Title: An optimised method for extraction and quantification of nucleotides and nucleotide sugars from mammalian cells

Short title: Optimised extraction and quantification of nucleotide sugars

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Subject Category: Chromatographic Techniques

Abstract

Glycosylation is a critical attribute of therapeutic proteins given its impact on the clinical safety and efficacy of these molecules. The biochemical process of glycosylation is inextricably dependent on metabolism and ensuing availability of nucleotides and nucleotide sugars (NSs) during cell culture. Herein, we present a comprehensive methodology to extract and quantify these metabolites from cultured cells. In order to establish the full protocol, two methods for the extraction of these compounds were evaluated for efficiency, and the requirement for quenching and washing the sample was assessed. A chromatographic method based on anion exchange has been optimised to separate and quantify eight nucleotides and nine NSs in less than 30 minutes. Degradation of nucleotides and NSs under extraction conditions was evaluated to aid in selection of the most efficient extraction protocol. We conclude that the optimised chromatographic method is quick, robust and sensitive for quantifying nucleotides and NSs. Furthermore, our results show that samples taken from cell culture should be treated with 50% v/v acetonitrile and do not require quenching or washing for reliable extraction of nucleotides and NSs. This comprehensive protocol should prove useful in determining the impact of nucleotide and NS metabolism on protein glycosylation.

Keywords: HPLC; Nucleotides; Nucleotide sugars; Protein glycosylation; CHO cells; metabolite extraction

Introduction

Approximately 60% of all currently manufactured therapeutic proteins are glycosylated [1]. Given the complexity and importance of this post-translational modification for biological activity, these glycoproteins are produced by culturing mammalian cells in order to ensure efficacy and safety. Several reports indicate that the presence and composition of the carbohydrates bound to glycoproteins greatly influence their therapeutic function by determining their serum half-life [2; 3], eliciting undesired immune response in patients [4] or by modulating the mechanisms by which they act *in vivo* [5; 6]. In addition, the influence of cell culture conditions has been shown to determine the extent of glycosylation as well as the monosaccharide composition of the carbohydrates bound to glycoproteins [7; 8; 9].

The two fundamental types of glycosylation are defined by the amino acid residue onto which carbohydrates are bound. In O-linked glycosylation, carbohydrates are bound to serine, threonine and tyrosine residues of proteins, whereas N-linked glycosylation refers to carbohydrates that are bound to the asparagine in the amino acid sequon Asn-X-Ser or Asn-X-Thr, where X is any amino acid except proline or asparagine. Despite differences during initiation of the process, both O- and N-linked glycosylation require the addition of monosaccharides to the protein-bound carbohydrate [10]. The addition of each monosaccharide is catalysed by a glycosyl-transferase enzyme which requires nucleotide sugars as co-substrates for the reaction. The nucleotide sugars most commonly involved in mammalian glycosylation are: uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), uridine diphosphate N-acetylgalactosamine (UDP-GalNAc), uridine diphosphate glucose (UDP-Glc), uridine diphosphate galactose (UDP-Gal), guanosine diphosphate mannose (GDP-Man), guanosine diphosphate fucose (GDP-Fuc), cytosine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac), uridine diphosphate glucoronic acid (UDP-GlcA) and cytosine monophosphate N-acetylglucoronic acid (CMP-Neu5Gc). Additional metabolites, such as ribonucleotide

triphosphates, are key metabolites for the biosynthesis of nucleotide sugars. These molecules are also prevalent determinants of nucleic acid synthesis, cellular growth and energy metabolism. The most common nucleotide triphosphates in living organisms are adenosine triphosphate (ATP), cytosine triphosphate (CTP), guanosine triphosphate (GTP) and uridine triphosphate (UTP). Other nucleotides which are closely involved in energy metabolism, nucleotide sugar biosynthesis and protein glycosylation are the mono and diphosphate versions of the nucleotides mentioned above: adenosine diphosphate (ADP), adenosine monophosphate (AMP), cytosine diphosphate (CDP), cytosine monophosphate (CMP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), uridine monophosphate (UMP) and uridine diphosphate (UDP).

Previous studies have found that the availability of nucleotide sugar donors determines the presence or absence of complex carbohydrates on the peptide backbone of the glycoprotein (macroheterogeneity) [11; 12] and also the monosaccharide composition of the carbohydrate bound to the protein (microheterogeneity) [13; 14; 15; 16]. Because of these effects, several attempts have been made to extract and quantify these compounds from cells in culture. Analytical techniques for nucleotide and nucleotide sugar analysis include high-performance anion exchange chromatography (HPAEC) [17; 18], reverse phase ion pairing high performance liquid chromatography (RP-IP-HPLC) [19; 20; 21; 22] and capillary electrophoresis (CE) [23; 24]. Despite the encouraging results from these reports, many of the analytical techniques have limitations regarding the length of analysis time [17; 18] (>50 minutes), detection of certain species [19; 20; 21] and, in some cases, are based on sophisticated detection or separation methods [22; 25], or require laborious preparation of the mobile phase(s) [21; 24].

In addition to the analytical method, recent reports suggest that cell culture sample treatment prior to extraction (washing and quenching) as well as the metabolite extraction protocol may greatly influence the measurement of intracellular metabolite pools [25; 26; 27; 28]. The objective of these procedures is to ensure that i) the extraction method allows for reproducible

retrieval of the desired metabolites, ii) the metabolite pools to be extracted are representative of the metabolic state of the cells at the moment of extraction, and iii) components from the cell culture medium do not contaminate the intracellular samples.

This article describes an optimised method for nucleotide and nucleotide sugar extraction and quantification from mammalian cells. Extraction with acetonitrile (ACN) and perchloric acid (PCA) were compared in order to define the most suitable protocol for nucleotide and NS recovery. The need for quenching and washing of the cell culture sample was also evaluated in order to assess the impact of these processes on the observed intracellular nucleotide and NS pools. Finally, an HPAEC method for nucleotide and NS quantification, based on that of Tomiya et al. [18], was optimised to increase throughput and resolution power. The result was a method able to resolve nine nucleotide sugars and eight nucleotides in under 30 minutes (including re-equilibration of the column). We report the validity of the optimised analytical method by discussing its linearity, reproducibility and sensitivity. The overall platform allows for rapid, convenient and accurate measurement of intracellular nucleotide and NS pools in the context of evaluating the effect of cellular metabolism on recombinant protein glycosylation.

Materials and Methods

HPAEC analysis

The HPAEC analysis was performed on an Alliance HPLC system (Waters, UK), which is composed of a 2695 series separations module coupled to a 2998 model photodiode array detector and controlled by the Empower 2 software (Waters, UK). Separation was carried out with a CarboPac PA-1 column with a PA-1 guard column (Dionex, US). The mobile phases used as eluents for the chromatographic method were: 3mM NaOH (E1) and 1.5M sodium acetate in 3mM NaOH (E2). These eluents were prepared on the day of analysis and filtered using 0.2μm filter units (Nalgene, UK). The column temperature was maintained at 30°C throughout

separation. Elution of the sample was carried out using the following gradient: t_{0min}: 20% E2; t_{6min}: 66% E2; t_{18min}: 66% E2; t_{23min}: 20% E2; t_{30min}: 20% E2. Detection was performed at 262.1nm and 271.6nm simultaneously in order to ensure maximum absorbance of all species present in the mixtures. Analysis of the chromatographic data was performed with the Empower 2 software (Waters, UK), and all peak assignment and integration was performed automatically with a user-defined data processing method on the software. The nineteen compounds which were used as standards for method optimisation and calibration were ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc, UDP-GlcA, AMP, ADP, CMP, GMP, UMP and UDP, and were all purchased from Sigma-Aldrich, UK. Calibration of the HPAEC method was achieved by serially diluting a stock mixture (each analyte at 0.08mM) in deionized water (18.2MΩ).

