CHAPTER SEVEN

Profiling of Sugar Nucleotides

Martin Rejzek, Lionel Hill, Edward S. Hems, Sakonwan Kuhaudomlarp, Ben A. Wagstaff, Robert A. Field[1]

John Innes Centre, Norwich, United Kingdom
[1]Corresponding author: e-mail address: rob.field@jic.ac.uk

Contents

Abstract

Sugar nucleotides are essential building blocks for the glycobiology of all living organisms. Detailed information on the types of sugar nucleotides present in a particular cell and how they change as a function of metabolic, developmental, or disease status is vital. The extraction, identification, and quantification of sugar nucleotides in a given sample present formidable challenges. In this chapter, currently used techniques for sugar nucleotide extraction from cells, separation from complex biological matrices, and detection by optical and mass spectrometry methods are discussed.

Methods in Enzymology, Volume 597
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2017.06.005

1. INTRODUCTION

In all living systems, the biosynthesis of glycans and glycoconjugates relies upon the activation of monosaccharides as sugar nucleotides, which are used as donor substrates by glycosyltransferases (Freeze & Elbein, 2009). The availability of sugar nucleotides is expected to be involved in the regulation of cellular glycosylation events, with impact on biological functions and capabilities. Determining which sugar nucleotides are present in a given cell or tissue, when and in what quantity are therefore central questions in glycobiology.

Profiling sugar nucleotides is motivated both by fundamental biological questions, as well as the need to control glycosylation events, for example, in biopharmaceutical production or in crop production. In mammalian systems, there is potential impact of protein glycosylation on biological activity, stability, and immunogenicity (Moremen, Tiemeyer, & Nairn, 2012). In green plants, sugar nucleotide pools impact on starch (Zeeman, Kossmann, & Smith, 2010) and cell wall biosynthesis (Bar-Peled, Urbanowicz, & O'Neill, 2012; Kumar, Campbell, & Turner, 2016; Nguema-Ona et al., 2014; Rosti et al., 2007), and on the heterologous expression of pharmaceutical glycoproteins (Piron, Santens, De Paepe, Depicker, & Callewaert, 2015). The glycosylation potential of insect cells has been studied in relation to production of therapeutic glycoproteins in these systems (Tomiya, Ailor, Lawrence, Betenbaugh, & Lee, 2001). The repertoire of bacterial sugar nucleotides is known to be particularly rich, including many rare compounds that are absent from mammals. Among others, these sugar nucleotides are used to synthesize outer membrane polysaccharides, such as O antigens (Samuel & Reeves, 2003), and to elaborate S-layer glycoproteins (Jarrell et al., 2014), often acting as virulence factors in Gram-negative or Gram-positive pathogens. The sugar nucleotide-processing enzymes involved in the biosynthesis of these polysaccharides are therefore of considerable interest from a drug discovery perspective; the identification and assay of these enzymes are greatly facilitated by detailed knowledge of the sugar nucleotide content and the metabolic processes of the organism in question.

A variety of different approaches have been used to separate, to detect, to characterize, and to quantify sugar nucleotides. Many methods have used UV absorbance for detection, with standard curves affording quantification; NMR detection has also been employed, either offline or in conjunction with chromatography. Mass spectrometry (MS) detection has seen

important advances in recent years, particularly in combination with liquid chromatography approaches (i.e., LC-MS), which allows definitive metabolite identification combined with accurate quantification. Most methods employed to date rely heavily on the use of authentic standards for calibration. However, only a limited number of sugar nucleotides are commercially available for direct comparison, although others can be obtained using chemical (Wagner, Pesnot, & Field, 2009) or enzymatic synthesis approaches (White-Phillip, Thibodeaux, & Liu, 2009). Herein we survey technical developments in sugar nucleotide profiling, with an emphasis on contemporary LC-MS-based techniques.

2. SUGAR NUCLEOTIDE EXTRACTION

The biosynthesis of sugar nucleotides in any cell is highly dynamic, governed by demands from a number of metabolic processes for activated monosaccharide building blocks. Any sugar nucleotide profile will therefore reflect the status of this metabolite pool at the time of cell lysis only. There are many published methods for the extraction and analysis of intracellular sugar nucleotides, most of which have been developed to extract both sugar nucleotides and nucleotides in a single step.

Successful extraction of intracellular nucleotides and sugar nucleotides requires efficient cell lysis and inactivation of enzymes capable of degrading or modifying these compounds (e.g., phosphatases, pyrophosphorylases, or sugar nucleotide-processing enzymes). The pool sizes of sugar nucleotides are modest, while the catalytic activities drawing on them can be significant; rapid inactivation of enzymes is therefore vital to ensure that the analytical results accurately reflect the situation in vivo.

Sugar nucleotides are prone to acid hydrolysis, but some species exhibit limited stability even at neutral and basic pH. In the more labile cases, UDP-α-D-apiofuranose (UDP-Api*f*) has been reported to have a half-life of 97 min at pH 8.0 and 25°C (Kindel & Watson, 1973), while CMP-D-ketodeoxyoctonate (CMP-KDO) has a half-life of 34 min at neutral pH and room temperature (Lin, Murray, Ollmann, & Wong, 1997). Given the relatively low stability of most sugar nucleotides, any extraction protocol and sample preparation have to be assessed with respect to degree of recovery.

Traditionally, extraction of intracellular sugar nucleotides and/or nucleotides has been achieved by the use of cold perchloric acid (PCA), which brings about concomitant protein precipitation. PCA is then precipitated

as potassium perchlorate and the precipitate is removed by centrifugation (Kochanowski et al., 2006). The supernatant, however, often contains significant amounts of residual potassium perchlorate which may further precipitate and cause blockage of analytical instrumentation. In addition, apart from being a hazardous oxidizing agent, PCA is a strong mineral acid and its use to extract the acid labile sugar nucleotides may result in poor recovery. A suitable alternative to PCA is trichloroacetic acid (TCA), which is a weaker acid and can be easily removed from extracts by partitioning between water and diethyl ether. TCA has been used to extract sugar nucleotides from mammalian cells (for example, Palmieri, Berry, Player, Rogers, & Segal, 1991). Formic acid is also an efficient extraction agent that can be easily removed by lyophilization. It has been used to extract nucleotides and sugar nucleotides from bacterial cells, for example (Payne & Ames, 1982). Aqueous ethanol is often used for extraction as it brings about cell lysis with a concomitant protein precipitation (MacRae et al., 2006; Turnock & Ferguson, 2007). The lysis is sometimes facilitated by sonication (Nakajima et al., 2010; Tomiya et al., 2001) or by heating (Seifar et al., 2009). Mixtures of organic solvents and water (e.g., chloroform–methanol–water; Urbaniak, Turnock, & Ferguson, 2006 or acetonitrile–water; del Val, Kyriakopoulos, Polizzi, & Kontoravdi, 2013) can also be used to similar effect. Hypotonic solutions of fluoride not only bring about cell lysis but also act as a potent phosphatase inhibitor (Pabst et al., 2010). On occasion, mixtures of detergents (such as Triton X-100) with organic solvents have been used to break up membrane structures and denature proteins at the same time (Feng, Wong, Wee, & Lee, 2008). Some less frequently used extraction methods include boiling in water (Sweeney, Mackintosh, & Mason, 1993), heating with 20 m*M* KOH in 10% EtOH (Lagunas & Diezmasa, 1994), or homogenization in 1% aqueous ammonia followed by solid-phase extraction (SPE) (Castilho et al., 2008). Plants, including seaweed tissues, usually require liquid nitrogen pulverization prior to sugar nucleotides extraction (Behmuller, Forstenlehner, Tenhaken, & Huber, 2014; Manley & Burns, 1991).

3. SUGAR NUCLEOTIDE PROFILING STRATEGIES

A major challenge in sugar nucleotide profiling is to separate structural isomers. Examples of particularly challenging isomeric pairs include: UDP-α-D-glucose (UDP-Glc)/UDP-α-D-galactopyranose (UDP-Gal*p*),

UDP-α-D-glucuronic acid (UDP-GlcA)/UDP-α-D-galacturonic acid (UDP-GalA), UDP-2-acetamido-2-deoxy-α-D-glucose (UDP-GlcNAc)/UDP-2-acetamido-2-deoxy-α-D-galactose (UDP-GalNAc), UDP-α-D-xylopyranose (UDP-Xyl*p*)/UDP-β-L-arabinopyranose (UDP-L-Ara*p*), UDP-L-Ara*p*/UDP-β-L-arabinofuranose (UDP-L-Ara*f*), or GDP-α-D-glucose (GDP-Glc)/GDP-α-D-mannose (GDP-Man). Detection by UV offers little or no selectivity between the sugar nucleotides and can be confounded by coeluting UV-active contaminants, such as nonsugar nucleotides. MS, which offers selectivity based on mass, will struggle to distinguish structural isomers such as UDP-Glc/UDP-Gal*p*. Reverse-phase chromatography, relying on nonpolar interactions, is a poor choice for sugar nucleotides, which are highly polar, and which do not differ sufficiently in polarity to allow separation of closely related isomers.

Later we describe a range of approaches that achieve separation in conjunction with detection by UV. We go on to describe how many of these approaches can be used in conjunction with MS. Coupling with MS requires use of volatile MS-compatible buffers. Alternatively, an efficient desalting step needs to be employed if use of nonvolatile buffer cannot be avoided.

3.1 Anion-Exchange Chromatography and UV Detection

Anion-exchange chromatography (AEC) is often used to separate complex mixtures of sugar nucleotides. For instance, sugar nucleotide pools of the red alga *Pterocladiella capillacea* were studied in relation to agar biosynthesis (Manley & Burns, 1991). Sugar nucleotides were extracted using formic acid (1 *M*) after pulverizing the algal tissue in liquid nitrogen. Crude extracts were prepurified by SPE on phenylsilane-bonded silica columns with elution using formic acid (1 *M*). The extraction method gave approximately 87% recovery of added standard UDP-Glc. An alternative extraction protocol based on PCA gave poorer standard recovery. AEC with UV detection was used to separate sugar nucleotides. Potassium phosphate buffer (50–250 m*M*, pH 3.5–4.5) was used for elution, but poor separation of both UDP- and GDP-sugars was observed. AEC separation facilitated identification of ADP-α-D-glucopyranose (ADP-Glc), UDP-Glc, and UDP-Gal*p*. GDP-Glc and GDP-Man coeluted under these conditions.

