B; the total run time: 45 min. The wavelength of UV detector was set at 260 nm. Temperature of the thermostat was controlled at 40 °C and the HPLC thermostat compartment was sealed to minimize heat dispersion. The UV spectrum was collected by the diode array detector. The backpressure during analytical runs remained under 400 bars. The injection volume was 5 μL if not otherwise indicated. After the analyses, the columns were washed with water to remove salt residues and stored in 30% methanol.

2.5 Peak annotation

The retention time for each nucleotide sugar was identified by running standards on HPLC. The retention time was used to annotate unknown peaks in the chromatographic trace of cell extract samples. The peak identities from cell extracts were further confirmed by the unique spectra of adenosine (A), uridine (U), cytidine (C), guanosine (G) compounds and tryptophan (since the tryptophan also absorbs at 260 nm). The spectra of these compounds are shown in **Fig. S1**, **supporting information**.

2.6 Calculations

The separation parameters calculated in the study include capacity factor (k'), theoretical plates (N), separation factor or selectivity (α) and resolution (Rs). The calculations were carried out by the following equations, where t_R represents retention time, t_0 represents the dead time of the column, $w_{0.5}$ represents peak width at half-peak height.

$$k' = \frac{t_R - t_0}{t_0} \quad \textit{(Eq. 1)}$$

$$N = 5.54 \left(\frac{t_R}{w_{0.5}}\right)^2 \quad \textit{(Eq. 2)}$$

$$\alpha = \frac{k_2'}{k_1'} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \quad \textit{(Eq. 3)}$$

$$R_s = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right) \quad \textit{(Eq. 4)}$$

3. Results

Fig.1 summarizes a workflow for developing the chromatographic method demonstrated in this work. A consequential flow was carried out to test different IR-RP parameters for the optimal analytical condition, and then a proof as concept was made by connecting columns in tandem to increase the separation power. Further modification was made to the method to achieve efficiency and reproducibility.

3.1 Parameters of IP-RP chromatography

To achieve optimal performance using IP-RP chromatography, the impacts of the analytical conditions were first evaluated using a single core-shell column; even though this could not resolve all the peaks, it provided a fast process to view the impacts of analytical conditions on chromatographic performance and to decide the optimal conditions for further testing using tandem core-shell columns to achieve better resolution and peak separations. The two buffer systems were adopted from pre-existing work: buffer A was 0.1 M potassium phosphate with 8 mM tetrabutylammonium phosphate, adjusted to pH 6.5 and buffer B was constituted by 70% mobile A and 30% methanol [9, 17, 19]. The standards of CMP-SA, UDP-Gal, UDP-Glc, GDP-Man, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc, GDP-Glc, CDP and tryptophan were mixed as a test sample. The CDP and tryptophan were added because of their presence noted in cell extracts,

absorptivity at 260 nm and close elution time with nucleotide sugars. The identification of the compounds in the result trace (shown in **Fig.2**) was assisted by UV spectra, as described in the Method section.

The first test examined the impacts of temperature on the separation outcome. The mixture of standards was separated respectively at the temperature of 30 ℃, 40 ℃ and 50 ℃, at isocratic buffer containing 5% buffer B and a flow rate of 0.4 mL/min. As shown in **Fig.2A**, the separation of mixture compounds was significantly affected by the temperature. As higher temperature shortened the retention time for all the compounds, the separation resolution was decreased significantly at 50 ℃. As a result, 40 ℃ was determined to be applied for obtaining reasonable separation and retention time.

In the next test, the standards were separated isocratically at different ratios between buffer B and A using a flow rate of 0.6 mL/min and 40 ℃; the different conditions tested contained 0%, 5%, 15%, 25% and 35% of buffer B respectively mixed with buffer A to constitute a 100% mobile phase. This gradient of ratios resulted in an increasing composition of methanol in the mobile phase. As shown in **Fig.2B**, a lower ratio of buffer B provided better separation of nucleotide sugars, in the meanwhile, a longer elution time.

As the retention for all the compounds decreased with the addition of buffer B, notably, the decrease was faster for the nucleotide sugars than the amino acid tryptophan. It was observed that the order of tryptophan (peak #8) and GDP-Glc (peak #9*) was switched as the percentage of buffer B in the mobile phase increased **(Fig. 2B)**: it was observed that tryptophan eluted earlier than GDP-Glc at lower percentage of buffer B; however, the peak of tryptophan shifted to the back of the GDP-Glc with the increased percentage of buffer B. The distance between these two compounds further expanded with the addition of buffer B. The phenomenon indicated a different

sensitivity between tryptophan and the nucleotide sugars responding to a change in the mobile phase composition, which will be later discussed as a mechanism of the IP-RP method.

3.2 Nucleotide sugar separation using multiple columns in tandem

From the above section, the optimal temperature was decided to be 40 °C and the mobile phase contain 95% A and 5% B. With these conditions, two or three columns were connected in tandem (respectively referred as 2X and 3X) to test the power for separating the same mixture of standards. As shown in **Fig.3A**, the separation was continuously improved with the number of columns connected. When 1X column was used, there was little space between UDP-GalNAc, UDP-GlcNAc and tryptophan (peak #6,7 and 8). When 2X columns was used, those compounds were completely separate. The 3X columns have further expanded the distance between these compounds.

For each of the tested conditions, the theoretical plates (*N*), capacity factor (*k'*), selectivity (α) and resolution (*Rs*) were calculated as per the equations (1) - (4) shown in the Method section. Resolution (*Rs*) is a result of the *N*, *k*' and α. As shown in **Fig.3B**, the number of theoretical plates (*N*) has increased with the number of columns connected, obtaining approximately 20000, 40000 and 60000 theoretical plates respectively at 1X, 2X and 3X columns. In the meantime, the two other chromatographic separation parameters, capacity factor (*k'*) and selectivity (α), remain unchanged. A higher resolution (*Rs*) was achieved as a result of the increase of theoretical plates.

The impact of the flow speed on the chromatographic separation was evaluated. The flow rates were chosen to keep the backpressure during the method to be under 400 bars. Using two columns connected (2X), an evaluation was made among the flow rates of 0.4, 0.6 and 0.8 mL/min. The results (**Fig.4**) showed that the separation resolution remained satisfactory from 0.4 to 0.6 mL/min but decreased at 0.8 mL/min.

3.3 Separation of nucleotide sugars in cell extracts

In this section, the cell extracts were tested in the analytical conditions obtained from the previous sections. The sample was an extract from 2.8 million CHO cells and spiked with GDP-Glc according to the Method section. A volume of 5 μL was injected to HPLC runs using 1X, 2X and 3X columns respectively. Considering that the backpressure increases as the number of columns increases, the tests used 0.6 mL/min for 2X columns as optimized above and used 0.8 and 0.4 mL/min respectively with 1X and 3X columns. The results are shown in **Fig.5**. According to the trace, CMP-SA, GDP-Man and GDP-Fuc were less abundant species compared to other nucleotide sugars. The separation was increasingly improved by the number of columns. Specifically, the peaks between UDP-GlcNAc, tryptophan and GDP-Glc (peak #6,7 and 8) were unable to be resolved within 1X column. The usage of 2X columns had successfully separated the peaks #6,7 and 8. The only unsolved nucleotide sugar was CMP-SA which was overlapped by an adjacent big peak (RT: 10 min). By further using 3X columns, the CMP-SA in cell extracts became also separated. Because of the extended flow path and the lowered flow rate limited by the backpressure, the elution time of nucleotide sugars was accordingly increased in the 1X, 2X and 3X columns.

