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Title	Periodic acid-Schiff's reagent assay for carbohydrates in a microtiter plate format
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Publication Date	2011
Publication Information	Kilcoyne, M, Gerlach, JQ, Farrell, MP, Bhavanandan, VP, Joshi, L (2011) 'Periodic acid-Schiff's reagent assay for carbohydrates in a microtiter plate format'. <i>Analytical Biochemistry</i> , 416 :18-26.
Link to publisher's version	http://dx.doi.org/10.1016/j.ab.2011.05.006
Item record	http://hdl.handle.net/10379/3641
DOI	http://dx.doi.org/DOI 10.1016/j.ab.2011.05.006

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Periodic acid-Schiff's reagent assay for carbohydrates in a microtitre plate format

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Short title: Periodic acid-Schiff's reagent microtitre plate assay

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Table of contents category: Carbohydrates

Abstract

Microtitre plate colourimetric assays are widely used for analysis of carbohydrates and glycoconjugates. However, mucins are often not easily detected, as they have low neutral sugar content. We have adapted and optimised the periodic acid-Schiff's reagent (PAS) staining for microtitre plate assay by examining five factors; concentration and volume of periodic acid, oxidation time, volume of Schiff's reagent and colour development time. This assay requires just 25 μL of sample, utilises standardised Schiff's reagent and has decreased assay time (140 minutes to completion). Seventeen monosaccharides (acidic, neutral, basic, phosphorylated and deoxy) and four disaccharides were assessed. PAS-positive carbohydrates (amino, *N*-acetylamino, deoxy and certain neutral monosaccharides and sialic acids) responded linearly within a 10 – 100 nmole range approximately, which varied for each carbohydrate. Assay response for fetuin and porcine gastric mucin (PGM) was linear up to 150 μg (highest concentration tested), with no response from non-glycosylated protein. A lower response for asialofetuin was observed, but desialylated PGM preparations were similar or higher in response than their sialylated counterparts. The simplicity and low sample consumption of this method makes it an excellent choice for screening or quantitation of chromatographic fractions containing carbohydrates and glycoconjugates, especially in the case of mucins.

Keywords: PAS assay, Schiff's reagent, periodate, mucins, glycoproteins

Colourimetric assays are widely used for both quantitative and qualitative analysis of carbohydrates occurring in polysaccharides, glycoproteins, glycolipids and proteoglycans. Measurement of UV absorbance at 280 nm is typically used to detect protein, but this approach is not suitable for detecting or differentiating the above molecules. For instance, polysaccharides and glycolipids do not absorb in the UV region and mucins absorb poorly, since they generally have no or negligible aromatic amino acid content [1;2]. Therefore, colourimetric assays are the preferred method for detection and analysis of complex carbohydrate molecules. Among the most popular of these assays are the phenol-sulfuric acid [3] and the Monsigny resorcinol [4] methods for neutral sugars, and to a lesser extent the Morgan-Elson method for free *N*-acetyl glycosylamines [5; 6] and the Park-Johnson assay for reducing sugars [7]. However, proteins and nucleic acids can interfere in these assays if present in high amounts. Further, the above assays are often unsuitable for assaying mucins and mucin-type glycoproteins and *O*-linked glycopeptides, as neutral sugars are not abundant in them and the *N*-acetyl glycosylamine constituents are occupied in glycosidic linkages.

Periodate oxidation of vicinal hydroxyl groups to aldehydes under aqueous conditions is a particularly suitable reaction for application to carbohydrates [8]. When this oxidation is paired with a subsequent reagent for colour development, e.g. Schiff's reagent, it is a useful method for qualitative or quantitative assay of carbohydrates. The periodic acid-Schiff's reagent (PAS)¹ stain is widely used in histology for visualising mucins [9; 10; 11], glycoproteins [12], glycogen [13] and other polysaccharides [14] in tissue and cells. PAS staining is also used for identifying the presence of polysaccharides, glycoproteins [15; 16] and mucins [17] in polyacrylamide or agarose gels and after transfer to membranes (blots). The PAS reaction has been adapted for a test tube method for screening chromatographic fractions [18; 19], but its use has been limited because of the relatively large volumes of sample (2 ml) and reagent required and the time-consuming reagent preparation.

We report here an optimised microtitre plate PAS assay, which is especially suitable for screening chromatographic fractions as it combines high sensitivity for sample detection with low reagent consumption. The commercial availability of stable Schiff's reagents has eliminated previous problems of in-house reagent inconsistency and has enabled development of a standardised, reproducible and reliable microtitre plate method with significantly decreased assay time.