Quantification of UDP-Glc and UDP-Gal*p* in human red blood cells was reported using AEC with UV detection (Palmieri et al., 1991). The sugar nucleotides were extracted with ice-cold TCA, followed by

partitioning between water and diethyl ether to remove excess TCA. The sugar nucleotides were separated on an anion-exchange column (CarboPac PA-1) using a potassium phosphate buffer (pH 4.5) gradient against water, and the analytes were detected by UV at 254 nm. Although baseline separation between UDP-Glc and UDP-Gal*p* was not achieved, integration of both species was still possible with reasonable accuracy. The limit of detection of this method was 12 pmol, with linearity over 30–240 pmol per injection.

Pool sizes of various UDP-sugars involved in glycosaminoglycan synthesis in mammalian chondrocytes were studied using AEC (Sweeney et al., 1993). UDP-sugars were released by boiling cell suspensions in water for 2 min, followed by freezing at −20°C. PCA (0.3 *M*) extraction followed by neutralization had to be avoided as UDP-GlcA was unstable under these conditions. The extracts were analyzed by strong anion-exchange (SAX) chromatography and detected by UV (254 nm). Phosphate buffer with varying ionic strength and pH was used for elution, providing good separation of UDP-GalNAc and UDP-GlcNAc. UDP-Glc and UDP-Gal*p* coeluted, but separated from UDP-Xyl*p*.

High-performance anion-exchange chromatography (HPAEC) has been used to determine cellular levels of sugar nucleotides and related nucleotides in insect cells (*Spodoptera frugiperda*, *Trichoplusia ni*) and mammalian cells [Chinese hamster ovary (CHO)] (Tomiya et al., 2001). Excellent separation of 9 sugar nucleotide species [CMP-*N*-acetyl-D-neuraminic acid (CMP-Neu5Ac), CMP-*N*-glycolyl-D-neuraminic acid (CMP-Neu5Gc), CMP-2-keto-3-deoxynonic acid (CMP-KDN), UDP-Gal*p*, UDP-Glc, UDP-GalNAc, UDP-GlcNAc, GDP-β-L-fucose (GDP-L-Fuc), GDP-Man] and 12 nucleotides was achieved with high sensitivity and reproducibility. Separation on an anion-exchange column was shown to be superior to C18 ion-pair reversed-phase (IP-RP) HPLC using tetrabutylammonium sulfate (TBAS) as the pairing reagent. The cells studied in this work were lysed by sonication in 75% EtOH and, after centrifugation and freeze drying, the samples were resuspended in phosphate buffer (pH 9.2) to stabilize CMP-sialic acids. The samples were analyzed on CarboPac PA-1 column (Dionex) using a concentration gradient of sodium acetate in sodium hydroxide. The analytes were detected by UV (260 nm). Injections of sugar nucleotides ranging from 1 pmol to 1 nmol showed a linear relationship between quantity and peak area monitored by UV; the limit of detection was around the 1 pmol level.

Mammalian cell extracts have been analyzed by AEC on IonPac AS11 columns (Dionex) using a KOH concentration gradient and dual detection by UV and suppressed conductivity (Ritter, Genzel, & Reichl, 2006). This method allowed analysis of a wide range of intracellular metabolites in a single chromatographic run; although four sugar nucleotides and some nucleotides were detected, their separation was limited.

An optimized method for sugar nucleotide and nucleotide extraction from mammalian cells has been reported recently (del Val et al., 2013). Aqueous acetonitrile (50%, v/v) extraction was found to be superior in terms of analyte recovery when compared to the PCA method. A previously reported method (Tomiya et al., 2001) based on HPAEC coupled with UV detection (262 nm) was used which, after some modifications, achieved separation and detection of nine sugar nucleotides and eight nucleotides.

3.2 IP-RP HPLC and UV Detection

A variety of ion-pairing reagents have been reported to facilitate C18 reversed-phase separation of sugar nucleotides (e.g., tetrabutylammonium salts; Kochanowski et al., 2006; Nakajima et al., 2010; Payne & Ames, 1982, and borate buffer containing tetra-, hexa-, and octadecyltrimethylammonium bromide; Lagunas & Diezmasa, 1994). These reagents have low volatility and form very stable ions, rendering them serious contaminants in mass spectrometry. Because of this, together with the fact that they are usually used in mixtures with nonvolatile buffers, methods using these reagents are generally incompatible with LC-MS techniques. Consequently, volatile ion-pairing reagents have also been employed in sugar nucleotide separations (e.g., triethylammonium acetate; Rabina et al., 2001).

Nucleosides, nucleotides, and sugar nucleotides extracted from two bacterial species, *Salmonella typhimurium* and *Escherichia coli*, were prepurified by boronate-affinity chromatography, followed by IP-RP HPLC and detected by UV (Payne & Ames, 1982). The cell extraction was performed using cold formic acid (1 *M*, pH 2), and the crude samples were enriched by boronate-affinity chromatography (Affi-Gel 601 boronate-derivatized polyacrylamide gel). Nucleotides and sugar nucleotides binding to the boronate gel were eluted with acetic acid (0.1 *M*). Subsequent IP-RP HPLC separation was performed using an acetonitrile gradient against potassium dihydrogen phosphate buffer (30 m*M*, pH 6.0) containing tetrabutylammonium phosphate (5 m*M*) and acetonitrile (4%). Eluting

compounds were detected by UV (254 nm) and identified by comparison with retention times of authentic standards.

Formation of UDP-2-deoxy-glucose and UDP-2-deoxy-galactose in *Saccharomyces cerevisiae* cells incubated with 2-deoxy-galactose was studied using C18 IP-RP HPLC (Lagunas & Diezmasa, 1994). The sugar nucleotides were extracted by heating the yeast cell pellet at 90°C for 2 min with 20 m*M* KOH in 10% EtOH. HPLC separation was achieved with borate buffer (0.55 *M*, pH 6.0) containing glycerol (2 *M*), hexadecyltrimethylammonium bromide (0.3 m*M*), and methanol (30%, v/v), and the sugar nucleotides were detected by UV (254 nm).

In relation to carrageenan biosynthesis studies, sugar nucleotides and nucleotides extracted from two species of algae (*Calliblepharis jubata* and *Solieria chordalis*) were analyzed and quantified using IP-RP HPLC (Goulard, Diouris, Deslandes, & Floc'h, 1999). Sugar nucleotides were extracted using formic acid (1 *M*) after pulverizing the algal tissue in liquid nitrogen (Manley & Burns, 1991). Formic acid extraction gave higher recovery of standards (ATP and UDP-Glc) than extraction protocols based on PCA or TCA. SPE (Oasis, Waters) was used to prepurify samples for analysis, followed by IP-RP HPLC separation with triethylammonium phosphate (40 m*M*, pH 6.5) used as the ion-pairing reagent and elution achieved with a gradient of acetonitrile. Sugar nucleotides and nucleotides were detected by UV (254 nm).

Stable recombinant *S. cerevisiae* strains expressing bacterial GDP-D-mannose dehydratase, GDP-4-keto-6-deoxy-D-mannose-4-reductase, or GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase/4-reductase were lysed, and the crude lysates were used to convert GDP-Man into either GDP-L-Fuc or GDP-α-D-rhamnose (GDP-D-Rha) (Rabina et al., 2001). In a similar manner crude lysate of *Pseudomonas aeruginosa* was supplied with GDP-Man and formation of GDP-D-Rha was observed. After incubation, SPE was used to remove proteins and to extract sugar nucleotides from the mixtures. The graphitized nonporous carbon SPE column used in this work strongly retains sugar nucleotides. Undesired contaminants were removed by a series of washes followed by a nearly quantitative elution of the sugar nucleotides using a mixture of ion-pairing reagent (50 m*M* triethylammonium acetate, pH 7.0) containing acetonitrile (25%, v/v). The SPE-extracted sugar nucleotide mixtures contained significant amounts of nucleotides that, if required, could be removed by treatment with alkaline phosphatase followed by anion-exchange purification. The mixtures were separated by C18 IP-RP HPLC using an acetonitrile gradient against triethylammonium acetate buffer (20 m*M*, pH 6.0). Sugar nucleotides were

detected by UV (254 nm) and identified using authentic standards and/or off-line MS analysis (MALDI-TOF) after HPLC purification. The SPE and HPLC methods reported in this chapter (Rabina et al., 2001) received significant attention by later authors, as both methods utilize a volatile ion-pairing agent compatible with LC-MS applications.

Analysis of intracellular nucleotide and nucleotide sugar content was performed to study the protein glycosylation capacity of mammalian cells. An IP-RP HPLC method was developed (Kochanowski et al., 2006) to quantify intracellular levels of nucleotides and nucleotide sugars in CHO cells. The cells were extracted using cold PCA (0.5 *M*) and centrifuged; the supernatants were neutralized by cold KOH (2.5 *M*), and precipitated potassium perchlorate was removed by centrifugation. The supernatant was then analyzed using IP-RP HPLC, with separation achieved at pH 6.5 using a methanol gradient against potassium phosphate buffer in the presence of tetrabutylammonium hydrogensulfate as the ion-pairing reagent. Analytes were detected by UV (254 nm), with sugar nucleotide and nucleotide identification based on retention times of authentic standards. UDP-Glc, UDP-Gal*p*, GDP-Man, UDP-GlcNAc, and UDP-GalNAc were separated and identified, together with eight nucleotide species. Depending on the structure, the limit of detection for examined analytes ranged from 7.5 to 150 pmol per injection, with linearity over a range of 7.5 to 6000 pmol (Kochanowski et al., 2006).