Cell culture

Suspension Chinese hamster ovary (CHO-S) cells (Invitrogen, UK) were cultured as indicated by the manufacturer. The culture media employed was CD-CHO (Invitrogen, UK) supplemented with 8mM glutamine (Invitrogen, UK) and 10mL/L of 100× HT supplement (Invitrogen, UK). The cells were cultured in 250mL shake flasks (Corning, UK) with a working volume of 50mL at 8% v/v CO_2 and 37°C in a temperature and CO_2 controlled incubator (NUAIRE, UK). The cells were maintained in suspension by stirring at 125rpm on an orbital shaker (Stuart, UK). Cell count and viability were determined by the trypan blue dye exclusion method.

Extraction of intracellular nucleotides and nucleotide sugars

The procedure for perchloric acid extraction was based on previous reports [21; 27] and was conducted as follows: 1.5×10^7 cells were removed from the culture and centrifuged (1000g, 1min). After discarding the supernatant, the pellet was resuspended in 200μL of ice-cold 0.5M PCA (Sigma-Aldrich, UK). At this point, 2μL of 20mM GDP-Glc were added as internal standard.

This NS was selected as internal standard because is not produced in living organisms. The pellet/PCA mixture was incubated on ice for 5 minutes and then centrifuged (0°C, 18,000g, 5min) in a refrigerated centrifuge (Hermle, Germany). The supernatant was transferred to a new microcentrifuge tube containing 40μL of ice-cold 2.5M KOH (Sigma-Aldrich, UK) in 1.1M K_2HPO_4 (Sigma-Aldrich, UK). This mixture was incubated on ice for 5 minutes, and then centrifuged (0°C, 18,000g, 5min). The supernatant from this sample was removed and stored at -80°C until HPAEC analysis. Immediately prior to HPAEC analysis, the sample was quickly thawed at room temperature and filtered using 0.2μm syringe filter units (Fisher Scientific, UK).

Acetonitrile (ACN) extraction, based on [27; 29], consisted of adding 3mL of ice-cold 50% v/v aqueous acetonitrile (Sigma-Aldrich, UK) to 1.5×10^7 cells. The resulting suspension was incubated on ice for 10 minutes, after which it was centrifuged (0°C, 18,000g, 5min). The supernatant was collected and dried using a SpeedVac (Savant, US). The dried extract was resuspended in 240μL of deionized water and stored at -80°C until HPAEC analysis. The sample was thawed at room temperature and filtered using 0.2μm syringe filter units (Fisher Scientific, UK) immediately prior to HPAEC analysis.

Cell pellet quenching and washing

Where applicable, the cell culture sample was either quenched or both quenched and washed prior to metabolite extraction. Based on previous findings, where different quenching methods were compared for nucleotide and NS extraction [27; 28], ice-cold 0.9% w/v of aqueous NaCl (Sigma-Aldrich, UK) was selected as the quenching and washing solution. For quenching alone, one volume of cell culture sample was added to four volumes of quenching solution. The mixture was centrifuged (1000g, 1min) and then extracted using one of the methods described above.

For samples that were both quenched and washed, the cell pellet obtained from the quenching procedure described above was resuspended in a second volume of quenching solution (ice-

cold 0.9% w/v NaCl). This suspension was centrifuged once more (1000g, 1min) and treated with one of the extraction protocols described previously.

Assessment of sample degradation

To evaluate PCA-associated degradation, 2mL of a 13 standard mixture containing ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc and UDP-GlcA (each analyte at 0.04mM) were incubated with 2mL 0.5M PCA. 200μL samples of this mixture were taken at 5, 10, 15, 20 and 30 minutes; along with a final sample at 2h. Immediately after being drawn, each of these samples was neutralised with 20μL of ice-cold 2.5M KOH in 1.1M K_2HPO_4. The samples were centrifuged (0°C, 18,000g, 5min) and the supernatant stored at -80°C until HPAEC analysis.

To assess ACN-associated degradation, 100μL of the same standard mixture was incubated with 3mL of ice-cold 50% v/v aqueous acetonitrile for 10min. The samples were then dried using the SpeedVac as described in the previous section. The dried sample was resuspended in 220μL of deionised water and stored at -80°C until HPAEC analysis.

An additional set of samples was prepared to distinguish potential degradation due to exposure to ACN from thermal degradation associated with the drying step performed in the SpeedVac. For these samples, 100μL of the standard mixture were combined with 120μL of deionised water. The samples were dried using the SpeedVac, resuspended in 220μL of deionised water and stored at -80°C until HPAEC analysis.

Potential degradation during the analytical method was also evaluated. 60μL of the 13 standard mixture (each analyte at 0.04mM) were incubated in 180μL of a 50% v/v mixture of the HPAEC eluents for 40 minutes at room temperature. The samples were then filtered and injected for HPAEC analysis.

Metabolite recovery

Recovery of nucleotide triphosphates and NSs was assessed for the ACN extraction protocol by spiking cellular pellets with known amounts of each species during metabolite extraction. Specifically, 60μL of a 13 standard mixture (analyte concentrations between 0.0275mM and 0.65mM) were added to 1.5×10^7 cells immediately prior to addition of the 50% v/v ACN extraction solution. The resulting spiked sample was incubated on ice for 10 minutes and subsequently centrifuged, dried and stored for HPAEC analysis as described for the ACN extraction procedure above.

Statistical analysis

In order to evaluate the statistical significance among the differences between treatments, variances were compared using Levene's test. Where they were observed to be equal, a one-way ANOVA was performed to evaluate differences between the means of treatments. At $p_{ANOVA}<0.05$, the data was subjected to Tukey's honestly significant difference (HSD) test for pairwise comparisons (* for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$). Where variances were observed to be unequal, a one-way Welch's ANOVA test was conducted. Finally, for data with $p_{Welch's}<0.05$, the Games-Howell *post-hoc* test was performed to assess pairwise significant differences. The aforementioned analysis was performed using the R statistical software package [30].

Results and Discussion

HPAEC method development

The method reported by Tomiya et al. [18] was taken as the starting point for the development of the HPAEC protocol. Initially, the gradient and eluent composition yielded co-elution of UDP-GlcNAc with UDP-GalNAc and UDP-GlcA with GTP. However, based on data regarding the

relationship between NaOH concentration and elution time of each species reported by this group, the concentration of NaOH present in E1 and E2 was increased from 1mM to 3mM. This concentration of NaOH generated adequate separation of 4 nucleotides and 9 NSs in 55 minutes.