Materials and methods

Materials

Concentrated (96%) acetic acid, periodic acid, *N*-acetylgalactosamine (GalNAc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), glucose (Glc), glucosamine (GlcN), mannose (Man), glucuronic acid (GlcU), cellobiose, lactose (Lac), xylose (Xyl), L-rhamnose (Rha), arabinose (Ara), trehalose, Schiff's reagent for aldehydes (cat. no. 84655), porcine gastric mucin type III (PGM), fetuin from fetal calf serum, asialofetuin (ASF), concentrated acetic acid (96%), cetylpyridinium chloride (CPC, hexadecylpyridinium chloride) and periodic acid were from Sigma-Aldrich Co. (Poole, U.K.). 2-Deoxy-D-galactose (dGal) and D-glucose-1-phosphate (Glc-1-P) disodium salt were obtained from Calbiochem (CA, USA). *N*-Acetyllactosamine (LacNAc), L-fucose (Fuc) and *N*-acetylneuraminic acid (Neu5Ac) were purchased from Dextra (Reading, U.K.). 3-Deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (Kdn), 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), *N*-glycolylneuraminic acid (Neu5Gc) were from Toronto Research Chemicals Inc. (Ontario, Canada). All carbohydrates were of the highest grade available. Coomassie Plus Assay Reagent was from Pierce (Thermo Fisher Scientific Inc.). Flat-bottomed 96-well microtitre plates and plastic seal covers for microtitre plates were from Sarstedt (Nümbrecht, Germany). A SpectraMax M5^e (Molecular Devices, CA, USA)

microplate reader with SoftMax® Pro software was used, which has PathCheck® Sensor technology to normalise the well absorbance automatically with respect to volume. All other chemicals were from Sigma-Aldrich Co. and were of the highest grade available.

Optimisation of microtitre plate-based PAS assay

The protocol was optimised using 25 µL of GalNAc solution per well (1 mg/ml, approximately 113 nmoles) and was initially based on the test tube method of Mantle [18]. Experiments for individual optimisation steps (as below) were carried out at least three times in a 96 well microtitre plate in triplicate. A 50% stock solution of periodic acid was made by dissolving 500 mg of periodic acid in 1 ml of water. This stock solution was stored at 4 °C for up to one week with good results.

Percentage periodic acid in 7% acetic acid. To 25 µL of GalNAc solution per well was added 100 µL of periodic acid solution in 7% acetic acid with mixing by pipette action. The percentage periodic acid ranged from 0 to 0.1%, prepared by adding 0 to 20 µL 50% periodic acid stock solution to 10 ml of 7% (v/v) concentrated acetic acid. (For the PAS assay, periodic acid in acetic acid solution was freshly prepared and used immediately). The mixture was incubated at 37 °C for 2 h and then 100 µL of Schiff's reagent was added with mixing by pipette action. The Schiff's reagent was stored at 4 °C, and an aliquot for use for the assay was allowed to come to room temperature 30 min before use and protected from light. The microtitre plate was shaken for 5 min and the purple colour allowed to develop for 25 min. An absorbance spectrum from 350 to 1000 nm was acquired and 550 nm was determined to be optimal for measuring absorbance for this assay. Absorbance was measured at 550 nm in all subsequent assays.

Volume of 0.06% periodic acid in 7% acetic acid. To 25 µL of GalNAc solution per well was added a range of 10 to 140 µL of 0.06% (2.6 mM) periodic acid solution in 7% acetic acid

with mixing by pipette action. The mixture was incubated at 37 °C for 2 h and then 100 µL of Schiff's reagent was added with mixing by pipette action. The microtitre plate was shaken for 5 min and allowed to develop for a further 25 min. Absorbance was then measured at 550 nm.

Optimal incubation time for oxidation of 0.06% periodic acid. To 25 µL of GalNAc solution per well was added 120 µL of 0.06% periodic acid solution in 7% acetic acid with mixing by pipette action. The mixture was incubated at 37 °C for various time intervals between 30 min to 3 h and then 100 µL of Schiff's reagent was added with mixing by pipette action. The microtitre plate was shaken for 5 min and allowed to develop for a further 25 min.

Absorbance was then measured at 550 nm.

Volume of Schiff's reagent added and time of colour development. To 25 µL of GalNAc solution per well was added 120 µL of 0.06% periodic acid solution in 7% acetic acid with mixing by pipette action. The mixture was incubated at 37 °C for 1.5 h and then a range of 20 to 120 µL of Schiff's reagent was added with mixing by pipette action. The microtitre plate was shaken for 5 min and allowed to develop. Absorbance at 550 nm was measured after a further 10, 25, 40 and 55 min.

Assessment of periodate reaction medium pH on colour development

Five carbohydrates (GalNAc, Neu5Gc, Gal, Lac and Fuc) were assayed in five replicates for response *versus* concentration using the optimised PAS assay (as above, absorbance was taken 45 min after addition of Schiff's reagent), except substituting 50 mM sodium acetate, pH 4 or sodium phosphate, pH 7 for 7% acetic acid. The absorbance spectrum was also measured (when possible) at 10 nm intervals between 350 and 1000 nm.