To study sugar nucleotide levels in breast and pancreatic cancer cell lines, a C18 IP-RP HPLC method was used (Nakajima et al., 2010). Cell lines were lysed by sonication in cold 70% ethanol and further enriched using EnviCarb SPE cartridges (Rabina et al., 2001). A set of 8 sugar nucleotide and 12 nucleotide standards was used to test the method. Inertsil ODS-3 and ODS-4 columns provided superior separation of all standards when compared to four other types of C18 columns. A gradient of acetonitrile against potassium phosphate buffer (100 m*M*, pH 6.4) supplemented with tetrabutylammonium hydrogensulfate (8 m*M*) as the ion-pairing reagent (Kochanowski et al., 2006) was used for separation. In particular, good separation of UDP-GlcNAc and UDP-GalNAc was achieved. The analytes were detected by UV (254 nm) and identified by comparison with standards; quantification was based on calibration curves for each standard.

3.3 Capillary Electrophoresis and UV Detection

Capillary electrophoresis (CE) with UV detection (262 nm) has been used to separate, detect, and quantify several sugar nucleotides (UDP-Glc,

UDP-GlcNAc, UDP-Gal*p*, UDP-GalNAc) extracted from mammalian cells (Lehmann et al., 2000). Optimal separation was achieved using 90 m*M* borate buffer (pH 9.0) at 18°C and 15.5 kV in an uncoated fused-silica capillary. The reported limit of detection was 180 fmol.

CE with UV detection has been used to separate 12 nucleotides and 7 sugar nucleotides extracted from CHO cells (Feng et al., 2008). The CHO cells in phosphate-buffered saline (PBS) solution were lysed using Triton X-100, and acetonitrile and lipids were removed from the aqueous layer by chloroform extraction. CE separation was achieved with 40 m*M* sodium tetraborate buffer (pH 9.5) containing 1% (w/v) polyethylene glycol. The nucleotides and sugar nucleotides were detected by UV (260 nm) and identified using authentic standards.

3.4 Liquid Chromatography-MS-Based Methods

Coupling liquid chromatography with mass spectrometry (LC-MS) is a particularly popular approach. These analytical techniques are complementary and orthogonal in the sense that they derive their specificity from different features of the analyte. Given the rich array of sugar nucleotides, it is difficult to find a single LC method that can separate them all, while many are isomers of the same mass and are therefore hard to distinguish by MS.

LC-MS-based sugar nucleotide profiling methods usually rely on electrospray ionization (ESI) detection in negative mode. Instruments operated in full-scan MS mode are used for explorative work, where unexpected metabolites may appear. The drawback is the complexity of the chromatograms and peak overlap with other charged species, particularly nucleotides.

Coupling LC with a triple-quadrupole tandem mass spectrometer (LC-MS/MS) provides much enhanced selectivity and is the basis for sensitive, targeted profiling of metabolites of interest in complex matrices. Targeted profiling uses either selected reaction monitoring (SRM) of single fragment ions, or multiple reaction monitoring (MRM) of multiple fragments, for quantification and confirmation of peak identity (Sage et al., 2013). Suitable SRM or MRM transitions for all sugar nucleotides of interest have to be established using authentic standards (e.g., see Table 1).

MRM mode is more sensitive than full-scan mode in quadrupole instruments, because the instrument can dwell for longer on each target mass, allowing more ions to reach the detector. The limit of detection is very often

Table 1 Relative Retention Times and MRM Transitions of Sugar Nucleotides Standards

Sugar Nucleotide	Relative Retention Time	MRM Transitions	Fragment
UDP-Glc	1.00	565 → 323	$[NMP\text{-}H]^-$
		565 → 79	$[H_3PO_4\text{-}H_3O]^-$
UDP-Gal*p*	0.92	565 → 323	$[NMP\text{-}H]^-$
		565 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-α-D-galactofuranose (UDP-Gal*f*)	1.10	565 → 323	$[NMP\text{-}H]^-$
		565 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-GlcNAc	0.98	606 → 385	$[NDP\text{-}H\text{-}H_2O]^-$
		606 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-GlcNAcA	0.89	620 → 403	$[NDP\text{-}H]^-$
		620 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-2-amino-2-deoxy-α-D-glucose (UDP-GlcN)	0.90	564 → 385	$[NDP\text{-}H\text{-}H_2O]^-$
		564 → 273	?
UDP-2-amino-2-deoxy-α-D-galactose (UDP-GalN)	0.86	564 → 385	$[NDP\text{-}H\text{-}H_2O]^-$
		564 → 273	?
UDP-2,3-diacetamido-2,3-dideoxy-α-D-glucuronic acid (UDP-GlcdiNAcA)	0.95	661 → 403	$[NDP\text{-}H]^-$
		661 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-GlcA	0.74	579 → 403	$[NDP\text{-}H]^-$
		579 → 323	$[NMP\text{-}H]^-$
UDP-2-deoxy-2-fluoro-α-D-galactose (UDP-2F-Gal)	0.94	567 → 385	$[NDP\text{-}H\text{-}H_2O]^-$
		567 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-β-L-rhamnose (UDP-L-Rha)	0.84	549 → 323	$[NMP\text{-}H]^-$
		549 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-L-Ara*f*	1.05	535 → 323	$[NMP\text{-}H]^-$
		535 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-L-Ara*p*	0.81	535 → 323	$[NMP\text{-}H]^-$
		535 → 159	$[H_4P_2O_7\text{-}H_3O]^-$
UDP-Xyl*p*	0.99	535 → 323	$[NMP\text{-}H]^-$
		535 → 159	$[H_4P_2O_7\text{-}H_3O]^-$

Continued

Table 1 Relative Retention Times and MRM Transitions of Sugar Nucleotides Standards—cont'd

Sugar Nucleotide	Relative Retention Time	MRM Transitions	Fragment
dTDP-α-D-glucose (dTDP-Glc)	1.39	563 → 321	$[NMP\text{-}H]^-$
		563 → 241	$[Glc\text{-}1\text{-}P\text{-}H\text{-}H_2O]^-$
dTDP-β-L-rhamnose (dTDP-L-Rha)	1.35	547 → 321	$[NMP\text{-}H]^-$
		547 → 225	$c[Rha\text{-}1\text{-}P\text{-}H\text{-}H_2O]^-$
GDP-Glc	1.56	604 → 362	$[NMP\text{-}H]^-$
		604 → 241	$c[Glc\text{-}1\text{-}P\text{-}H\text{-}H_2O]^-$
GDP-β-L-galactose (GDP-L-Gal)	1.51	604 → 442	$[NDP\text{-}H]^-$
		604 → 423	$[NDP\text{-}H\text{-}H_2O]^-$
GDP-Man	1.43	604 → 442	$[NDP\text{-}H]^-$
		604 → 424	$[NDP\text{-}H\text{-}H_2O]^-$
GDP-L-Fuc	1.60	588 → 442	$[NDP\text{-}H]^-$
		588 → 344	$[NMP\text{-}H\text{-}H_2O]^-$
GDP-α-D-arabinopyranose (GDP-Ara*p*)	1.53	574 → 442	$[NDP\text{-}H]^-$
		574 → 362	$[NMP\text{-}H]^-$
GDP-β-L-xylopyranose (GDP-L-Xyl*p*)	1.52	574 → 442	$[NDP\text{-}H]^-$
		574 → 424	$[NDP\text{-}H\text{-}H_2O]^-$
ADP-Glc	1.65	588 → 346	$[NMP\text{-}H]^-$
		588 → 241	$c[Glc\text{-}1\text{-}P\text{-}H\text{-}H_2O]^-$
5″-(Adenosine 5′-pyrophosphoryl)-D-ribose (ADP-Rib)	1.64	558 → 346	$[NMP\text{-}H]^-$
		558 → 159	$[H_4P_2O_7\text{-}H_3O]^-$

set by the signal-to-noise ratio. Not only is the signal potentially stronger than from a scanning quadrupole instrument, but the background noise is generally very low, because of the specificity of the mass transition. This effective removal of background noise is an important advantage for analysis of sugar nucleotides by LC-MS/MS.

Due to analogous MS fragmentation pattern and mass transitions, most isobaric sugar nucleotides may appear in each other's MRM

chromatograms, a phenomenon referred to as "bleed-through" (Turnock & Ferguson, 2007). For example, a MRM chromatogram for UDP-Glc may also show other UDP-hexoses present in the sample. In these instances, the final identification needs to be based on retention time. Occasionally, structural isomers of sugar nucleotides can be distinguished by their characteristic fragmentation (Turnock & Ferguson, 2007). For example, C2 isomers such as GDP-Glc/GDP-Man provide different fragmentation patterns.

The accuracy of quantification by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) is limited by various factors. Not only can the efficiency of extraction be variable but, in contrast to detection by UV absorbance, detection efficiency by electrospray can vary. Ionization efficiency depends on the degree of contamination of the instrument, and on other coeluting compounds (cosuppression), which of course may not be visible in an MRM-based method. The use of internal standards can compensate for these problems. An internal standard is a chemically similar analyte, not found naturally in the sample, which is added early during extraction. If it is sufficiently similar to the target compound(s), it should suffer from identical losses during extraction. Its ionization efficiency should vary in the same way as endogenous material, so although the overall signal may fluctuate, the ratio of analyte signal to that of internal standard should remain constant. The perfect internal standard is an isotopically labeled version of the target analyte (e.g., ^{2}H or ^{13}C isotope). For sugar nucleotides this will rarely be available at present. An alternative is to use one or more sugar nucleotides not found in the tissue to be analyzed. It must be kept in mind that since this internal standard will not coelute with the target compound(s), it will not suffer from the same cosuppression effects; and therefore, there is a risk that measurements will be influenced by contaminating, coeluting compounds.