3.4 Column variability

Since the method involves usage of multiple columns, the variability of single columns was tested. In the test, four columns from different manufacturing lots (including the ones used in the above studies) were tested individually using a cell extract sample. The results are shown in **Fig.S2**, **supporting information**. The overall separation pattern was comparable. However, the resolution for the closely eluting compounds had shown some variability (as shown in the red boxes on Fig. S2).

3.5 Post-isocratic separation

After the nucleotide sugars were isocratically separated, stickier compounds like triphosphates were still bound to the stationary phase and could present as residues to the next runs and therefore needed to be flushed out of the columns. To evaluate the retention duration of other compounds in the cell extracts, a test was carried out by running a cell extract sample using the isocratic condition along one column at a flow rate of 0.8 mL/min, until all the compounds were eluted **(Fig. 6A)**. Two late-eluting compounds were found to be abundant in the cell extract, identified to be ADP and ATP. ATP was the last peak and took 8.3 times longer than GDP-Glc to elute (Retention time: 55 min for ATP versus 6.6 min for GDP-Glc). A modification to the method was made by adding a stringent wash using 100% B after the main peaks of interest were eluted (**Fig.6B**). It was found that ATP was eluted within five column volumes of wash. After the rinse, another five column volumes of starting mobile phase condition was applied to equilibrate the columns, and runs following this loop were found to be free of carryover and the traces could be consistent from consecutive runs. The cycle was similarly adapted to 2X columns.

3.6 Linearity and Reproducibility

The linearity of the method was tested using 2X columns by injecting a series of volumes of a standard mixture. As shown in **Fig.7**, the chromatographic peak area and the nucleotide sugar amount (from 1 to 100 pmol) formed a linear relationship. The baseline noise from a blank run was under 0.1 mAu.

To test the reproducibility of the HPLC method, a same volume of mixture standard was injected for a total of 8 times across different days. The tests used 2X columns and were conducted at the optimal conditions from the study. The variation of the retention time and peak area of each compound is reported in **Table 1**.

Five aliquots of 2.8 million cells were extracted in parallel to examine the variation from cell extraction steps. At each extraction, the internal control of GDP-Glc was spiked. Each extracted sample was tested using the HPLC method with 2X columns. The peak area obtained for each compound is shown in **Table 2**. The peak area of each peak was normalized to the peak area of GDP-Glc.

4. Discussion

4.1 About using multiple columns in tandem

It has been demonstrated that enhancing separation and selectivity must be required for tackling the challenges of the similar isomers of nucleotide sugars and the additional interfering compounds present in cell extracts. An improvement to the chromatographic separation was focused by means of increasing the number of theoretical plates. The conventional approach by using one analytical column and a length within a range between 5 cm and 25 cm inevitably has a ceiling on how much the separation can be extended. Here, the strategy applies core-shell columns and connecting multiple columns in tandem as a mean to extend separation power. Using multiple columns could probably raise a concern of peak broadening; therefore, a sacrifice of resolution. However, this study shows that the increasing number of theoretical plates by connecting columns have resulted in a continuously improvement of resolution. Core-shell stationary phase technology has been reported to generate a better separation efficiency than porous packing columns [21]. A Kinetex® C18 column is specified with 264,600 plates/meter by the manufacture. Our work has empirically observed 400,000- 500,000 plates by connecting two columns, which were found to be needed to separate the majority of the nucleotide sugars in CHO cells, including UDP-sugar isomers. All the seven nucleotide sugars were completely resolved in the cell extracts by using three columns coupled.

One drawback in this strategy is that HPLC run time increases with the number of columns. Therefore, there is a tradeoff between separation resolution and the method run time. Increased flow rate can be potentially used to reduce the method run time; however, the speed could be limited by the pressure tolerance of a liquid chromatographic system, for which the highest backpressure occurs during the method when the columns are rinsed using 100% buffer B. The flow rates can impact the separation efficiency, depending on column packing, particle size and others. In this work, a reasonable flow rate was 0.6 mL/min when using two columns connected and required 45 min including re-equilibration time to complete a run. A better balance between the separation resolution and run time may be obtained by using an ultra-performance liquid chromatography (UPLC) system, while the best flow rate in the regime of UPLC needs to be evaluated.

As shown in our work, single columns would result in variations of separation resolution. Therefore, the robustness of enough separation can be subject to column variations from different manufacturing lots (or column usage condition) and different systems. In this work, such possible variability was less problematic because the separation power was improved by connecting multiple columns. It will thus be practical to apply this method across different laboratories and analytical systems to achieve satisfactory results regardless of the variations existing in single columns.

4.2 About the mechanisms of IP-RP

IP-RP chromatography is known to be a method mixed by different types of separation mechanism. Two theories for the binding between polar compounds and the stationary phase have been previously proposed: (1) the ion-pair reagent first binds with the analytes and then takes the analytes to the stationary phase via its own nonpolar end and (2) the ion-pair reagent is first coated

on the stationary phase and forms a charged surface where analytes are captured via the attraction between counter ions. In this work, the second mechanism was preferred for the observed chromatographic behavior. A theory is proposed in **Fig.8** to explain the chromatographic behavior throughout a method cycle, and accounts for the different retention time of molecules seen in **Fig. 2B** responding to different amount of buffer B used in the mobile phase.

At equilibration stage, a layer of ion-pair reagent (tetrabutylammonium) is formed near the stationary phase via the hydrophobic interaction with the C18 stationary phase. The new surface is both hydrophobic and charged by the presence of N+ cation provided by the tetrabutylammonium. When cell extracts are applied, molecules in the samples are attracted to the stationary phase via two different modes of interactions: counter-ion absorption and hydrophobic interaction. Nucleotide sugars are molecules with negative charge on phosphate(s), and the charge strength is dependent on the number of phosphates contained. Once entering the columns, nucleotide sugars are temporarily absorbed on the stationary phase via the attraction between negative charged phosphate anions and the N+ cation near the stationary surface. There is an increase in retention time with the compounds that have more phosphates. In contrast, the tryptophan is a neutral molecule and has no interaction with the charge near the stationary surface; however, tryptophan is a hydrophobic molecule and can be attracted to the stationary phase by the hydrophobic interaction. At the wash stage when mobile phase B is increased to 100%, the mobile phase becomes more hydrophobic; thus, part of the ion-pair reagents tetrabutylammonium along with highly absorbed phosphate ions are eluted from the stationary phase. By re-equilibration, the tetrabutylammonium is re-coated onto the surface of the stationary phase.