In order to increase the throughput of the method, we set out to reduce the analysis runtime. In anion exchange chromatography, analytes are adsorbed onto the stationary phase through coulombic interactions and a second anion (which in this case is CH_3COO^-) is then flushed through the system and, due to competing interactions, the analytes are eluted differentially from the stationary phase. By employing sharper elution gradients we found that the chromatograms contracted in time without compromising the resolution of seventeen analytes. In order to sharpen the gradients even further, the concentration of CH_3COONa in E2 was increased from 1M to 1.5M. The higher initial concentration of CH_3COONa in E2 allowed for shallower volumetric gradients of E2 while achieving higher CH_3COONa concentrations to elute the species bound to the column. It is worth noting that the concentration of CH_3COONa flowing through the column never exceeded 1M (corresponding to a volumetric flowrate of 67% E2) to comply with the maximum CH_3COONa concentration for CarboPac PA1 columns recommended by their manufacturer. Under these conditions, separation of four nucleotide triphosphates (ATP, CTP, GTP and UTP), nine NSs (CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc and UDP-GlcA) and four additional nucleotides (CMP, AMP, UMP, ADP) was achieved. With this method, GMP and UDP were found to coelute and prevented baseline resolution of UDP-Glc. This will be further discussed in the following section. A sample chromatogram showing separation of seventeen species in 20 minutes is presented in Figure 1.

Reproducibility, linearity and sensitivity

The optimised method described in preceding sections was assessed for reproducibility, sensitivity, and quantification linearity. As a means to establish reproducibility, the retention times for each analyte and its variation over 90 injections is presented in Table 1. For all species

except GDP-Glc, GTP and UDP-GlcA, the coefficient of variation (CV) is below 1%. The retention times for GDP-Glc, GTP and UDP-GlcA have a CV of 1.0%, 1.9% and 1.6% respectively. These CVs are low and were never observed to interfere with peak resolution or quantification. The 90 injections considered for Table 1 were performed over a six month period, with different batches of eluents and also include cellular extracts. The data suggests that, with respect to peak identification and separation, the chromatographic method, along with the preparation of the different eluents, is consistent and reproducible.

The range of linear quantification with the improved HPAEC method was determined by generating a series of calibration curves generated by injecting serial dilutions of the standard mixture and measuring the area below the corresponding peaks obtained after chromatographic separation. The maximum concentration of each standard was selected to encompass the range of intracellular values for each species previously reported in mammalian cells [18; 21; 31]. In order to populate the calibration curves further, different volumes (30μL, 25μL, 20μL, 15μL and 10μL) of each dilution were injected. All calibration curves were performed in triplicate to enable statistical assessment of the variation in measured concentration. The area under each peak was plotted against the known amount of each analyte added to the standard mixtures. Table 1 presents the values obtained for the slope, $\boldsymbol{m}$, along with the coefficient of determination, $\boldsymbol{R^2}$, which is a measure of how well the data fit the linear equation. The value of $\boldsymbol{R^2}$ is above 0.99 for all species, and these values confirm that for the range tested, quantification with the optimised method is linear and accurate. Samples of obtained calibration curves are presented in Figure 2A (nucleotides) and Figure 2B (NSs).

Tomiya et al. reported significant degradation of nucleotide sugars when exposed to NaOH concentrations above 4mM [18]. In order to determine whether the nucleotides and NSs degraded during the HPAEC procedure, a mixture of 13 standards (ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc and UDP-GlcA) devoid of nucleotide mono and diphosphates was incubated in a 50 % v/v mixture of both

eluents used for HPAEC analysis at room temperature for 40 minutes. These samples were subsequently subjected to HPAEC analysis and compared with control standard mixtures which had not been incubated in the eluent mixture. No differences were observed between the samples and controls, and, importantly, no degradation products (AMP, ADP, CMP and GMP/UDP) were observed. These results suggest that under the optimised HPAEC conditions no degradation of any of the analytes occurs.

Method sensitivity was assessed by determining the limit of detection (LoD) and limit of quantitation (LoQ) using the standard deviation of the response and slope as recommended in [32]. This method determines the lowest concentration that can be detected and measured accurately when the dispersion of the analytical measurements is considered. In this method, LoD is considered to be three times the residual standard deviation of the regression divided by the slope of the standard curve, whereas LoQ is defined as ten times this ratio. The results for LoD and LoQ are summarized in Table 1. There we see that these range from 2.2 to 12.9pmol for LoD and 7.3 to 42.9 pmol for LoQ. The highest LoD and LoQ correspond to UDP-Glc, whereas the lowest correspond to CMP-Neu5Ac. The LoQ for UDP-Glc is the highest likely because of its partial coelution with GMP/UDP. Despite this, the obtained LoQ value for UDP-Glc is approximately 10-fold lower than the intracellular concentrations of this NS measured for CHO cells in this work and nearly 15-fold lower than those reported previously [21]. This confirms that despite not having baseline resolution for UDP-Glc, this method can reliably quantify this NS in CHO cell culture. All other values for LoD and LoQ presented in Table 1 are considerably lower than the intracellular concentrations obtained in this work (all below 25%). These intracellular concentrations will be discussed further in subsequent sections. Finally, we must highlight that the optimised analytical method presented here is between 40 and 50% quicker than previously reported analytical methods. The shorter run time lowers the risk of analyte degradation during the chromatographic step and also increases throughput. This, in turn, could lead to more populated intracellular time-courses for nucleotides and NSs and aid in further understanding the metabolism of these compounds.

Extraction of intracellular nucleotides and nucleotide sugars

Dietmair and collaborators [27] recently published a comparison of 3 quenching methods and 12 extraction protocols for recovery of intracellular glucose, amino acids, nucleotides and two nucleotide sugars (UDP-Glc and UDP-GlcA). This group found that quenching with ice-cold 0.9% w/v NaCl led to 40% higher recovery of ATP and seven-fold lower leakage of this nucleotide to the supernatant when compared to quenching with 60% aqueous MeOH buffered with ammonium bicarbonate (AMBIC) at -40°C. Based on these results, ice-cold 0.9% w/v NaCl was selected as the quenching solution. To assess the effect of quenching on the intracellular pools of nucleotides and nucleotide sugars, CHO cells were not quenched (NQ), quenched (Q) or quenched and washed (QW) prior to metabolite extraction.

Dietmair *et al.* also explored different extraction protocols, which included acetonitrile (ACN), MeOH freeze, cold 50% MeOH, MeOH/chloroform, hot 80% MeOH, cold 100% MeOH, hot EtOH, hot EtOH/HEPES, cold EtOH, hot H_2O, potassium hydroxide (KOH) and perchloric acid (PCA). From the data reported by this group, seven protocols achieved high recovery of nucleotide and NS standards, with cold 50% v/v ACN extraction observed to be the most efficient [27]. Here, we compare ACN with PCA extraction given that these methods were previously only evaluated for two nucleotide sugars (UDP-Glc and UDP-GlcA) [27] and that PCA has been the most commonly used protocol for nucleotide and NS extraction [17; 18; 19; 21].