Another mode of data acquisition in a triple-quadrupole tandem mass spectrometry analysis is precursor ion scanning. This mode is particularly suitable for monitoring of sugar nucleotides known to produce common fragment ions specific to the different nucleotide carriers (Soo et al., 2004). In precursor scanning the final quadrupole is fixed to select the mass of an interesting fragment, and the first quadrupole is scanned to find all possible precursor ions that give rise to this fragment. For example, if the final quadrupole is set to select $[UDP\text{-}H]^-$ or fragments thereof, then scans of the first quadrupole should find the precursors UDP-Glc, UDP-GalNAc, and any other (potentially unexpected) UDP-sugars that may be present.

3.4.1 AEC-MS-Based Methods

High salt concentration required to elute analytes in AEC usually renders this method incompatible with direct MS analysis unless efficient desalting is employed. As an alternative to salt, a pH gradient is much more amenable to LC-MS applications (Veltkamp et al., 2006). HPAEC on an IonPac AS11 column coupled with tandem mass spectrometry (Quattro-Premier, Waters) was used to separate and quantify a number of polar metabolites from plant tissues (*Arabidopsis thaliana* and *Trigonella foenum-graecum*), including sugar nucleotides involved in cell wall biosynthesis (Alonso, Piasecki, Wang, LaClair, & Shachar-Hill, 2010). Before MS analysis, NaOH used for elution had to be removed with a postcolumn anion suppressor ASRS 300 (Dionex). Mass spectra were acquired using ESI in negative mode and MRM. Using this method of separation, detection, and quantification of nine sugar nucleotides, including several isobaric species, was accomplished, although GDP-Man and GDP-Glc coeluted under these conditions.

3.4.2 IP-RP HPLC-MS-Based Methods

In vitro enzymatic transformation of GDP-Man into GDP-D-Rha was studied using LC-MS and online stopped flow LC-NMR (Ramm, Wolfender, Queiroz, Hostettmann, & Hamburger, 2004). C18 IP-RP HPLC with triethylammonium acetate or tripropylammonium acetate (pH 6.0) as the ion-pairing reagents and either isocratic or gradient acetonitrile elution separated up to 11 different sugar nucleotides. ESI-MS detection was performed in negative mode on a single-quadrupole mass spectrometer with turbo ion spray interface (PE Sciex API 165, Applied Biosystems). Increasing buffer concentration was shown to reduce signal intensity of sugar nucleotides in negative ESI mode. Too low a buffer concentration, however, led to insufficient retention and separation; the concentration of buffer used had to be a compromise between optimal ionization conditions and optimal separation. Online NMR detection was performed on a 500-MHz spectrometer equipped with a flow-probe cell. The water suppression enhanced through T1 effects (WET) pulse sequence for multiple signals suppression (Smallcombe, Patt, & Keifer, 1995) was of critical importance to suppress signals originating from acetonitrile, water, and triethylammonium acetate. An estimated 40–80 μg of a sugar nucleotide present in the flow-probe cell was required to give a satisfactory signal-to-noise ratio.

Sugar nucleotide profiling methods based on LC-ESI-MS/MS using MRM were developed to study sugar nucleotide pools that fuel the biosynthesis of cell surface glycoconjugates of trypanosomatid parasites

(MacRae et al., 2006; Turnock & Ferguson, 2007; Urbaniak et al., 2006). Cells of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major* were lysed using 70% ethanol (MacRae et al., 2006; Turnock & Ferguson, 2007) or chloroform–methanol–water (2:4:1) (Urbaniak et al., 2006). In both cases, prior to cell lysis, a known amount of GDP-Glc was added as an internal standard to facilitate quantification of the detected sugar nucleotides. The samples were then partitioned between water and butan-1-ol to remove lipids, and sugar nucleotides were extracted using an EnviCarb graphitized carbon column, as described previously (Rabina et al., 2001). IP-RP HPLC was used to separate sugar nucleotides before MS analysis; triethylammonium acetate, a volatile ion-pairing MS-compatible reagent, was employed as previously reported (Rabina et al., 2001). Solvent addition was required postcolumn, producing a solvent content (approximately 50% acetonitrile) compatible with stable ESI. The sugar nucleotide ESI-MS analysis was performed on a triple-quadrupole mass spectrometer (Quattro Ultima, Waters) in negative mode, operated in MRM mode. This LC-ESI-MS/MS method allowed identification and quantification of 11 sugar nucleotide species with a limit of detection of about 1 pmol and linearity over a range of 10–1000 pmol per injection. To validate the LC-ESI-MS/MS method, sugar nucleotide levels in CHO cells were analyzed (Turnock & Ferguson, 2007) and the results were found to be in good agreement with data obtained by HPAEC and UV detection published earlier (Tomiya et al., 2001).

3.4.3 Hydrophilic Interaction Liquid Chromatography-MS-Based Methods

Hydrophilic interaction liquid chromatography (HILIC) coupled to ESI-MS is gaining increasing interest in relation to profiling of a range of polar metabolites (Antonio et al., 2008) including sugar nucleotides (Bajad et al., 2006). A systematic optimization approach based on HILIC methods identified aminopropyl column (Luna NH_2) at pH 9.45 as an effective tool for the separation of polar cellular metabolites, including many nucleotides and some examples of sugar nucleotides (Bajad et al., 2006). The elution of metabolite extracts was performed using volatile buffer [ammonium acetate (20 m*M*) with ammonium hydroxide (20 m*M*) in 95:5 water:acetonitrile] against a decreasing concentration of acetonitrile. Detection was achieved by ESI-MS/MS operated in SRM mode on a triple-quadrupole instrument.

An LC-ESI-MS/MS method for targeted profiling of intracellular metabolites including sugar nucleotides and nucleotides using silica-based zwitterionic (ZIC) stationary phase in the HILIC mode was reported

(Preinerstorfer, Schiesel, Lammerhofer, & Lindner, 2010). Samples were analyzed using a ZIC-HILIC stationary phase, with a gradient of water against acetonitrile, both containing ammonium formate (20 m*M*, pH 3.5) used for the elution. Limited separation of isobaric species was observed. Compounds were detected by tandem mass spectrometry on a triple-quadrupole instrument (Applied Biosystems 4000 QTRAP) in the SRM mode; specific SRM transitions for 258 metabolites were collated. The method was validated by analyzing extracts of beta-lactam antibiotic fermentation broths, showing among other things four sugar nucleotide species but again, with no separation of isobaric species.

Another ZIC-HILIC-based LC-ESI-MS/MS method was used to analyze sugar nucleotides involved in plant cell wall biosynthesis (Ito et al., 2014). Tandem mass spectrometry (5500 QTRAP, AB Sciex) operating in SRM mode was used to detect and quantify 12 sugar nucleotides at low fmol quantities, with linear responses ranging from 10 fmol to 25–250 pmol, depending on the sugar nucleotide species. Eight sugar nucleotides were separated on a ZIC-HILIC column using a gradient of decreasing acetonitrile concentration against ammonium acetate (10 m*M*, pH 7.0). Some structural isomers, however, failed to separate (UDP-Glc/UDP-Gal*p*, UDP-GlcNAc/UDP-GalNAc) under these conditions.

3.4.4 Porous Graphitic Carbon LC-MS-Based Methods

Several recently published LC-MS-based methods make use of the unique ability of a porous graphitic carbon (PGC) stationary phase to retain polar and ionic analytes. PGC properties and retention mechanisms have been summarized by Jansen, Rosing, Schellens, and Beijnen (2009) and reviewed in detail by West, Elfakir, and Lafosse (2010). The major drawback of PGC-based LC is retention time instability (Bapiro, Richards, & Jodrell, 2016). This arises from the fact that the stationary phase can undergo redox reactions. The oxidation state of the material will impact on retention of analytes such as sugar nucleotides and nucleotides. Various reducing (Pabst et al., 2010) and oxidizing agents have been used to tackle this problem (Jansen et al., 2009). Recently, a simple wash with aqueous methanol has been shown to fully restore the original retention capacity of the PGC column (Bapiro et al., 2016).

In relation to heterologous expression of pharmaceutical glycoproteins in plants, transgenic *A. thaliana* was generated expressing enzymes required for synthesis of CMP-Neu5Ac (Castilho et al., 2008). *In planta* synthesis of CMP-Neu5Ac was studied using LC-ESI-MS/MS. To extract the sugar

nucleotide, plant leaves were homogenized in 1% aqueous ammonia solution, centrifuged, and subjected to SPE (C18 followed by HyperSep Hypercarb SPE cartridges). The samples were analyzed using a surface-conditioned PGC column (Hypercarb) with an acetonitrile gradient elution against ammonium formate (65 m*M*, pH 3.0). CMP-Neu5Ac was detected by an in-line ESI-Q-TOF mass spectrometer (Ultima Global) operating in MS/MS mode.

Building on previous results, Pabst et al. (2010) reported a new PGC-based LC-ESI-MS method to profile nucleotides and sugar nucleotides. Target compounds were extracted from CHO cells and from *E. coli* using ice-cold sodium fluoride (80 m*M*). Plant tissues (*A. thaliana* and *Nicotiana benthamiana*) had to be homogenized first under liquid nitrogen with a ball mill before treatment with sodium fluoride (40 m*M*). The lysates underwent SPE on a Hypercarb SPE column. Using an acetonitrile gradient against ammonium formate buffer (pH 9.0), 40 various species of nucleotides and 35 sugar nucleotides were then successfully separated on a surface-conditioned PGC column (Hypercarb capillary column I.D. 0.32 mm). Compounds were detected in negative ESI mode on Q-TOF Ultima instrument operating in either full-scan or MS/MS mode. The PGC stationary phase provided adequate retention of nucleotides and sugar nucleotides in the absence of ion-pairing reagents, but suffered from retention time instability. To mitigate this issue, the PGC column had to be treated with reducing agent (sodium sulfite) and hydrochloric acid. Electrical grounding of the capillary column and high ionic strength of the eluent were also essential. Peaks in the LC-MS chromatograms were identified using commercial standards and MS/MS experiments enabling discrimination of isobaric species; UDP-GalNAc and UDP-GlcNAc were found to coelute on PGC. The limit of detection determined for a group of representative sugar nucleotides and nucleotides was around 25 fmol. In terms of retention time reproducibility, absolute values were found to vary significantly; however, relative retention times varied with a mean standard deviation of less than 1%. PGC separates nucleotides and sugar nucleotides in a manner orthogonal to IP-RP HPLC, with the nature of the base as well as the attached sugar impacting on retention much more than the number of phosphates.