In the scenario shown in **Fig. 2B**, both nucleotide sugars and tryptophan have reduced retention time when higher composition of buffer B was used. However, the impacts caused by the mobile

phase were diverse. First, the fraction of ion-pair reagent coated on the stationary phase was reduced at higher presence of buffer B and the stationary phase became less charged. This had a major effect on the affinity to the nucleotide sugars. Second, the solvent had also become more hydrophobic which resulted in the competition with the stationary phase for tryptophan. Because of the distinct effects, the rates of retention change occurring with nucleotide sugars and tryptophan appeared to be different.

The study reveals that temperature and mobile phase composition are two critical parameters in the method and can significantly change the behavior of the chromatographic separation. The analytical condition must be equilibrated sufficiently with the starting condition (containing 95% A and 5% B) to maintain consistency across runs. Previous studies suggested a need of long-equilibration for IP-RP chromatography [22]. In this study, five column volumes were found sufficient for satisfactory consistency in consecutive runs. The temperature as another parameter of substantial influence on IP-RP chromatography should be tightly controlled for run to run consistency.

5. Conclusion

We have proposed and comprehensively investigated an approach of using core-shell columns and multiple columns in tandem to solve the shortage in the chromatographic separation for nucleotide sugars, especially UDP-sugar isomers. Key parameters affecting chromatographic results were identified and a workflow has been introduced to tune the method as needed for analysis. We anticipate that this method can help expedite the studies in biopharmaceutical process and other biological fields where the knowledge of the nucleotide sugar metabolism is to grow [23] .

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Conflict of interest

The authors claim no conflict of interest.

Reference

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Tables

Table 1: Multi-injection reproducibility (n=8) from different days

Compounds	RT			Peak area		
	Mean	SD	CV%	Mean	SD	CV%
UDP-Gal	10.990	0.795	7.237	98.263	5.786	5.888
UDP-Glc	11.754	0.666	5.669	228.888	13.197	5.766
GDP-Man	13.068	0.386	2.954	104.500	6.106	5.843
UDP-GalNAc	13.586	0.264	1.945	81.188	8.479	10.443
UDP-GlcNAc	14.197	0.211	1.487	14.840	2.805	18.904
GDP-Fuc	17.230	0.259	1.502	6.963	0.940	13.494
GDP-Glc *	15.437	0.197	1.278	168.313	10.530	6.256

Table2 Reproducibility of cell extractions (n=5)

Compounds	Peak area (Normalized to GDP-Glc*)		
	Average	SD	CV%
UDP-Gal	0.0216	0.0014	6.3622
UDP-Glc	0.0715	0.0003	0.3846
GDP-Man	0.0020	0.0003	13.5238
UDP-GalNAc	0.0327	0.0015	4.4742
UDP-GlcNAc	0.0773	0.0100	12.8691
GDP-Fuc	0.0041	0.0003	7.6218

* Peak area of compounds/peak area of GDP-Glc

Figure legends

Figure 1 Workflow of the method development for nucleotide sugar separation.

Figure 2 The impacts of analytical parameters on chromatographic separation. (A) Effects of temperature; (B) Effects of mobile phase composition. The IDs of compounds in the chromatograms are labeled numerically as followings: (1) CMP-Sialic acid; (2) CDP; (3) UDP-Gal; (4) UDP-Glc; (5) GDP-Man; (6) UDP-GalNAc; (7) UDP-GlcNAc; (8) Trp; (9) GDP-Glc* and (10) GDP-Fuc.

Figure 3 Separation of mixture standards from 1X, 2X and 3X columns. (A) Chromatograms generated using 1X, 2X and 3X columns. The IDs of compounds in the chromatograms are labeled numerically as followings: (1) CMP-Sialic acid; (2) CDP; (3) UDP-Gal; (4) UDP-Glc; (5) GDP-Man; (6) UDP-GalNAc; (7) UDP-GlcNAc; (8) Trp; (9) GDP-Glc* and (10) GDP-Fuc. (B) The calculation of theoretical plates (N), capacity factor (k'), selectivity (α) and resolution (Rs) using 1X, 2X and 3X columns.

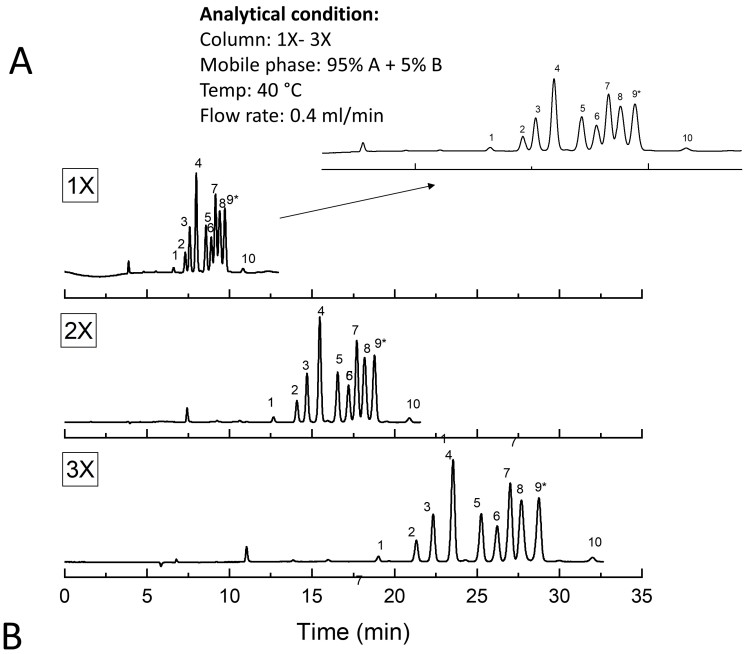
Figure 4 Tests of chromatographic separation using different flow rates. The IDs of compounds in the chromatograms are labeled numerically as followings: (1) CMP-Sialic acid; (2) CDP; (3) UDP-Gal; (4) UDP-Glc; (5) GDP-Man; (6) UDP-GalNAc; (7) UDP-GlcNAc; (8) Trp; (9) GDP-Glc*, (10) GDP-Fuc and (11) UDP.

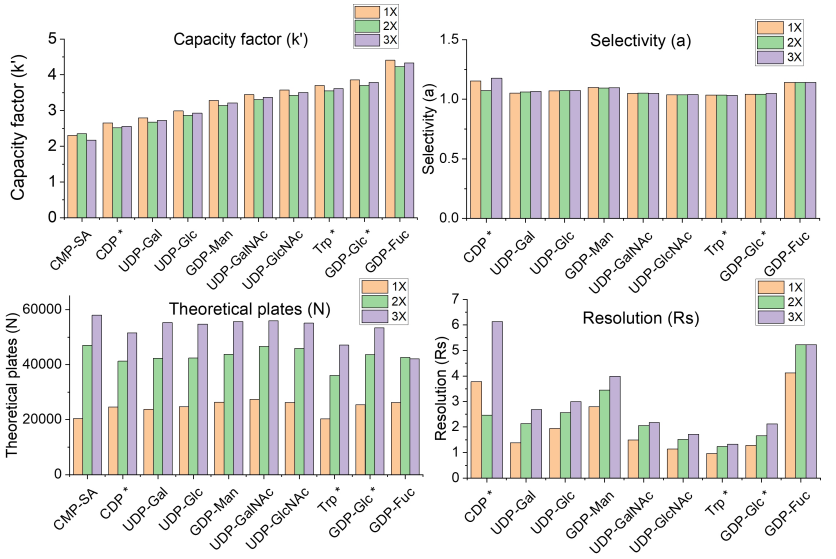
Figure 5 Separation of nucleotide sugars from cell extracts using 1X, 2X and 3X columns. The IDs of compounds in the chromatograms are labeled numerically as followings: (1) CMP-Sialic acid; (2) CDP; (3) UDP-Gal; (4) UDP-Glc; (5) GDP-Man; (6) UDP-GalNAc; (7) UDP-GlcNAc; (8) Trp; (9) GDP-Glc*; (10) GDP-Fuc; (11) UDP and (12) GMP.