Figure 3 shows a comparison between the different extraction and quenching procedures where the data has been normalised to the highest amount of extracted metabolite for each treatment. The data reported is the average of six replicates (n=6) for each quenching/extraction protocol performed on two parallel flasks of CHO cells to ensure that only the impact of extraction and quenching was determined.

No statistical difference was observed for any of the quenching treatments (NQ, Q or QW) performed on PCA extracted samples. These results suggest that quenching and washing have

no impact on the observed intracellular pools of nucleotides and nucleotide sugars when extraction is performed with PCA. Since no statistical differences were observed among the quenching and washing results for PCA extraction, this data was averaged for comparison with the ACN extraction treatments. The validity of this finding can be confirmed by the relatively small error bars shown as "PCA Avg." in Figure 3.

In contrast to results obtained for PCA, the different quenching and washing treatments were observed to generate statistical differences for ACN extracted samples. For ATP, CTP, GTP, UTP, UDP-GalNAc, UDP-GlcNAc, GDP-Fuc and UDP-GlcA, higher amounts were recovered from non-quenched (NQ) samples. For UDP-Gal, UDP-Glc and GDP-Man, a mild or no statistical difference was observed between NQ, Q and QW treated samples. Finally, there is no species for which Q or QW yielded higher extraction. These results are consistent with previous work where quenching and washing has been reported as reducing metabolite recovery in CHO cells [26; 28]. However, it must be noted that quenching may play a crucial role in quantifying nucleotides and NSs in organisms with higher metabolic turnover (e.g. yeast and bacterial cell lines).

When comparing the different extraction protocols, Figure 3 shows that there is either no statistical difference, or that extraction yield is lower for PCA. The increase in metabolite recovery with ACN-NQ is particularly marked for UDP-GalNAc, UDP-GlcNAc, UDP-Glc, GDP-Fuc, GDP-Glc (internal standard) and UDP-GlcA, where ACN-NQ extracts (mean ± one standard deviation) 44%±8%, 34%±9%, 56%±3%, 73%±3%, 37%±4% and 62%±8% more, respectively. The single exception is CTP, which seems to be extracted more efficiently with PCA than ACN, although the confidence level for the difference is low ($p = 0.05$) and the increase in the amount extracted with PCA over ACN-NQ is only 16%±12%.

Figure 3 shows that considerably less internal standard (GDP-Glc) is recovered in the PCA-extracted samples. In order to further compare both extraction methods and account for this difference, the measured quantities of all species were normalised with respect to the amount of GDP-Glc recovered. When rearranging the data this way, all quenching treatments (NQ, Q and

QW) prior to PCA extraction yielded statistically similar results and NQ yielded statistically similar or higher recovery of all species when ACN was used for extraction. Although these results are consistent with those shown in Figure 3 (data normalised to the maximum amount extracted for each metabolite in every experiment), differences can be seen in relative recovery between the different extraction procedures. Table 2 shows that when normalising to internal standard recovery, PCA achieves a higher relative extraction of CTP, GTP and UTP with differences of (mean ± one standard deviation vs. ACN-NQ) 37.5%±7.2%, 12.5%±7.8% and 16.9%±8.6%, respectively. Conversely, higher relative extraction is achieved with ACN-NQ for all NS species, except UDP-Gal where no statistical difference is observed. The measured differences are (mean ± one standard deviation): 25.3%±6.1% for UDP-GalNAc, 12.3%±7.5% for UDP-GlcNAc, 41.0%±15.1% for UDP-Glc, 65.5%±6.2% for GDP-Fuc and 49.5%±4.8% for UDP-GlcA.

Overall, the data presented in Figure 3 and Table 2 suggests that ACN-NQ yields the highest total extraction of nucleotides and nucleotide sugars from CHO cells. By coupling these extraction processes with the HPAEC analytical method described above, the following intracellular concentrations of nucleotides and nucleotide sugars is obtained in units of *fmol/viable cell* ± one standard deviation: ATP (3.978±0.172), CTP (0.108±0.006), GTP (1.381±0.057), UTP (2.569±0.075), UDP-GalNAc (0.125±0.003), UDP-GlcNAc (0.286±0.004), UDP-Gal (0.252±0.002), UDP-Glc (0.620±0.013), GDP-Fuc (1.039±0.015), UDP-GlcA (0.433±0.003), AMP (0.447±0.053), ADP (1.312±0.036), CMP (0.105±0.017), UMP (0.181±0.012). Where they have been reported previously, the concentrations of metabolites we have measured are similar [21; 31]. In addition, we are able to quantify two additional nucleotide sugars (GDP-Fuc and UDP-GlcA). In the context of bioprocessing, robust quantification of GDP-Fuc is desired given the impact of Fc glycan core fucosylation on the therapeutic efficacy of mAbs [33]. Finally, it was not possible to determine CMP-Neu5Ac concentration in any of the intracellular samples, irrespective of the extraction method, due to the presence of a contaminating peak at t_{ret} = 3.80 min.

Sample degradation

A final element to consider regarding the extraction protocols examined in this work is the possible degradation of analytes during manipulation. In order to evaluate these effects for PCA extraction, a mixture of 13 standards (containing ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc and UDP-GlcA at 0.02mM each) was incubated with 0.5M PCA. Samples were drawn at 5, 10, 15, 20 and 30 minutes with a final sample taken at 2 hours. To evaluate degradation during ACN extraction, a sample of the same mixture was subjected to ACN extraction, including the drying step described in the Materials and Methods above.

None of the nucleotides were found to degrade significantly and only CMP-Neu5Ac and GDP-Fuc were observed to become depleted when incubated in presence of 0.5M PCA. Of these, CMP-Neu5Ac was shown to degrade the most (40.7%±1.7%) in 2 hours, followed by GDP-Fuc with 15.9%±2.4%. However, because incubation in PCA does not usually exceed 10 minutes, degradation at this incubation time is more relevant. The relevant values at 10 minutes are 6%±1% (CMP-Neu5Ac) and no significant degradation for GDP-Fuc. Although a small effect is observed for the 10 minute samples, the overall degradation results suggest that during PCA extraction, differences in incubation time may lead to undesired variation and must be avoided. Interestingly, GDP-Glc was not found to degrade when treated with PCA. This result, in combination with the lower recoveries observed for this NS from PCA-extracted cells, suggests that this component may precipitate or degrade only in the presence of cellular components and not solely with potassium perchlorate as proposed previously [21]. Overall, this data further confirms ACN as a more suitable extraction method for nucleotides and NSs from CHO cells.

As with PCA, nucleotides were not observed to degrade when subjected to ACN extraction and only two NSs, UDP-Gal and UDP-Glc, were found to significantly degrade when incubated with ACN. 5.2%±3.0% ($p=0.027$) of UDP-Gal and 4.1%±2.3% ($p=0.015$) of UDP-Glc were found to be

lost in this case, and although relatively modest, this degradation could contribute to undesired variability in measured intracellular pools of these NSs.