In a study looking at UDP-glucose dehydrogenase genes involved in UDP-sugar metabolism, sugar nucleotides were extracted from *A. thaliana* cells and analyzed using LC-ESI-MS (Behmuller et al., 2014). Fresh plant material was pulverized in liquid nitrogen and extracted with chloroform–methanol–water. The crude extracts were further enriched

by SPE with a graphitized nonporous carbon column (EnviCarb, Supelco) (Rabina et al., 2001) providing 80%–90% extraction efficiency. Separation of sugar nucleotides was achieved on a PGC column (Hypercarb) using a gradient of acetonitrile against 0.3% (v/v) formic acid adjusted to pH 9.0 with ammonia. To ensure stable retention times, the column effluent had to be electrically grounded during the LC-MS analyses and before each sequence a 3-h regeneration wash with 80% (v/v) acetonitrile–water containing 0.1% (v/v) trifluoroacetic acid was employed. MS detection was performed on an Orbitrap mass spectrometer (Exactive, Thermo Fisher), and isobaric UDP-sugars were identified based on their LC retention times. Six different UDP-sugars, including UDP-Glc, UDP-Gal*p*, UDP-L-Ara*p*, UDP-Xyl*p*, UDP-GlcA, and UDP-GalA were detected and quantified using UDP as internal standard. The limit of detection for the individual UDP-sugars was around 100–200 fmol per injection.

An optimized elution program for PGC chromatography has recently been used to obtain very stable retention times for a group of nucleosides and nucleotides (Bapiro et al., 2016). Binding of the analytes was performed in ammonium acetate (10 m*M*) buffer pH 10 followed by gradient elution with acetonitrile. A short maintenance step consisting of a 95% aqueous methanol wash restored the original retention capacity of the PGC column. Finally, a short equilibration step prepared the stationary phase for the next injection. The use of this maintenance step integrated into each run enabled consistent column performance and obviated the need for complicated and lengthy regeneration procedures.

3.5 CE-MS-Based Methods

CE coupled to ESI-MS has been used to profile intracellular pools of sugar nucleotides in *Campylobacter jejuni* (Soo et al., 2004). Cells were lysed in ammonium bicarbonate (50 m*M*, pH 8.0) by sonication, and cellular debris was removed by centrifugation. Ice-cold ethanol was added to the lysates, and precipitated material was again removed by centrifugation. Sugar nucleotides were extracted using SPE (Isolute PE-AX strong anion-exchange cartridges) with elution using ammonium acetate (0.1 *M*, pH 9.0). Electrophoretic separation (Crystal 310 CE system) was performed in bare, fused-silica capillaries using morpholinium formate buffer (30 m*M*, pH 9.0) containing 5% methanol (v/v) and a separation voltage of 20 kV. Negative mode precursor ion scanning for fragment ions specific to the different nucleotide carriers CMP, UDP, GDP, and ADP enabled selective sugar

nucleotide detection. Further, to determine the nature of the sugar part of the detected sugar nucleotides a MS/MS positive mode product ion scanning was used to detect oxonium fragment ions (Soo et al., 2004). This approach enabled precise characterization of unexpected sugar nucleotides involved in the biosynthesis of pseudaminic acid.

3.6 High-Throughput Methods

To study the impact of feed supplementation on antibody *N*-glycosylation, profiling of intracellular sugar nucleotides and nucleotides in cultured CHO cells was carried out using a high-throughput method based on matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) (Villiger et al., 2016). The extraction of sugar nucleotides and nucleotides from washed cell pellets was performed in a 96-deep-well plate using 50% (v/v) aqueous acetonitrile (del Val et al., 2013). An internal standard ($^{13}C^{15}N$-ATP) was added to the pellet before extraction. A modified microarray for mass spectrometry was used as a sample target slide (Pabst et al., 2013) to acquire MALDI-TOF-MS and MS/MS spectra. The crude extracts were cocrystallized with the matrix 9-aminoacridine, which is known to provide sensitive MALDI detection of phosphorylated metabolites. All sugar nucleotides and nucleotides were detected directly without the need for chromatographic separation, although isobaric sugar nucleotides cannot generally be distinguished. Supplementation of media additives was shown to alter the intracellular sugar nucleotide and nucleotide profiles resulting in changes of antibody *N*-glycosylation pattern.

4. SUGAR NUCLEOTIDE PROFILING PROTOCOL

In connection with our transcriptome data analysis of the single-celled Eukaryote alga *Euglena gracilis* (O'Neill, Saalbach, & Field, 2016; O'Neill, Trick, Henrissat, & Field, 2015; O'Neill, Trick, Hill, et al., 2015), we had a need to profile sugar nucleotides in this species to better understand its glycosylation capacity. We therefore set about establishing extraction and profiling protocols, details of which follow.

4.1 Cell Culture and Lysis

In order to generate meaningful profiling data, contamination of studied cell cultures by other organisms had to be avoided. In the case of *E. gracilis* grown under autotrophic conditions, an axenic cell culture was produced following

the protocol published by the Culture Collection of Algae and Protozoa (CCAP) (http://www.ccap.ac.uk/). To ensure reproducibility, the *E. gracilis* culture was always harvested in mid-log phase. The wet weight of the pellet was determined. Low temperature storage following flash freezing generally resulted in a loss of the nucleoside diphosphate sugars (NDP-sugars). Consequently, lysis was performed immediately following cell harvesting. An internal standard can be added at this stage. For example, a sugar nucleotide not detected in the studied organism can conveniently be used for this purpose.

Example protocol:

1. *E. gracilis var. saccharophila* Klebs (strain 1224/7A) was obtained from the CCAP. To produce an axenic cell culture, treat the stock culture with antibiotics as suggested by CCAP (https://www.ccap.ac.uk/documents/Antibiotic_treatment.pdf). Treat the stock culture with 0%, 0.5%, and 1% of the antibiotic mixture containing cefotaxime 500 mg/L, carbenicillin 500 mg/L, and kanamycin 200 mg/L only in the recommended EG:JM medium for *E. gracilis* (1:1, EG: *E. gracilis* medium, JM: Jaworski's medium, https://www.ccap.ac.uk/media/documents/EG_JM.pdf) and subsequently inoculate into fresh EG:JM medium (1:1) at the following time intervals: 24, 48, and 72 h. Examine the culture by microscopy and plating on EG:JM medium (1:1) agar to confirm the production of axenic culture. Maintain the culture by subculturing into EG:JM medium (1:1) using aseptic technique.
2. Monitor growth curve of *E. gracilis* (in duplicate) by measuring OD_{600} values at regular periods. Inoculate sterile EG:JM medium (1:1, 600 mL) in a 1-L flask (read background OD_{600}) with the axenic culture of *E. gracilis* (1 mL). Grow cells in Algem photobioreactor (Algenuity) for 14 days at 22°C on a 14:10 light cycle with a light intensity of 100 μmol/m^2/s with shaking at 120 rpm. From the growth curve determine OD value at which the mid-log phase is reached (OD = 1.1).
3. Grow *E. gracilis* culture as above (in triplicate) and harvest cells when the culture reaches mid-log phase (OD = 1.1 in about 6 days).
4. Pellet the cells by centrifugation (6750 × *g* for 20 min at 4°C). Resuspend the pellet in ice-cold PBS (200 mL) and centrifuge again (6750 × *g* for 20 min at 4°C). Transfer the pellet into tared centrifuge vial (Oak Ridge) using PBS (25 mL), centrifuge (6750 × *g* for 20 min at 4°C), and carefully decant the supernatant before weighing out

the wet pellet. Lyse the cells straight away without flash freezing and/or cold storage!

5. If required, add an internal standard at this stage. For *E. gracilis* grown under autotrophic conditions add UDP-2-acetamido-2-deoxy-α-D-glucuronic acid (UDP-GlcNAcA) (Mw = 687.28, 3 Na salt, 1 μg = 1.46 nmol per 1 g of wet pellet) (Rejzek, Mukhopadhyay, Wenzel, Lam, & Field, 2007).
6. Lyse the cell pellet with cold (−20°C) 70% ethanol (20 mL) in an ice bath for 1 h with occasional shaking/vortexing. Remove the cell debris by centrifugation (12,857 × *g*, 20 min, 4°C) and transfer the supernatant into a glass round bottom flask (100 mL). Evaporate ethanol at reduced pressure and ambient temperature and freeze dry the aqueous residue. This can be stored at −80°C for any length of time before the next step.
7. Remove lipophilic components by partitioning the sample between water and butan-1-ol (Turnock & Ferguson, 2007): dissolve the resulting solid in 9% aqueous butan-1-ol (3 × 2 mL) and transfer it into a glass vial (10 mL volume). Extract the solution with 90% butan-1-ol (3 × 2 mL) to remove lipids (but also chlorophyll and insoluble paramylon forming middle layer). Collect bottom layer and extract again. Use centrifugation to speed up the separation of the layers (200 × *g*, 4°C, 5 min). Collect the clear aqueous layer and freeze dry in a pear-shaped flask (do not use small vials as foaming may appear under vacuum). Store at −80°C before the next step.

4.2 Sample Preparation

SPE of sugar nucleotides was performed essentially as described by Rabina et al. (2001).