Figure 4 A complete run including a wash step after isocratic separation. (A) a run with cell extract at an entirety of isocratic condition (5% B). (B) a run with isocratic separation using 5% B, followed by a wash using 100% B, and re-equilibration by 5% B.

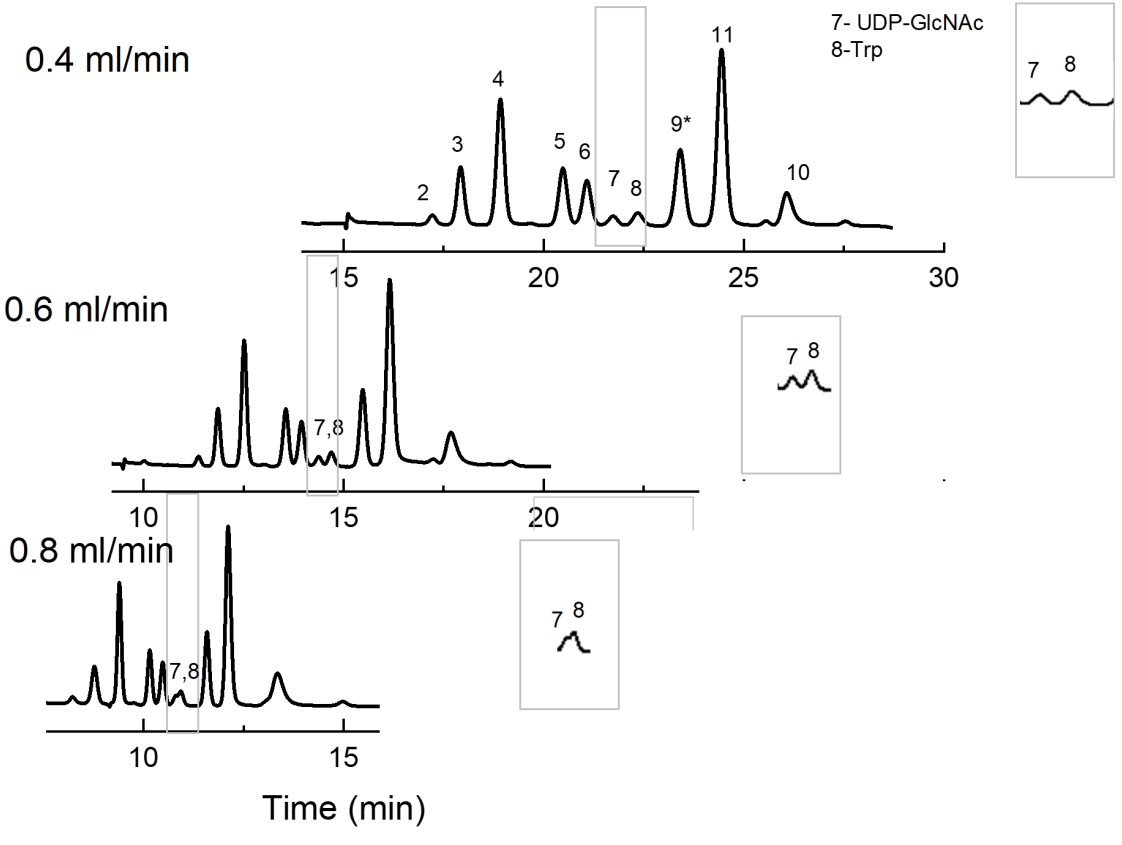
Figure 7 Method linearity tested with 2X columns and nucleotide sugar standards.

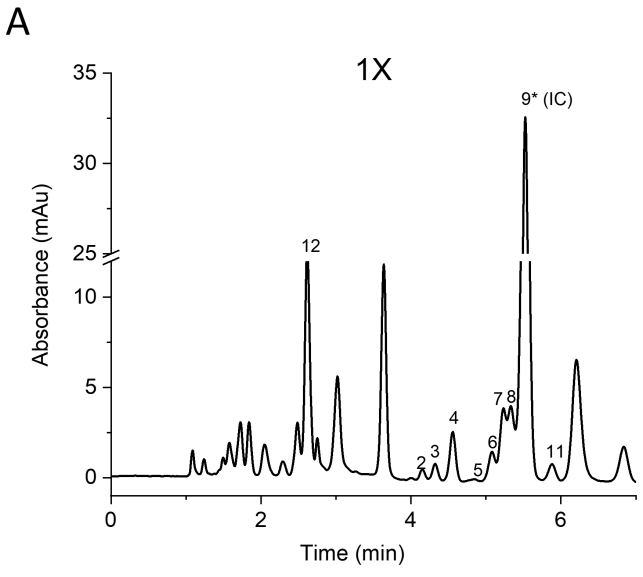
Figure 8 Hypothetic theory of the chromatographic behavior during a cycle of the method. (A) isocratic separation; (B) wash stage: (C) re-equilibration.

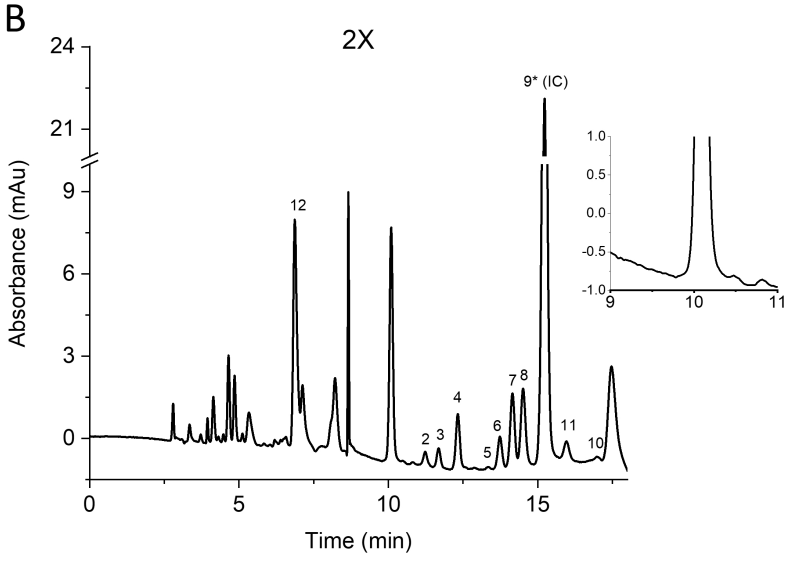

A
Analytical condition:
Column: 1X- 3X
Mobile phase: 95% A + 5% B
Temp: 40 °C
Flow rate: 0.4 ml/min
1X
2X
3X
Time (min)