An additional sample was tested to evaluate whether degradation was due to the presence of ACN, or if it occurred thermally during the drying process. In this case, 220μL of the standard mixture was set to dry in the SpeedVac. In contrast to the ACN case, all nucleotides in these SpeedVac-only (SV) samples were found to degrade with respect to the control. The observed losses were 19.0%±5.7% for ATP, 17.0%±5.6% for CTP, 16.9%±5.6% for GTP and 17.3%±5.5% for UTP. In addition, depletion of three NSs was observed: 3.4%±1.0% (CMP-Neu5Ac), 7.7%±1.8% (UDP-Gal) and 4.8%±1.4% (UDP-Glc). When comparing these results with those reported for the ACN degradation samples described above, it seems likely that the degradation observed in both cases is of thermal origin and that ACN, if anything, contributes to lower degradation of nucleotides. Speculatively, this may be because the nucleotides remain in solution longer when dissolved in water (SV) than when dissolved in 50% ACN during the drying step, a conclusion further supported by the higher volatility of the H_2O/ACN mixture at the employed drying conditions.

Having observed that ACN exhibits higher net extraction of most nucleotides and NSs, a series of spiking experiments were performed to assess metabolite recovery from cellular samples with this procedure. This was done by adding 60μL of a mix of 13 standards (containing ATP, CTP, GTP, UTP, CMP-Neu5Ac, UDP-GalNAc, UDP-GlcNAc, UDP-Gal, UDP-Glc, GDP-Fuc, GDP-Man, GDP-Glc and UDP-GlcA at concentrations close to those observed intracellularly) to cell pellets prior to ACN extraction. In parallel, non-spiked samples were also subjected to ACN extraction to determine the intracellular concentration of nucleotides and NSs. After extraction and drying, the spiked and non-spiked samples were resuspended in 240μL of deionised water. These samples, along with the spiking mixture were subjected to HPAEC analysis. Table 3 shows a comparison between the observed intracellular concentration (IC), the amount of each species that was added in the spiking mixture (Added) and the measured concentration of each

metabolite in the spiked intracellular sample (Measured). Recovery was calculated as the measured amount in the spiked samples divided by the sum of intracellular concentration with the amount added with the spiking mixture. Five species were found to have statistically lower recoveries: GTP (91.0%±4.2%), UTP (82.9%±5.3%), CMP-Neu5Ac (83.2%±4.5%), UDP-Gal (90.7%±5.1%) and GDP-Glc (91.1%±2.9%). These results are consistent with the data for thermal degradation during drying presented above, and although modest, should be considered when performing this extraction protocol. Despite the slightly lower recovery of these species, ACN-NQ yields better extraction than any of the other methods examined in this work. Finally, because the lower recovery of these species is likely due to thermal degradation, it may be corrected by reducing the drying time by increasing the vacuum on the SpeedVac or by reducing sample temperature during drying (although this would likely increase drying time).

Finally, the losses observed through degradation associated with the ACN extraction protocol are minor compared to the lower extraction observed for PCA. Therefore, the optimal method for extraction of nucleotides and NSs would be ACN without the need for quenching or washing (ACN-NQ). A sample chromatogram obtained from CHO cell culture samples prepared with this optimised method is presented in Figure 4.

Conclusions

A robust and sensitive analytical method based on anion exchange chromatography has been optimised to separate and quantify eight nucleotides and nine NSs in 30 minutes. Two protocols have been evaluated for efficiency in extracting nucleotides and NSs from CHO cells. In addition, the need for quenching and washing cell pellets was also assessed. Finally, degradation associated with the extraction protocols was determined. The obtained results lead us to conclude that the most robust method for quantifying nucleotides and NSs from cell culture involves performing metabolite extraction using 50% v/v acetonitrile and that no quenching or

washing steps are required. Finally, the comprehensive extraction/quantification protocol concluded from the work presented herein is a robust methodology to reliably extract and quantify nucleotides and nucleotide sugars from cells in culture. Given the simplicity of the extraction protocol, along with the short analysis time, we believe this platform will prove useful in the assessment of metabolic effects on the glycosylation of therapeutic proteins.

Acknowledgments

We gratefully acknowledge the financial support of U.K. BBSRC and EPSRC and industrial members of the Bioprocessing Research Industry Club (BRIC). KMP and CK thank Research Councils U.K. for their Fellowships. CK thanks LONZA Biologics for their financial support. We also acknowledge Philip M. Jedrzejewski for his assistance with the HPLC.

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Tables

Table 1. Reproducibility, linearity and sensitivity parameters of the chromatographic method t_{ret} is retention time, *CV* is coefficient of variance, *m* is slope, R^2 is coefficient of determination, *LoD* is Limit of Detection and *LoQ* is Limit of Quantitation.

	Reproducibility			Linearity		Sensitivity	
Component	t_{ret} *(min.)*	t_{ret} *SD (min.)*	*CV (%)*	*m (mV sec/pmol)*	R^2	*LoD (pmol)*	*LoQ (pmol)*
ATP	9.224	0.043	0.5%	899	0.9999	4.1	13.8
CTP	7.044	0.016	0.2%	543	0.9999	6.5	21.8
GTP	14.496	0.269	1.9%	449	0.9999	10.1	33.7
UTP	9.697	0.068	0.7%	233	0.9999	6.2	20.7
ADP[a]	7.849	0.005	0.1%	783	0.9999	3.5	11.7
AMP[a]	5.370	0.004	0.1%	666	0.9999	3.5	11.6
CMP[a]	3.531	0.006	0.2%	670	0.9996	2.2	7.3
UDP/GMP[a]	8.591	0.005	0.1%	919	0.9999	6.7	22.2
UMP[a]	6.861	0.012	0.2%	415	0.9999	3.6	12.1
CMP-Neu5Ac	3.805	0.015	0.4%	644	0.9999	2.2	7.4
UDP-GalNAc	8.029	0.021	0.3%	359	0.9999	6.0	20.0
UDP-GlcNAc	8.224	0.022	0.3%	353	0.9999	9.1	30.2
UDP-Gal	8.420	0.025	0.3%	258	0.9999	8.9	29.8
UDP-Glc[a]	8.717	0.059	0.7%	339	0.9999	12.9	42.9
GDP-Fuc	12.304	0.104	0.8%	670	0.9996	7.0	23.3
GDP-Man	12.700	0.096	0.8%	543	0.9999	10.4	34.6
GDP-Glc	13.284	0.129	1.0%	644	0.9997	8.0	26.8
UDP-GlcA	16.315	0.263	1.6%	330	0.9997	9.5	31.6

[a] The parameters for these species were obtained from 30 injections, whereas the others were obtained from 90 injections as mentioned in the text.

Table 2. Comparison of quenching, washing and extraction procedures
The results were obtained from six replicate experiments (n = 6) and all data has been normalised to recovery of the internal standard (GDP-Glc).