Example protocol:

1. Condition a graphitized carbon column (EnviCarb, Supelco, 250 mg, 3 mL) by washing it with 80% aqueous acetonitrile, 0.1% trifluoroacetic acid (3 mL) followed by water (2 mL).
2. Dissolve the sample in ammonium bicarbonate (5 m*M*, 500 μL) and apply on column. Wash the column with water (2 mL), followed by 25% aqueous acetonitrile (2 mL), and 50 m*M* triethylammonium acetate buffer (pH 7.0, 2 mL). Finally, elute the sugar nucleotides with 50 m*M* triethylammonium acetate buffer pH 7.0 containing 25% acetonitrile (1.5 mL). Filter the sample using 0.45 μm disc filters (PTFE) and freeze dry. Store samples at −80°C prior to LC-MS/MS analysis.

4.3 ESI-MS/MS on Xevo TQ-S

LC-MS/MS was performed on a Xevo TQ-S tandem quadrupole mass spectrometer (Waters). MRM transitions for sugar nucleotide standards in negative ESI mode were generated using IntelliStart software. Typically, two most prominent transitions were selected and used for analysis, one transition as an identifier and a second one as a quantifier. Examples of MRM transitions of some sugar nucleotides are shown in Table 1.

If a standard sugar nucleotide is not available, the MRM transitions can still be predicted reasonably well (Turnock & Ferguson, 2007). MRM functions can be set up for generic groups such as NDP-hexoses, NDP-hexuronic acids, NDP-*N*-acetylhexosamines, NDP-6-deoxyhexoses, or NDP-pentoses. In the negative ESI mode, most NDP-sugars release the sugar as either a neutral loss or sometimes as sugar-1,2-cyclic phosphates and produce either $[NDP\text{-}H]^-$ or $[NMP\text{-}H]^-$ ions. These are frequently accompanied by loss of water giving rise to $[NDP\text{-}H_2O\text{-}H]^-$ or $[NMP\text{-}H_2O\text{-}H]^-$, respectively. There is a tendency for sugar nucleotides with axial sugar C2 hydroxyl groups to give rise to nucleotide diphosphate fragments $[NDP\text{-}H]^-$, probably assisted by the loss of neutral sugar-1,2-epoxides. For sugar nucleotides with equatorial sugar C2 hydroxyl groups the tendency is to give rise to nucleotide monophosphate fragments $[NMP\text{-}H]^-$ which may be assisted by the formation of sugar-1,2-cyclic phosphates detectable in negative ESI mode as $c[Sugar\text{-}1\text{-}P\text{-}H_2O\text{-}H]^-$ ions. NDP-HexNAc species tend to eliminate NDP which is probably assisted by the formation of HexNAc 1,2-oxazoline derivatives (Turnock & Ferguson, 2007).

Xevo TQ-S ESI-MS/MS

1. Perform ESI-MS/MS in negative ion mode using a source with a capillary voltage of 1.5 kV, 500°C desolvation temperature, 1000 L/h desolvation gas, 150 L/h cone gas, and 7 bar nebulizer pressure.
2. Establish mass transitions for sugar nucleotide standards. Introduce samples (10 μ*M*) at 10 μL/min combined with a flow from the HPLC pump typical of an LC run. Use IntelliStart software to automatically optimize transitions (Table 1).
3. Once retention times of standards have been established, collect the mass transitions in time-windows centered on the relevant peaks, to avoid collecting excessive numbers of transitions simultaneously.
4. Operate the mass spectrometer in MRM mode for a number of predetermined mass transitions.
5. Use MassLynx software (Waters) to collect, to analyze, and to process data.

4.4 Surface-Conditioned PGC LC-MS/MS

Separation of sugar nucleotides was achieved on a PGC column (Hypercarb, Thermo Scientific, dimensions 1 × 100 mm, particle size 5 μm) equipped with a column guard (Hypercarb, 5 μm, 1 × 10 mm). Although between runs there were significant differences in absolute retention times of standards, relative retentions were reasonably reproducible (Table 1). To ensure retention time (R_f) stability, after a batch of samples, the PGC column had to be regenerated and its performance was tested using UDP-Glc as a standard. The regeneration and column performance steps were performed using a standard HPLC (Ultimate 3000, Dionex) system equipped with UV detector.

Buffers and reagents

- Mobile phase A: Formic acid 0.3% brought to pH 9.0 with ammonia
- Mobile phase B: Acetonitrile
- Mobile phase C: Acetonitrile 80%, water 20%, TFA 0.1%
- Reducing solvent: Freshly prepared and filtered (0.45 μm) aqueous solution of sodium sulfite (Na_2SO_3, 100 m*M*)

PGC LC-MS/MS

1. Use the following multistep gradient at a flow rate 80 μL/min: 0 min: 2% B; 20 min: 15% B; 26 min: 50% B; 27 min: 90% B; 30 min: 90% B; 31 min: 2% B; 50 min: 2% B.
2. Inject available sugar nucleotide standards (5 μL, 10 μ*M*) to determine retention time (Table 1).
3. Using serial dilution of UDP-Glc determine limit of detection (10 fmol on column).
4. Reconstitute samples of extracted sugar nucleotides in A (25 μL) and inject 5 μL (20% of total) using partial loop injection. Analyze three biological replicates.
5. Where in doubt, coinject sample with appropriate standard sugar nucleotide for positive identification.
6. Process data using MassLynx (Waters) software.

PGC column regeneration

1. Test column performance at flow rate 80 μL/min by injecting UDP-Glc (5 μL, 10 μ*M*) standard.
2. Wash for at least 3 h with C (Behmuller et al., 2014) followed by MQ water (five column volumes).
3. Reduce column with sodium sulfite (100 m*M*) for 24 h (Pabst et al., 2010) followed by MQ water (five column volumes).

4. Wash with high acetonitrile (90% B, 10% A) for 30 min then equilibrate with 2% B, 98% A for 10 min at 80 µL/min.
5. Test column performance by injecting UDP-Glc (5 µL, 10 µ*M*) standard.
6. Store column in 2% B, 98% A.

4.5 Example of Results

MRM chromatograms of selected standards injected at 30–50 pmol level show good separation on the Hypercarb column (Fig. 1).

Examination of *E. gracilis* extracts revealed a number of sugar nucleotides, examples of which are given in Fig. 2. A known amount of internal standard (UDP-GlcNAcA) was added to enable quantification.

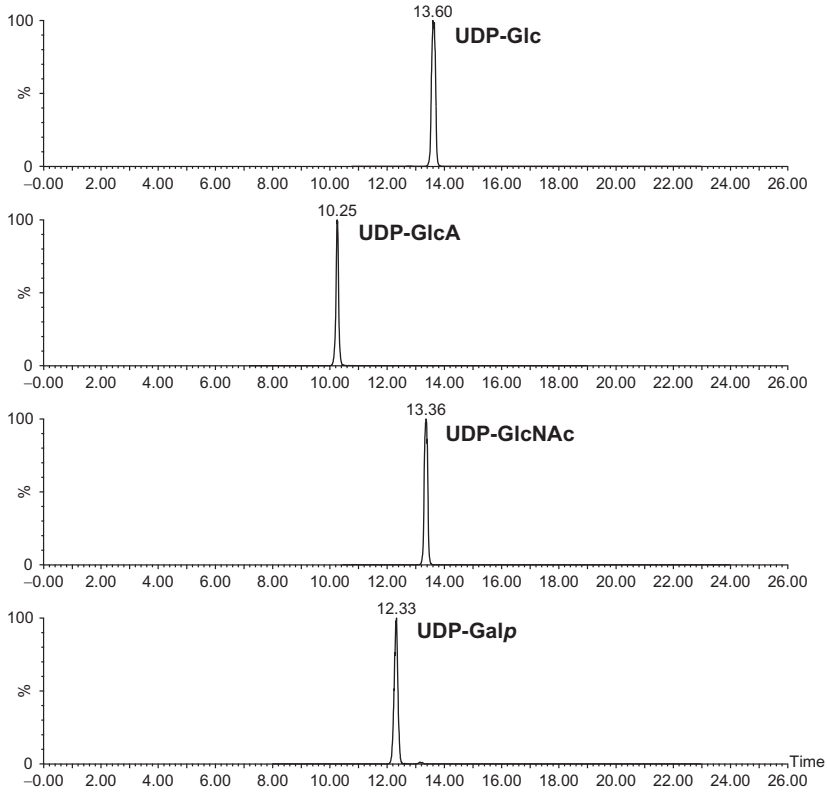

Fig. 1 Selected MRM chromatograms of standards (10 µ*M*). *Top* to *bottom*: UDP-Glc (R_f = 13.60 min, injection of 50 pmol), UDP-GlcA (10.25 min, 30 pmol), UDP-GlcNAc (13.36 min, 50 pmol), and UDP-Gal*p* (12.33 min, 30 pmol).

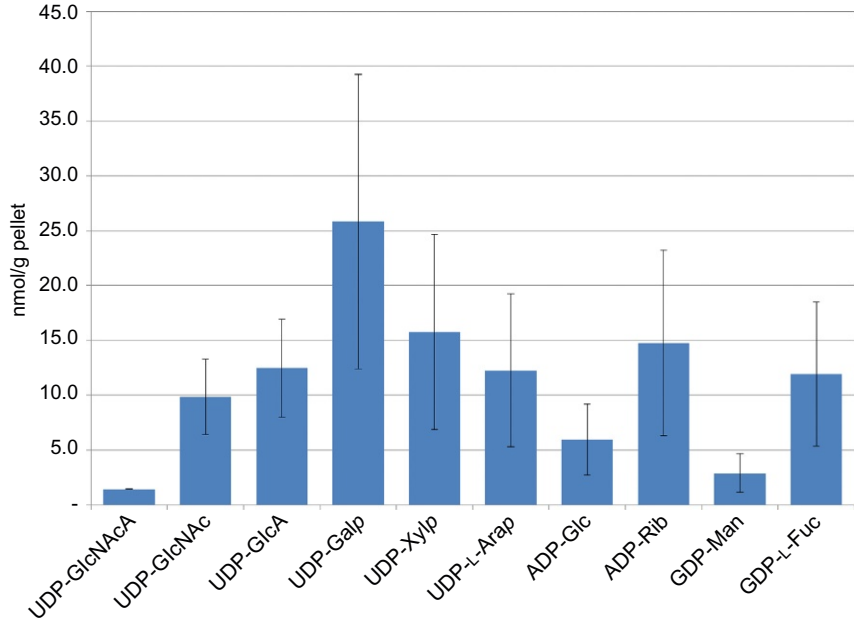

Fig. 2 Intracellular sugar nucleotide profile of *Euglena gracilis* grown at 22°C on a 14:10 light cycle with a light intensity of 100 µmol/m^2/s. Mid-log-phase cultures were harvested. The data (selected examples) are mean of three biological replicates; *error bars* indicate standard error. UDP-GlcNAcA was used as internal standard (added at 1.46 nmol/g wet pellet).