B
Capacity factor (k')
Selectivity (a)
Theoretical plates (N)
Resolution (Rs)
1X
2X
3X
CMP-SA
CDP *
UDP-Gal
UDP-Glc
GDP-Man
UDP-GalNAc
UDP-GlcNAc
Trp *
GDP-Glc *
GDP-Fuc

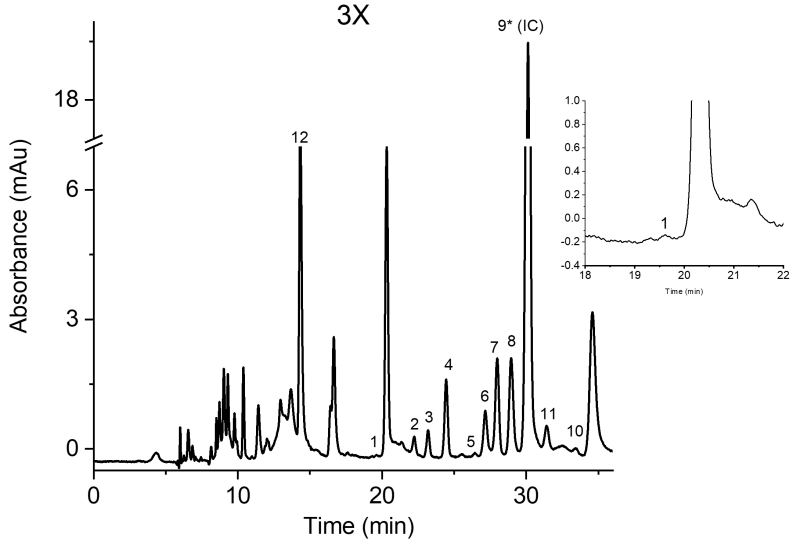
Analytical condition:
Columns: 2X
Mobile phase: 95% A + 5% B (isocratic)
Temp: 40 °C