Component	PCA[a] (Mean ± S.D.)	ACN (Mean ± S.D.)		
		Non-Quenched	Quenched	Quenched & Washed
ATP	95.7% ± 2.5%	85.9% ± 7.9%	70.8% ± 4.3%	77.8% ± 17.8%
CTP	97.1% ± 2.7%	59.7% ± 6.6%	44.0% ± 11.3%	44.4% ± 4.9%
GTP	97.2% ± 2.3%	84.8% ± 7.4%	69.6% ± 5.3%	76.6% ± 18.6%
UTP	97.2% ± 2.5%	80.2% ± 8.2%	65.9% ± 2.5%	74.9% ± 16.5%
UDP-GalNAc	73.2% ± 6.0%	98.5% ± 1.3%	73.3% ± 13.0%	69.8% ± 15.3%
UDP-GlcNAc	85.4% ± 7.2%	97.7% ± 2.1%	73.8% ± 14.3%	69.8% ± 14.9%
UDP-Gal	90.8% ± 10.9%	72.8% ± 20.8%	69.7% ± 21.0%	65.9% ± 20.4%
UDP-Glc	57.8% ± 15.0%	98.8% ± 1.8%	89.3% ± 7.4%	80.7% ± 3.6%
GDP-Fuc	33.0% ± 6.0%	98.5% ± 1.5%	48.3% ± 5.0%	35.2% ± 4.2%
UDP-GlcA	48.7% ± 3.8%	98.2% ± 2.8%	78.8% ± 7.4%	75.2% ± 12.5%

[a]All PCA treatments have been averaged because they were found to be similar ($p>0.05$) based on the statistical analysis performed as described in the Materials and Methods.

Table 3. Metabolite recovery with the optimised method

Component	*IC*[a] *(nmol)*	*Added*[b] *(nmol)*	*Expected*[c] *(nmol)*	*Measured*[d] *(nmol)*	*Recovery (%)*
ATP	2.62 ± 0.18	1.20 ± 0.01	3.82 ± 0.18	3.95 ± 0.24	103.3% ± 8.0%
CTP	0.80 ± 0.12	0.80 ± 0.00	1.59 ± 0.12	1.43 ± 0.08	89.6% ± 8.4%
GTP	1.02 ± 0.02	1.43 ± 0.01	2.45 ± 0.02	2.23 ± 0.10	91.0% ± 4.2%
UTP	1.36 ± 0.11	3.49 ± 0.01	4.84 ± 0.11	4.01 ± 0.24	82.9% ± 5.3%
CMP-Neu5Ac	0.05 ± 0.00	0.06 ± 0.00	0.12 ± 0.00	0.10 ± 0.01	83.2% ± 4.5%
UDP-GalNAc	0.94 ± 0.01	0.73 ± 0.00	1.66 ± 0.01	1.64 ± 0.09	98.5% ± 5.5%
UDP-GlcNAc	2.28 ± 0.03	2.77 ± 0.00	5.05 ± 0.03	4.99 ± 0.23	98.7% ± 4.6%
UDP-Gal	0.09 ± 0.01	0.20 ± 0.00	0.29 ± 0.01	0.26 ± 0.01	90.7% ± 5.1%
UDP-Glc	0.21 ± 0.02	0.50 ± 0.00	0.70 ± 0.02	0.66 ± 0.04	93.9% ± 6.0%
GDP-Fuc	0.81 ± 0.00	0.15 ± 0.00	0.96 ± 0.01	0.97 ± 0.04	101.3% ± 4.0%
GDP-Man	N.D.[e]	0.63 ± 0.01	0.63 ± 0.01	0.61 ± 0.02	97.5% ± 4.0%
GDP-Glc	N.D.[e]	0.65 ± 0.00	0.65 ± 0.00	0.59 ± 0.02	91.1% ± 2.9%
UDP-GlcA	0.24 ± 0.01	0.32 ± 0.00	0.60 ± 0.01	0.58 ± 0.03	97.6% ± 5.6%

Notes: All values are mean ± S.D. for n = 3

[a] Measured intracellular concentration.

[b] Amount of standard that was added to spike the intracellular sample.

[c] Sum of measured intracellular concentration and added standard.

[d] Measured concentration in spiked intracellular samples.

[e] Not Detected.