5. SUMMARY

The protocols presented herein are based on a short review of published approaches to sugar nucleotide profiling and on experience in our group. Profiling methods using UV detection are particularly convenient when rapid and accurate quantification of a limited number of well-defined sugar nucleotides is required. LC-MS/MS-based approaches are better suited for exploratory work where unusual sugar nucleotides can be expected. The separation of sugar nucleotides on PGC columns is excellent, but it suffers from retention time instability and an efficient regeneration method has still to be found. We expect the PGC LC-MS/MS to become a powerful tool in advancing fundamental areas of glycobiology research.

ACKNOWLEDGMENTS

The authors gratefully acknowledge support from the UK BBSRC Institute Strategic Programme Grant on Understanding and Exploiting Metabolism (MET) [BB/J004561/1], the Royal Thai Government Scholarship programme, and The John Innes Foundation.

REFERENCES

Alonso, A. P., Piasecki, R. J., Wang, Y., LaClair, R. W., & Shachar-Hill, Y. (2010). Quantifying the labeling and the levels of plant cell wall precursors using ion chromatography tandem mass spectrometry. *Plant Physiology*, *153*(3), 915–924. http://dx.doi.org/10.1104/pp.110.155713.

Antonio, C., Larson, T., Gilday, A., Graham, I., Bergstrom, E., & Thomas-Oates, J. (2008). Hydrophilic interaction chromatography/electrospray mass spectrometry analysis of carbohydrate-related metabolites from *Arabidopsis thaliana* leaf tissue. *Rapid Communications in Mass Spectrometry*, *22*(9), 1399–1407. http://dx.doi.org/10.1002/rcm.3519.

Bajad, S. U., Lu, W. Y., Kimball, E. H., Yuan, J., Peterson, C., & Rabinowitz, J. D. (2006). Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1125*(1), 76–88. http://dx.doi.org/10.1016/j.chroma.2006.05.019.

Bapiro, T. E., Richards, F. M., & Jodrell, D. I. (2016). Understanding the complexity of porous graphitic carbon (PGC) chromatography: Modulation of mobile-stationary phase interactions overcomes loss of retention and reduces variability. *Analytical Chemistry*, *88*(12), 6190–6194. http://dx.doi.org/10.1021/acs.analchem.6b01167.

Bar-Peled, M., Urbanowicz, B. R., & O'Neill, M. A. (2012). The synthesis and origin of the pectic polysaccharide rhamnogalacturonan II—Insights from nucleotide sugar formation and diversity. *Frontiers in Plant Science*, *3*, 92. http://dx.doi.org/10.3389/fpls.2012.00092.

Behmuller, R., Forstenlehner, I. C., Tenhaken, R., & Huber, C. G. (2014). Quantitative HPLC-MS analysis of nucleotide sugars in plant cells following off-line SPE sample preparation. *Analytical and Bioanalytical Chemistry*, *406*(13), 3229–3237. http://dx.doi.org/10.1007/s00216-014-7746-3.

Castilho, A., Pabst, M., Leonard, R., Veit, C., Altmann, F., Mach, L., et al. (2008). Construction of a functional CMP-sialic acid biosynthesis pathway in arabidopsis. *Plant Physiology*, *147*(1), 331–339. http://dx.doi.org/10.1104/pp.108.117572.

del Val, I. J., Kyriakopoulos, S., Polizzi, K. M., & Kontoravdi, C. (2013). An optimized method for extraction and quantification of nucleotides and nucleotide sugars from mammalian cells. *Analytical Biochemistry*, *443*(2), 172–180. http://dx.doi.org/10.1016/j.ab.2013.09.005.

Feng, H. T., Wong, N., Wee, S., & Lee, M. M. (2008). Simultaneous determination of 19 intracellular nucleotides and nucleotide sugars in Chinese Hamster ovary cells by capillary electrophoresis. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *870*(1), 131–134. http://dx.doi.org/10.1016/j.jchromb.2008.05.038.

Freeze, H. H., & Elbein, A. D. (2009). Glycosylation precursors. In A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, & M. E. Etzler (Eds.), *Essentials of glycobiology*. (2nd ed.). . Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Goulard, F., Diouris, M., Deslandes, E., & Floc'h, J. Y. (1999). Nucleotides, nucleoside sugars and UDP-glucose-4-epimerase activity in the iota-carrageenophytes *Solieria chordalis* and *Calliblepharis jubata* (Rhodophyceae). *European Journal of Phycology*, *34*(1), 21–25. http://dx.doi.org/10.1017/s0967026298001711.

Ito, J., Herter, T., Baidoo, E. E. K., Lao, J. M., Vega-Saanchez, M. E., Smith-Moritz, A. M., et al. (2014). Analysis of plant nucleotide sugars by hydrophilic interaction liquid chromatography and tandem mass spectrometry. *Analytical Biochemistry*, *448*, 14–22. http://dx.doi.org/10.1016/j.ab.2013.11.026.

Jansen, R. S., Rosing, H., Schellens, J. H. M., & Beijnen, J. H. (2009). Retention studies of 2′-2′-difluorodeoxycytidine and 2′-2′-difluorodeoxyuridine nucleosides and nucleotides on porous graphitic carbon: Development of a liquid chromatography-tandem mass spectrometry method. *Journal of Chromatography A*, *1216*(15), 3168–3174. http://dx.doi.org/10.1016/j.chroma.2009.02.002.

Jarrell, K. F., Ding, Y., Meyer, B. H., Albers, S. V., Kaminski, L., & Eichler, J. (2014). N-linked glycosylation in archaea: A structural, functional, and genetic analysis. *Microbiology and Molecular Biology Reviews*, *78*(2), 304–341. http://dx.doi.org/10.1128/mmbr.00052-13.

Kindel, P. K., & Watson, R. R. (1973). Synthesis, characterization and properties of uridine 5′-(alpha-D-apiofuranosyl pyrophosphate). *Biochemical Journal*, *133*(2), 227–241.

Kochanowski, N., Blanchard, F., Cacan, R., Chirat, F., Guedon, E., Marc, A., et al. (2006). Intracellular nucleotide and nucleotide sugar contents of cultured CHO cells determined by a fast, sensitive, and high-resolution ion-pair RP-HPLC. *Analytical Biochemistry*, *348*(2), 243–251. http://dx.doi.org/10.1016/j.ab.2005.10.027.

Kumar, M., Campbell, L., & Turner, S. (2016). Secondary cell walls: Biosynthesis and manipulation. *Journal of Experimental Botany*, *67*(2), 515–531. http://dx.doi.org/10.1093/jxb/erv533.

Lagunas, R., & Diezmasa, J. C. (1994). Separation and analysis of 4′-epimeric UDP-sugars by ion-pair reversed-phase HPLC. *Analytical Biochemistry*, *216*(1), 188–194. http://dx.doi.org/10.1006/abio.1994.1024.

Lehmann, R., Huber, M., Beck, A., Schindera, T., Rinkler, T., Houdall, B., et al. (2000). Simultaneous, quantitative analysis of UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, UDP-glucose and UDP-galactose in human peripheral blood cells, muscle biopsies and cultured mesangial cells by capillary zone electrophoresis. *Electrophoresis*, *21*(14), 3010–3015. http://dx.doi.org/10.1002/1522-2683(20000801)21:14<3010::aid-elps3010>3.0.co;2-c.

Lin, C. H., Murray, B. W., Ollmann, I. R., & Wong, C. H. (1997). Why is CMP-ketodeoxyoctonate highly unstable? *Biochemistry*, *36*(4), 780–785. http://dx.doi.org/10.1021/bi962055c.

MacRae, J. I., Obado, S. O., Turnock, D. C., Roper, J. R., Kierans, M., Kelly, J. M., et al. (2006). The suppression of galactose metabolism in *Trypanosoma cruzi* epimastigotes causes changes in cell surface molecular architecture and cell morphology. *Molecular and Biochemical Parasitology*, *147*(1), 126–136. http://dx.doi.org/10.1016/j.molbiopara.2006.02.011.

Manley, S. L., & Burns, D. J. (1991). Formation of nucleoside diphosphate monosaccharides (NDP-sugars) by the agarophyte *Pterocladia capillacea* (Rhodophyceae). *Journal of Phycology*, *27*(6), 702–709. http://dx.doi.org/10.1111/j.0022-3646.1991.00702.x.

Moremen, K. W., Tiemeyer, M., & Nairn, A. V. (2012). Vertebrate protein glycosylation: Diversity, synthesis and function. *Nature Reviews Molecular Cell Biology*, *13*(7), 448–462. http://dx.doi.org/10.1038/nrm3383.

Nakajima, K., Kitazume, S., Angata, T., Fujinawa, R., Ohtsubo, K., Miyoshi, E., et al. (2010). Simultaneous determination of nucleotide sugars with ion-pair reversed-phase HPLC. *Glycobiology*, *20*(7), 865–871. http://dx.doi.org/10.1093/glycob/cwq044.