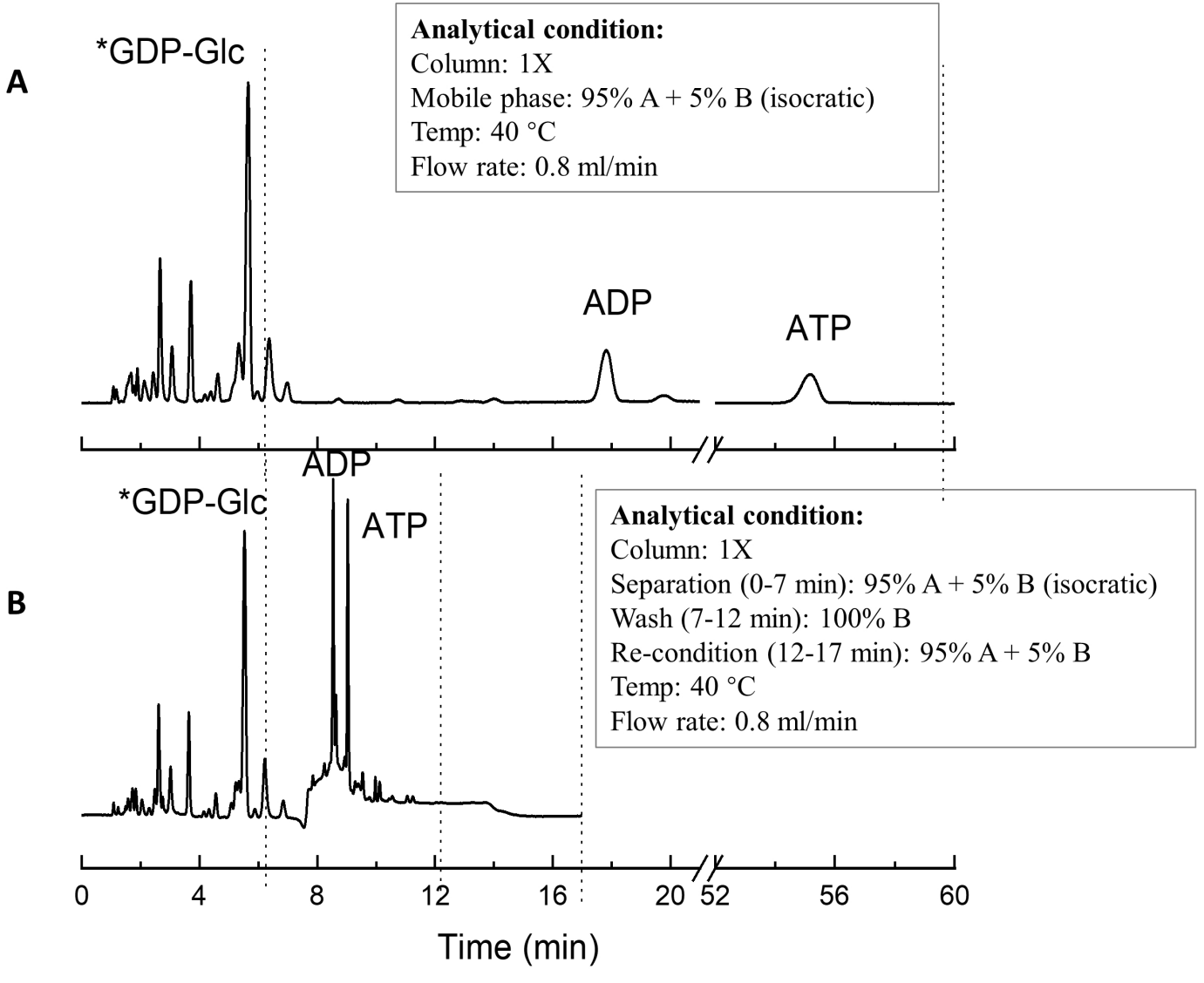


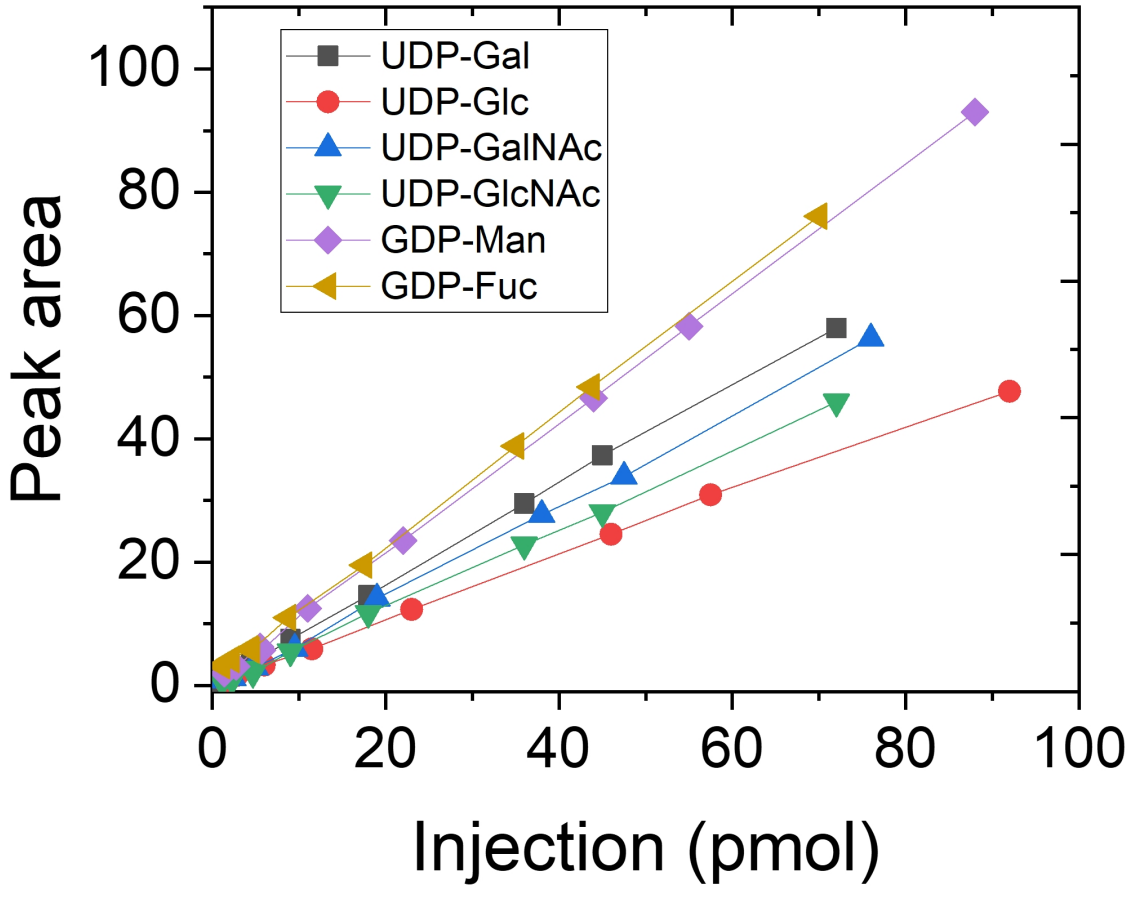


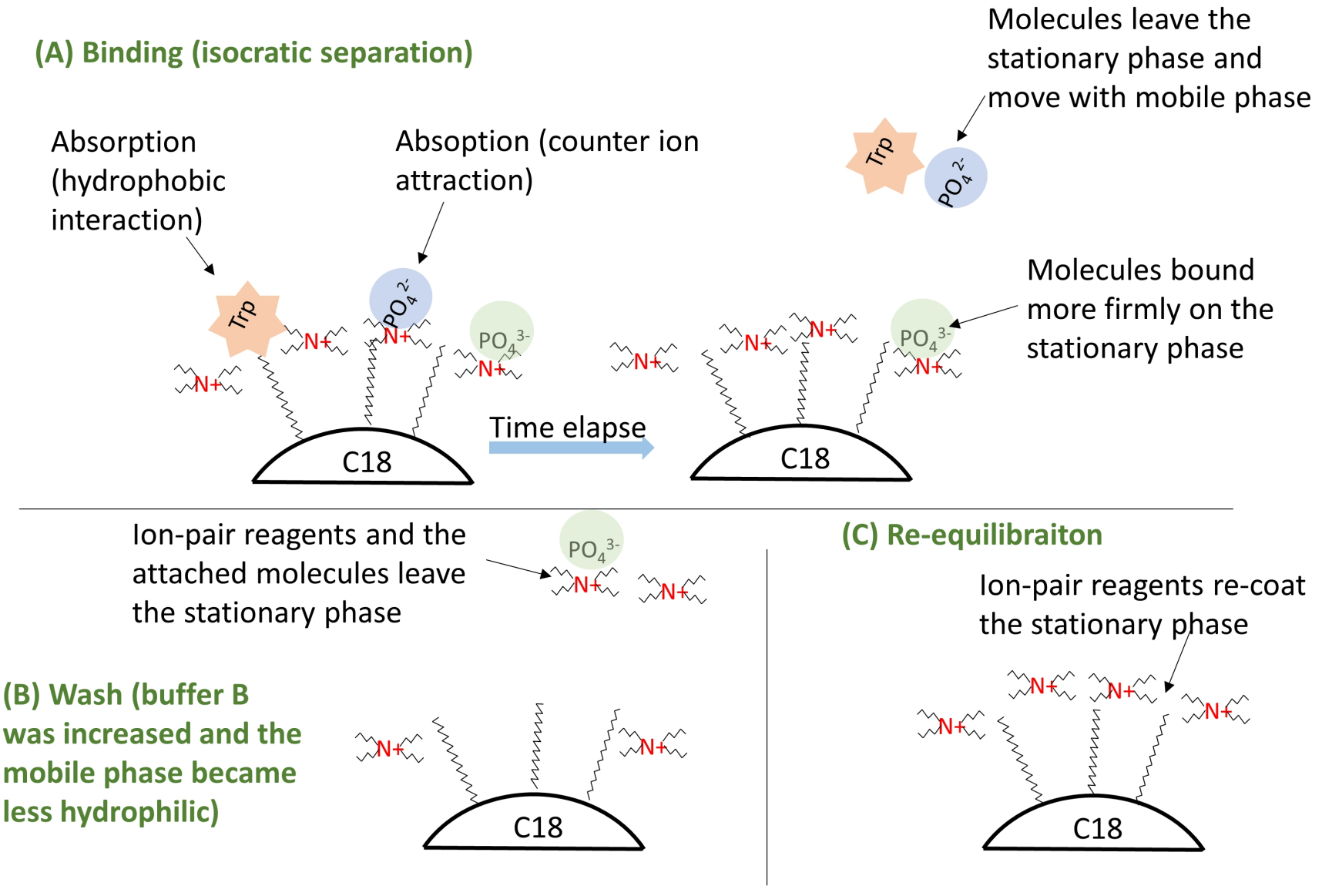


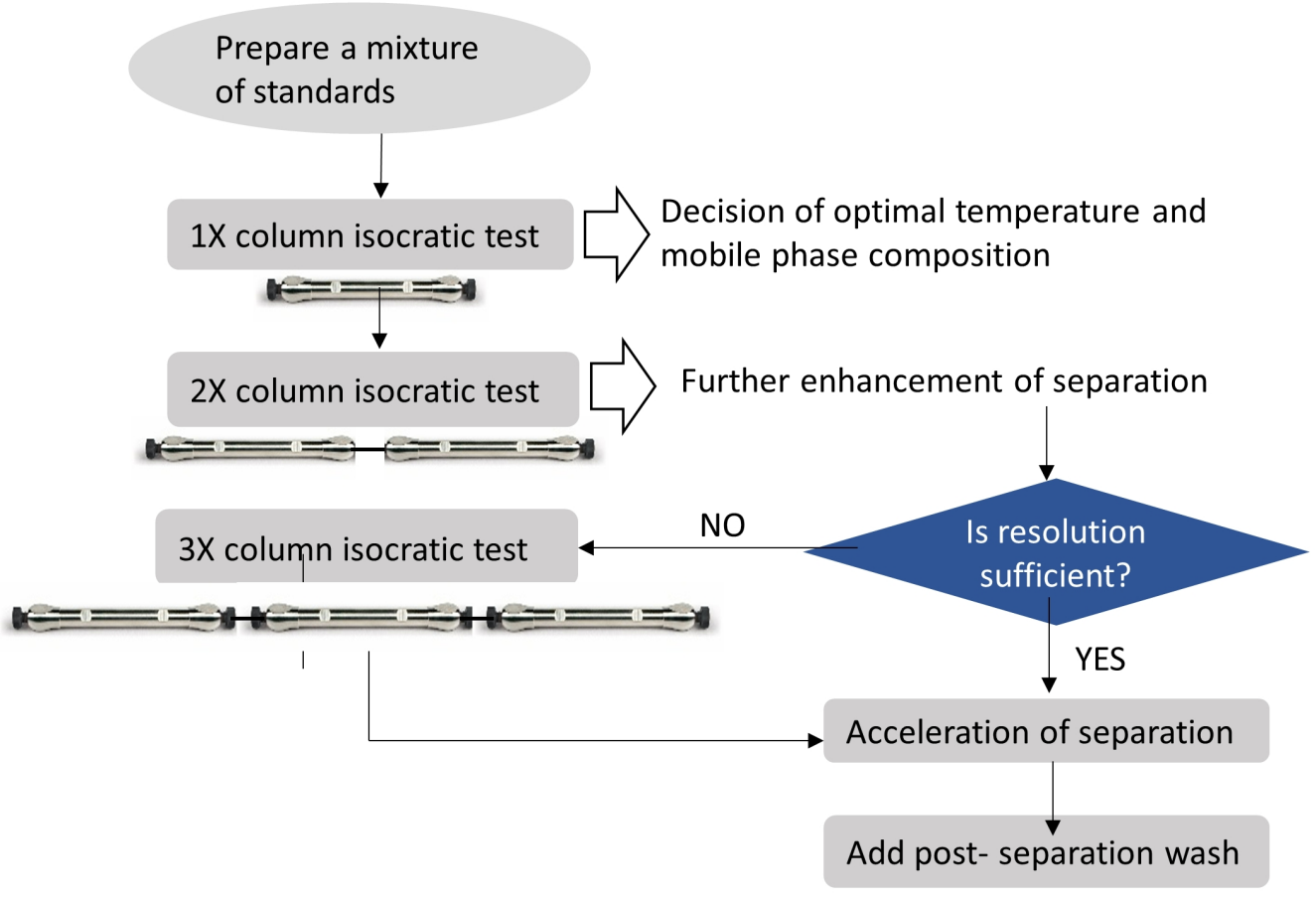
Analytical condition:
Column: 1X to 3X
Mobile phase: 95% A + 5% B
Temp: 40 °C
Flow rates:
0.8 ml/min (1X);
0.6 ml/min (2X);
0.4 ml/min (3X)




A
*GDP-Glc
ADP
ATP
Analytical condition:
Column: 1X
Mobile phase: 95% A + 5% B (isocratic)
Temp: 40 °C
Flow rate: 0.8 ml/min
B
ADP
*GDP-Glc
ATP
Analytical condition:
Column: 1X
Separation (0-7 min): 95% A + 5% B (isocratic)
Wash (7-12 min): 100% B
Re-condition (12-17 min): 95% A + 5% B
Temp: 40 °C
Flow rate: 0.8 ml/min
0
4
8
12
16
20
52
56
60
Time (min)

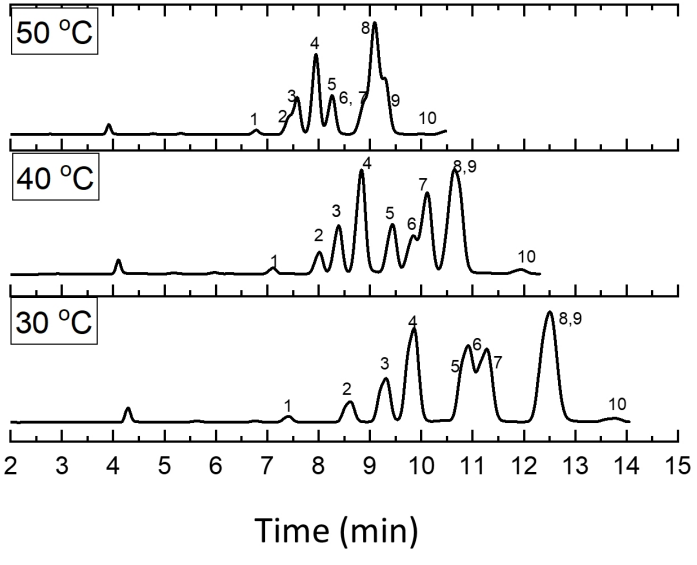

UDP-Gal
UDP-Glc
UDP-GalNAc
UDP-GlcNAc
GDP-Man
GDP-Fuc
Peak area
Injection (pmol)
0
20
40
60
80
100


(A) Binding (isocratic separation)
Absorption (hydrophobic interaction)