Figures

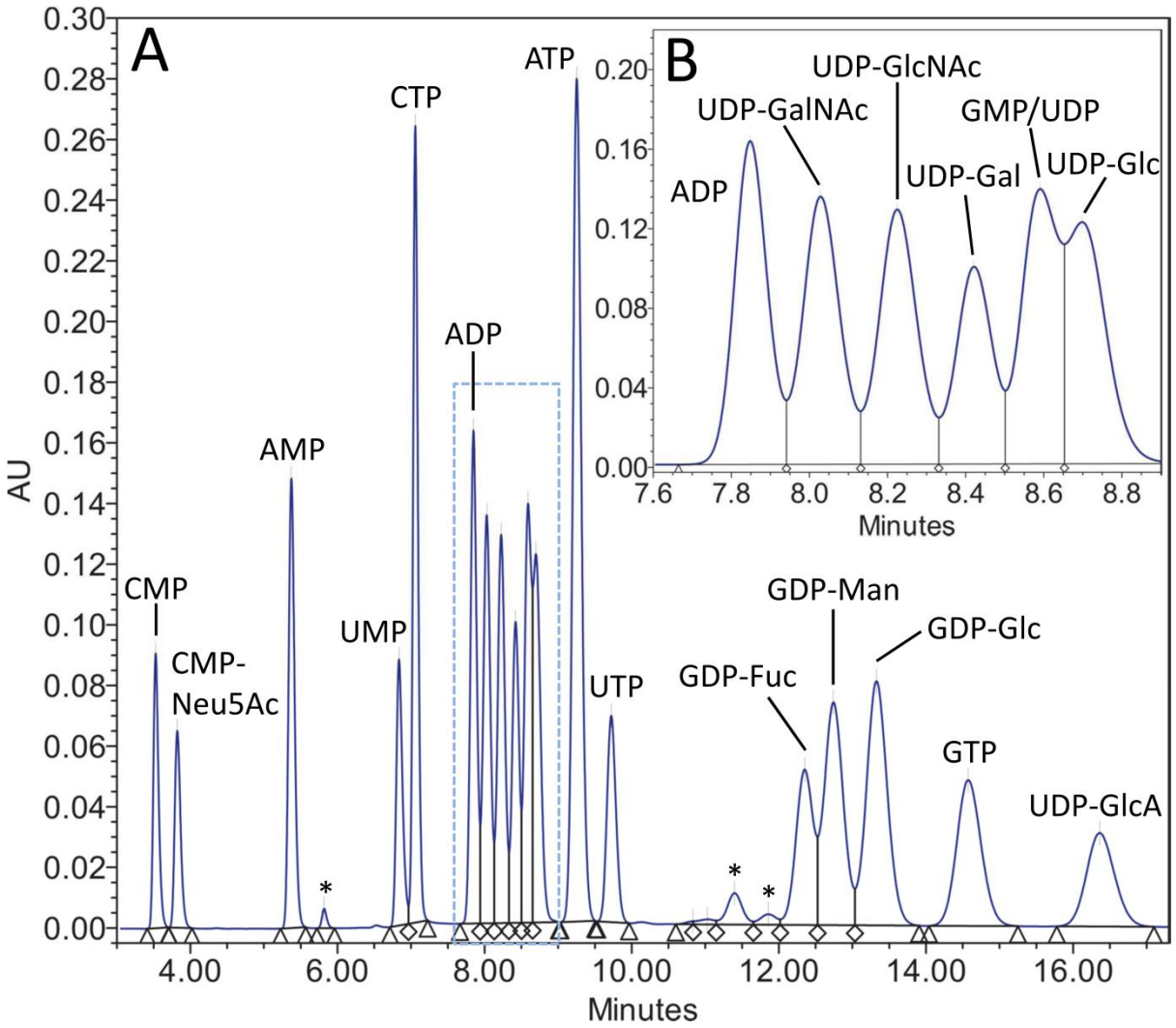

Figure 1. The chromatogram was produced by injecting 30μL of a mixture containing 9 nucleotides and 9 nucleotide sugars (between 0.04 and 0.08mM each) and shows separation of 17 species in under 20 minutes. Eluents used were 3mM NaOH (E1) and 3mM NaOH containing 1.5M sodium acetate (E2). Separation was performed at 30°C on a CarboPac PA-1 column, with the following elution profile: t_{0min}: 20% E2; t_{6min}: 66% E2; t_{18min}: 66% E2; t_{23min}: 20% E2; t_{30min}: 20% E2. Detection was done by absorbance at 262.1nm. (A) shows the full chromatogram and (B) shows the peaks that elute between 7.6 and 9 minutes (enclosed in the dashed box). Asterisks denote peaks that have not been assigned.

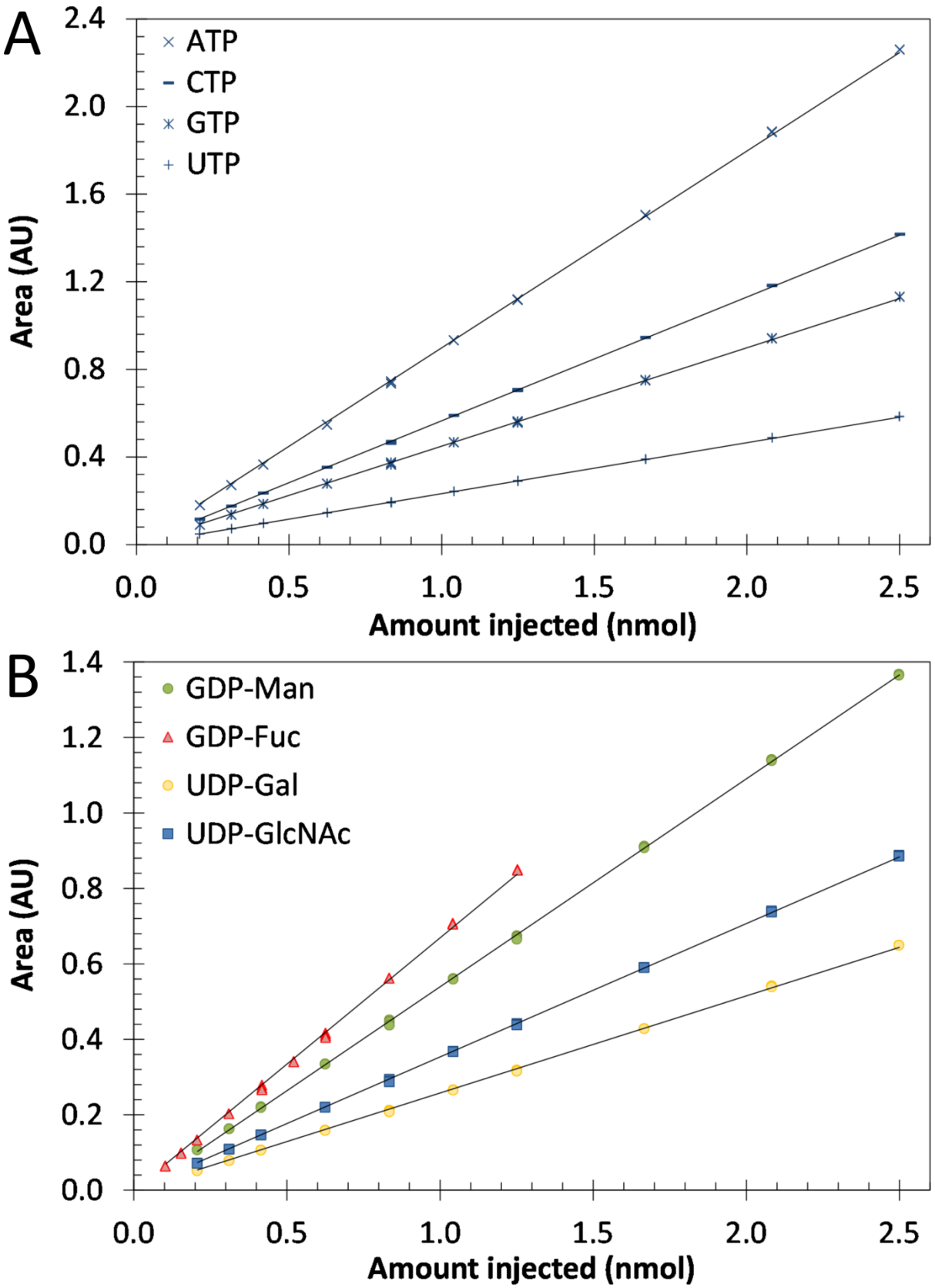

Figure 2. Calibration curves for 4 nucleotides (A) and 4 NSs (B)
The amounts injected range from 2.5 to 0.2nmol. A clear linear trend can be seen for all eight compounds presented. The slope and R^2 for the linear regression for each compound is presented in Table 1. (B) Only shows four nucleotides for the sake of clarity – the calibration curves of the remaining 5 NSs are similar to that of UDP-GlcNAc.