Nguema-Ona, E., Vicre-Gibouin, M., Gotte, M., Plancot, B., Lerouge, P., Bardor, M., et al. (2014). Cell wall O-glycoproteins and N-glycoproteins: Aspects of biosynthesis and function. *Frontiers in Plant Science*, *5*, 499. http://dx.doi.org/10.3389/fpls.2014.00499.

O'Neill, E. C., Trick, M., Henrissat, B., & Field, R. A. (2015). Euglena in time: Evolution, control of central metabolic processes and multi-domain proteins in carbohydrate and natural product biochemistry. *Perspectives in Science*, *6*, 84–93. http://dx.doi.org/10.1016/j.pisc.2015.07.002.

O'Neill, E. C., Trick, M., Hill, L., Rejzek, M., Dusi, R. G., Hamilton, C. J., et al. (2015). The transcriptome of *Euglena gracilis* reveals unexpected metabolic capabilities for carbohydrate and natural product biochemistry. *Molecular BioSystems*, *11*(10), 2808–2820. http://dx.doi.org/10.1039/c5mb00319a.

O'Neill, E. C., Saalbach, G., & Field, R. A. (2016). Gene discovery for synthetic biology: Exploring the novel natural product biosynthetic capacity of eukaryotic microalgae. *Methods in Enzymology*, *576*, 99–120. http://doi.org/10.1016/bs.mie.2016.03.005.

Pabst, M., Fagerer, S. R., Kohling, R., Kuster, S. K., Steinhoff, R., Badertscher, M., et al. (2013). Self-aliquoting microarray plates for accurate quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Analytical Chemistry*, *85*(20), 9771–9776. http://dx.doi.org/10.1021/ac4021775.

Pabst, M., Grass, J., Fischl, R., Leonard, R., Jin, C. S., Hinterkorner, G., et al. (2010). Nucleotide and nucleotide sugar analysis by liquid chromatography-electrospray ionization-mass spectrometry on surface-conditioned porous graphitic carbon. *Analytical Chemistry*, *82*(23), 9782–9788. http://dx.doi.org/10.1021/ac 101975k.

Palmieri, M. J., Berry, G. T., Player, D. A., Rogers, S., & Segal, S. (1991). The concentration of red-blood-cell UDP-glucose and UDP-galactose determined by high-performance liquid-chromatography. *Analytical Biochemistry*, *194*(2), 388–393. http://dx.doi.org/10.1016/0003-2697(91)90246-p.

Payne, S. M., & Ames, B. N. (1982). A procedure for rapid extraction and high-pressure liquid-chromatographic separation of the nucleotides and other small molecules from bacterial cells. *Analytical Biochemistry*, *123*(1), 151–161. http://dx.doi.org/10.1016/0003-2697(82)90636-4.

Piron, R., Santens, F., De Paepe, A., Depicker, A., & Callewaert, N. (2015). Using GlycoDelete to produce proteins lacking plant-specific N-glycan modification in seeds. *Nature Biotechnology*, *33*(11), 1135–1137. http://dx.doi.org/10.1038/nbt.3359.

Preinerstorfer, B., Schiesel, S., Lammerhofer, M. L., & Lindner, W. (2010). Metabolic profiling of intracellular metabolites in fermentation broths from beta-lactam antibiotics production by liquid chromatography-tandem mass spectrometry methods. *Journal of Chromatography A*, *1217*(3), 312–328. http://dx.doi.org/10.1016/j.chroma.2009.11.051.

Rabina, J., Maki, M., Savilahti, E. M., Jarvinen, N., Penttila, L., & Renkonen, R. (2001). Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography. *Glycoconjugate Journal*, *18*(10), 799–805. http://dx.doi.org/10.1023/a:1021107602535.

Ramm, M., Wolfender, J. L., Queiroz, E. F., Hostettmann, K., & Hamburger, M. (2004). Rapid analysis of nucleotide-activated sugars by high-performance liquid chromatography coupled with diode-array detection, electrospray ionization mass spectrometry and nuclear magnetic resonance. *Journal of Chromatography A*, *1034*(1–2), 139–148. http://dx.doi.org/10.1016/j.chroma.2004.02.023.

Rejzek, M., Mukhopadhyay, B., Wenzel, C. Q., Lam, J. S., & Field, R. A. (2007). Direct oxidation of sugar nucleotides to the corresponding uronic acids: TEMPO and platinum-based procedures. *Carbohydrate Research*, *342*(3–4), 460–466. http://dx.doi.org/10.1016/j.carres.2006.10.016.

Ritter, J. B., Genzel, Y., & Reichl, U. (2006). High-performance anion-exchange chromatography using on-line electrolytic eluent generation for the determination of more than 25 intermediates from energy metabolism of mammalian cells in culture. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *843*(2), 216–226. http://dx.doi.org/10.1016/j.jchromb.2006.06.004.

Rosti, J., Barton, C. J., Albrecht, S., Dupree, P., Pauly, M., Findlay, K., et al. (2007). UDP-glucose 4-epimerase isoforms UGE2 and UGE4 cooperate in providing UDP-galactose for cell wall biosynthesis and growth of *Arabidopsis thaliana*. *Plant Cell*, *19*(5), 1565–1579. http://dx.doi.org/10.1105/tpc.106.049619.

Sage, A., Wolff, C., Mussell, C., Neville, D., Lord, G., Saeed, M., et al. (2013). Guide to achieving reliable quantitative LC-MS measurements, In M. Sargent (Ed.), *RSC Analytical Methods Committee* (1st ed) (61 pp). Teddington, UK: LGC Limited. Retrieved from http://www.rsc.org/images/AMC%20LCMS%20Guide_tcm18-240030.pdf.

Samuel, G., & Reeves, P. (2003). Biosynthesis of O-antigens: Genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydrate Research*, *338*(23), 2503–2519. http://dx.doi.org/10.1016/j.carres.2003.07.009.

Seifar, R. M., Ras, C., van Dam, J. C., van Gulik, W. M., Heijnen, J. J., & van Winden, W. A. (2009). Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry. *Analytical Biochemistry*, *388*(2), 213–219. http://dx.doi.org/10.1016/j.ab.2009.02.025.

Smallcombe, S. H., Patt, S. L., & Keifer, P. A. (1995). WET solvent suppression and its applications to LC NMR and high-resolution NMR spectroscopy. *Journal of Magnetic Resonance Series A*, *117*(2), 295–303. http://dx.doi.org/10.1006/jmra.1995.0759.

Soo, E. C., Aubry, A. J., Logan, S. M., Guerry, P., Kelly, J. F., Young, N. M., et al. (2004). Selective detection and identification of sugar nucleotides by CE-electrospray-MS and its application to bacterial metabolomics. *Analytical Chemistry*, *76*(3), 619–626. http://dx.doi.org/10.1021/ac034875i.

Sweeney, C., Mackintosh, D., & Mason, R. M. (1993). UDP-sugar metabolism in Swarm rat chondrosarcoma chondrocytes. *Biochemical Journal*, *290*, 563–570.

Tomiya, N., Ailor, E., Lawrence, S. M., Betenbaugh, M. J., & Lee, Y. C. (2001). Determination of nucleotides and sugar nucleotides involved in protein glycosylation by high-performance anion-exchange chromatography: Sugar nucleotide contents in cultured insect cells and mammalian cells. *Analytical Biochemistry*, *293*(1), 129–137. http://dx.doi.org/10.1006/abio.2001.5091.

Turnock, D. C., & Ferguson, M. A. J. (2007). Sugar nucleotide pools of *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major*. *Eukaryotic Cell*, *6*(8), 1450–1463. http://dx.doi.org/10.1128/ec.00175-07.

Urbaniak, M. D., Turnock, D. C., & Ferguson, M. A. J. (2006). Galactose starvation in a bloodstream form *Trypanosoma brucei* UDP-glucose 4'-epimerase conditional null mutant. *Eukaryotic Cell*, *5*(11), 1906–1913. http://dx.doi.org/10.1128/ec.00156-06.

Veltkamp, S. A., Hillebrand, M. J. X., Rosing, H., Jansen, R. S., Wickremsinhe, E. R., Perkins, E. J., et al. (2006). Quantitative analysis of gemcitabine triphosphate in human peripheral blood mononuclear cells using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry. *Journal of Mass Spectrometry*, *41*(12), 1633–1642. http://dx.doi.org/10.1002/jms.1133.

Villiger, T. K., Steinhoff, R. F., Ivarsson, M., Solacroup, T., Stettler, M., Broly, H., et al. (2016). High-throughput profiling of nucleotides and nucleotide sugars to evaluate their impact on antibody N-glycosylation. *Journal of Biotechnology*, *229*, 3–12. http://dx.doi.org/10.1016/j.jbiotec.2016.04.039.

Wagner, G. K., Pesnot, T., & Field, R. A. (2009). A survey of chemical methods for sugar-nucleotide synthesis. *Natural Product Reports*, *26*(9), 1172–1194. http://dx.doi.org/10.1039/b909621n.

West, C., Elfakir, C., & Lafosse, M. (2010). Porous graphitic carbon: A versatile stationary phase for liquid chromatography. *Journal of Chromatography A*, *1217*(19), 3201–3216. http://dx.doi.org/10.1016/j.chroma.2009.09.052.

White-Phillip, J., Thibodeaux, C. J., & Liu, H. W. (2009). Complex enzymes in microbial natural product biosynthesis, part B: Polyketides, aminocoumarins and carbohydrates: Enzymatic synthesis of TDP-deoxysugars. *Methods in Enzymology*, *459*, 521–544. http://dx.doi.org/10.1016/S0076-6879(09)04621-7.

Zeeman, S. C., Kossmann, J., & Smith, A. M. (2010). Starch: Its metabolism, evolution, and biotechnological modification in plants. *Annual Review of Plant Biology*, *61*, 209–234. http://dx.doi.org/10.1146/annurev-arplant-042809-112301.