Absoption (counter ion attraction)
Molecules leave the stationary phase and move with mobile phase
Trp
PO_4^{2-}
PO_4^{3-}
N+
C18
Time elapse
Molecules bound more firmly on the stationary phase
Ion-pair reagents and the attached molecules leave the stationary phase
(B) Wash (buffer B was increased and the mobile phase became less hydrophilic)
(C) Re-equilibraiton
Ion-pair reagents re-coat the stationary phase


Prepare a mixture of standards
1X column isocratic test
Decision of optimal temperature and mobile phase composition
2X column isocratic test
Further enhancement of separation
Is resolution sufficient?
NO
3X column isocratic test
YES
Acceleration of separation
Add post- separation wash

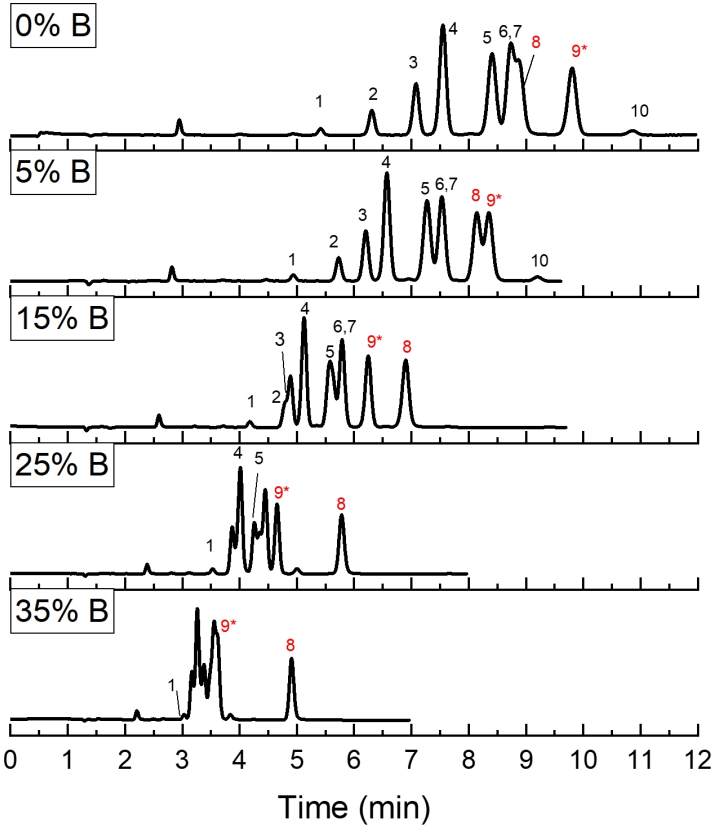
(A) Effects of temperature

Analytical condition:
Column: 1X
Mobile phase: 95% A + 5% B
Flow rate: 0.4 mL/min