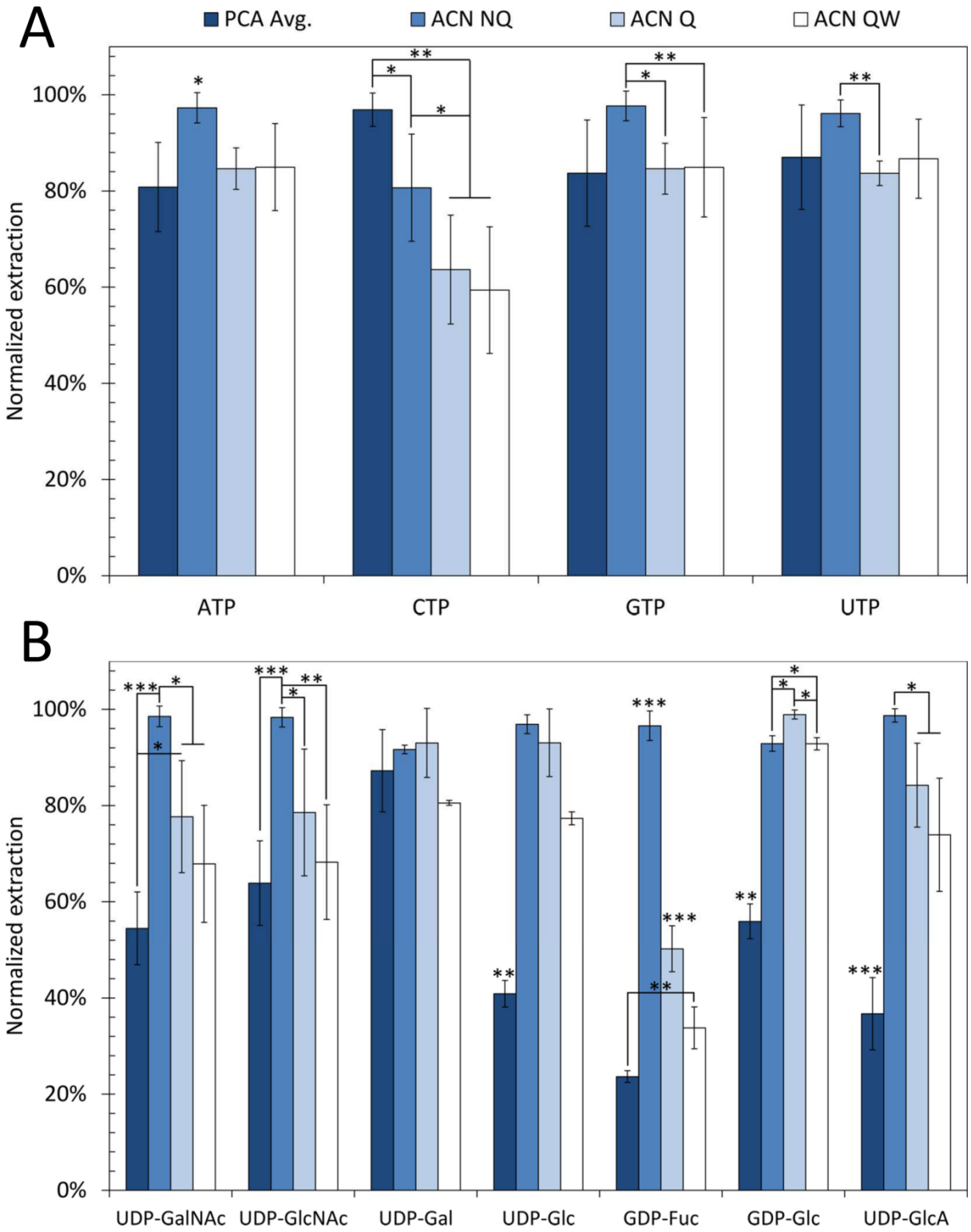

Figure 3. Comparison of quenching, washing and extraction procedures with data normalised to the maximum recovery of each species

Data for nucleotides (A) and NSs (B) is presented. Data for averaged PCA treatments (■), non-quenched acetonitrile (■), quenched acetonitrile (□) and quenched and washed acetonitrile (□) is shown. Data for all PCA treatments was averaged because they were found to be statistically similar. Six samples for each treatment were monitored (n=6), and the statistical procedure described in the Materials and Methods was applied to the data. A significant difference with a $p<0.001$ is represented by (***), $p<0.01$ with (**) and $p<0.05$ with (*).

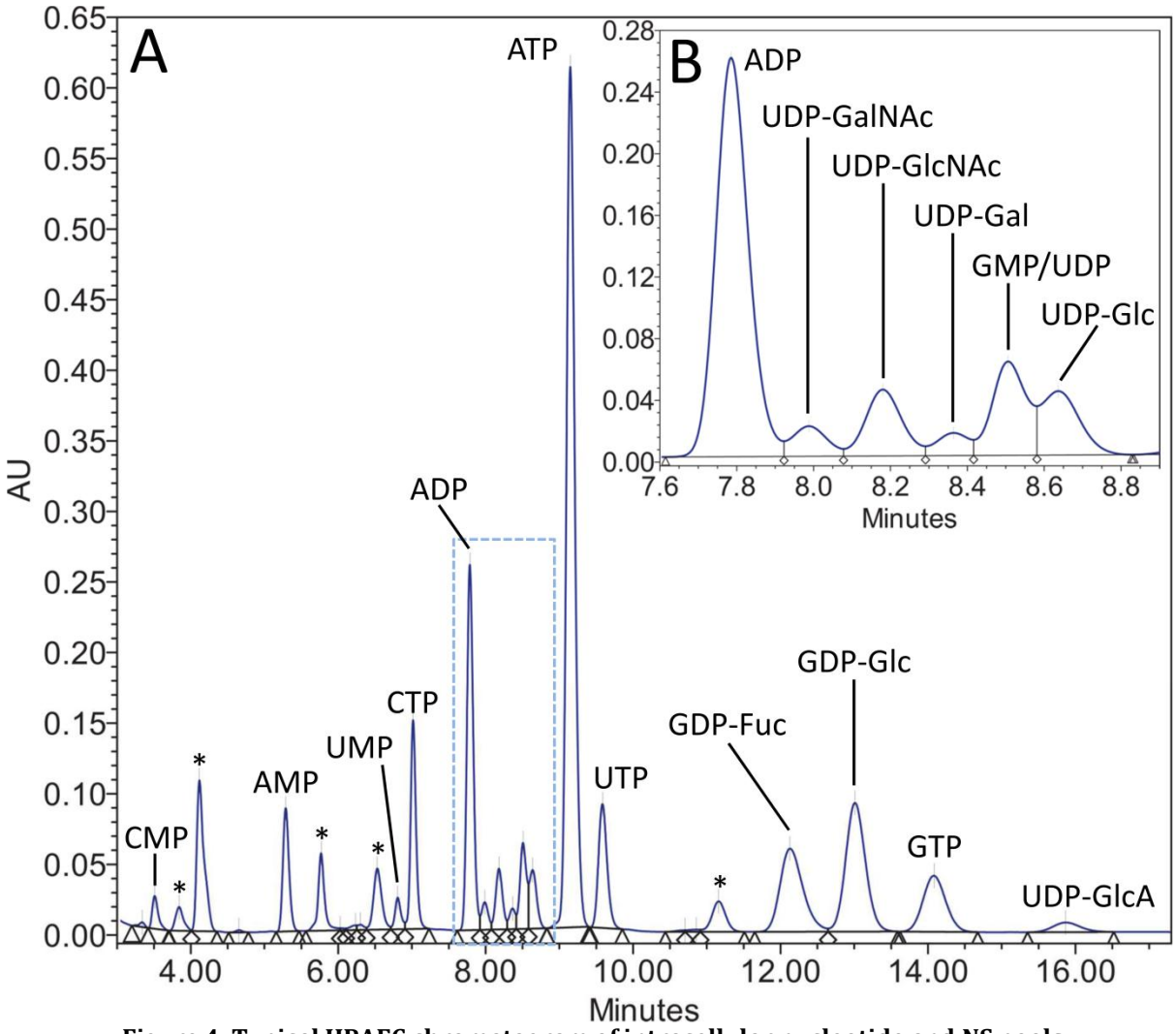

Figure 4. Typical HPAEC chromatogram of intracellular nucleotide and NS pools
The chromatogram shows adequate separation of eight nucleotides and seven NSs that have been obtained from CHO cells using non-quenched ACN extraction. The full chromatogram is shown as A, and a detail of the chromatogram between 7.6 and 9 minutes (marked by the dashed box) is shown to confirm adequate separation of the NSs that elute between those times (B). Peaks that were not assigned are marked with asterisks.