(B) Effects of mobile phase composition

Analytical condition:
Column: 1X
Temperature: 40 °C
Flow rate: 0.6 mL/min



Supporting material

A High Resolution Measurement of Nucleotide Sugars by using Ion-Pair Reverse Chromatography and Tandem Columns

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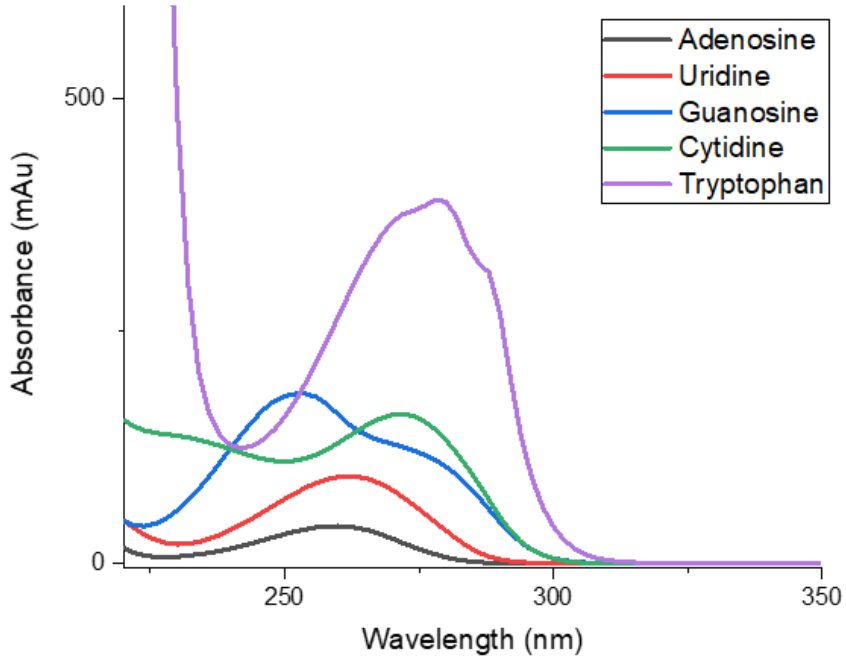

Fig.S1 Spectra of compounds containing adenosine, uridine, guanosine and cytidine groups and the spectrum of tryptophan.

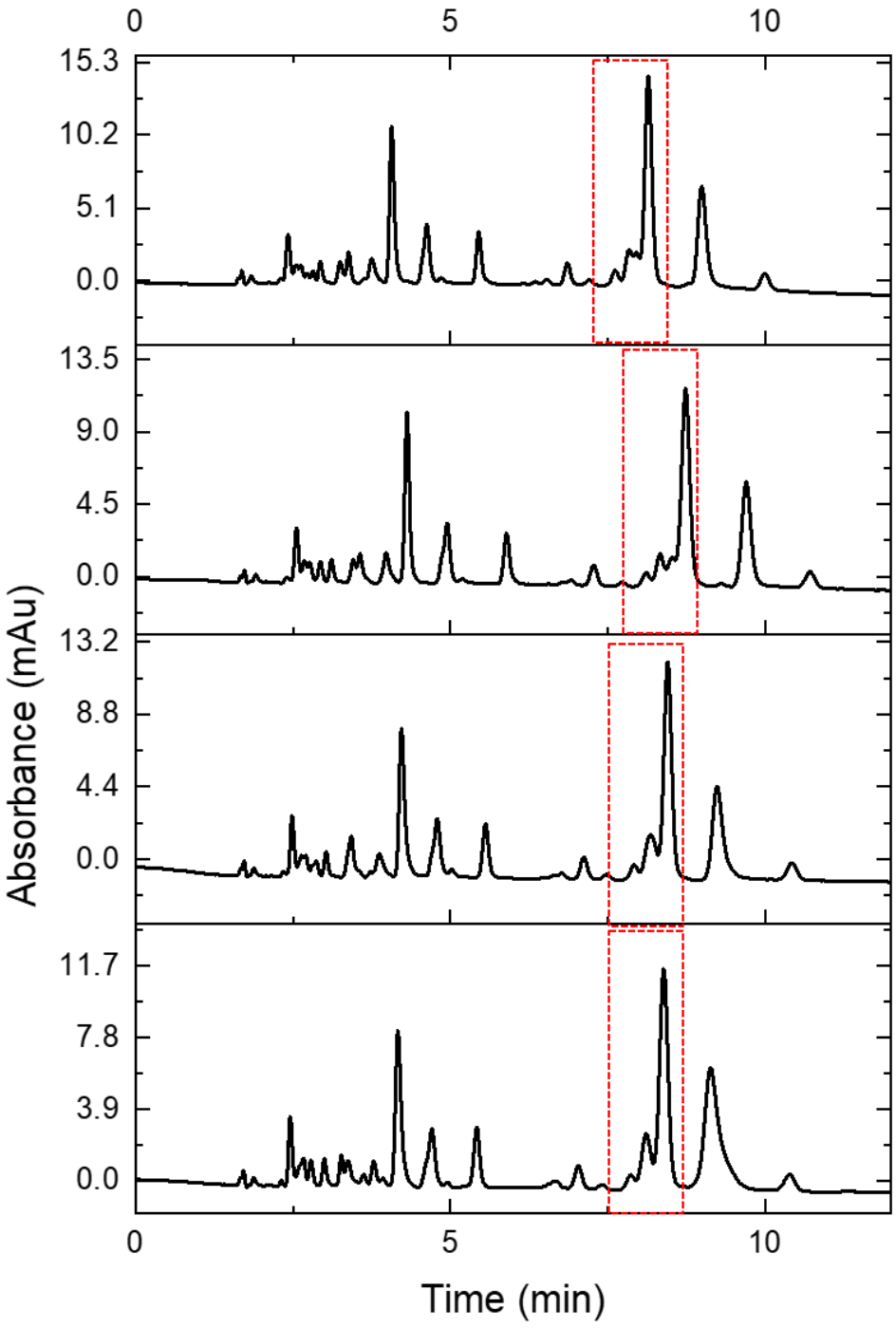

Fig.S2 Column variability shown by running a cell extract sample on single columns obtained from different manufacturing lots.