Precipitation of PGM and preparation of asialo glycoconjugates

PGM was dissolved in 100 mM NaCl at 10 mg/ml and a 10% suspension of CPC was added at 5 mg/ml, shaken and left for 1 h at room temperature to allow mucin to precipitate. The precipitate was pelleted at 2,000 rpm for 10 min, the supernatant was taken off and the pellet was re-dissolved in minimum volume 1 M NaCl. The redissolved pellet was precipitated by adding ethanol to 80% (v/v), left for 30 min at room temperature and precipitate was collected again by centrifugation. The mucin was washed with 90% ethanol and lyophilised [20].

A portion of PGM supernatant (PGM Sp) and pellet (PGM Pel) were treated with 0.1 N HCl at 80 °C for 1 h to generate asialoPGM Sp (aPGM Sp) and asialoPGM Pel (aPGM Pel). The desialylated PGM preparations were neutralised with NaOH, dialysed against water to remove the released sialic acid and lyophilised. In addition, the ASF was also dialysed against water and then lyophilised.

Linearity and absorbance spectra

Fetuin, ASF, PGM, PGM Sp, PGM Pel, aPGM Sp, aPGM Pel and other carbohydrates were assayed in five replicates for linearity of response *versus* concentration using the optimised PAS assay (as above). The absorbance spectrum was also measured (when possible) at 10 nm intervals between 350 and 1000 nm. Data were compared after export to Excel (Microsoft).

Results

To develop a microtitre plate assay based on the PAS reaction the following five factors were optimised: (i) the percentage of periodic acid, (ii) the volume of periodic acid solution, and (iii) the incubation time required for optimal carbohydrate oxidation, (iv) the volume of Schiff's reagent and (v) incubation time needed for maximal colour development. A sample

volume of 25 μL was selected, as the consumption of only a small quantity of chromatographic fractions for screening is desirable, and GalNAc was chosen as the test carbohydrate, as it is a ubiquitous component of mucin-type oligosaccharides.

(i) Percentage periodic acid mixture- A range of 0 to 0.1% periodic acid in 7% acetic acid was tested to determine the optimal concentration for colour development of carbohydrate while minimising background interference. The use of acetic acid alone did not produce any colour while colour development measured by absorbance at 550 nm increased with increasing periodic acid concentration and reached a plateau at 0.09% periodic acid (Fig. 1A). All concentrations of periodic acid gave good colour evolution when the periodic acid solutions were used within 10 minutes of preparation, but higher concentrations of periodic acid solutions tested (0.25, 0.5, 1, 2.5 and 5%) also all resulted in false positives in the blanks. In addition, when the periodic acid solutions of the lower concentration range (0 to 0.1%) were used one hour after preparation, high blank values were obtained. Therefore 0.06% periodic acid was selected for this assay and the periodic acid mixture was used within 10 minutes of preparation for reproducibility.

(ii) Volume of periodic acid and (iii) incubation time- Different volumes (10 to 140 μL) of 0.06% periodic acid (2.6 mM) in 7% acetic acid were tested. Higher absorbance was observed with higher volumes (Fig. 1B). Since the assay was carried out in a microtitre plate, the maximum total volume was limited to 250 μL per well, therefore 120 μL of the periodic acid solution was chosen for the assay. Optimum colour development was reached at 1.5 hours incubation after addition of periodic acid (Fig. 1C).

(iv) *Volume of Schiff's reagent and (v) incubation time*- Schiff's reagent was allowed to reach room temperature prior to addition to facilitate a speedier reaction time. After addition of Schiff's reagent, a transient yellow colour was observed which dissipated upon mixing the solution by pipette action. When lower volumes of Schiff's reagent were used, colour was evolved immediately, even in the blank (water) (Fig. 2A), which indicates that an excess of Schiff's reagent is required to drive the reaction forward and prevent the reaction stopping at the formation of an intermediate. The colour response for water and carbohydrate varied according to the amount of Schiff's reagent added, but at 80 μL and above no colour evolved in the blank and a true positive was apparent in the wells containing GalNAc. A longer incubation time post-Schiff's reagent addition resulted in increased response (Fig. 2B).

Final optimised microplate periodic acid/Schiff's reagent assay

To 25 μL of sample in a microtitre plate well, 120 μL of freshly prepared solution of 0.06% periodic acid in 7% acetic acid was added and mixed by pipette action. The microtitre plate was covered with a plastic seal and incubated at 37 °C for 1.5 hours. The mixture was allowed to cool to room temperature and 100 μL of Schiff's reagent at room temperature was added with mixing by pipette action. The microtitre plate was covered with a plastic seal, shaken for 5 minutes and colour allowed to develop at room temperature for a further 40 minutes. The plastic seal was removed and absorbance read at 550 nm. The whole assay takes approximately 2 hours and 20 minutes.

Relative absorbance and absorption spectra

Seventeen monosaccharides of different classes (acidic, neutral, basic, phosphorylated and deoxy sugars) and four disaccharides were assessed for colour response (see Table 1).

Absorbance spectra from 350 to 1000 nm were recorded for sugars that produced a coloured

product (Fig. 3A and B). No colour developed in the cases of Fuc, Lac, LacNAc, trehalose, cellobiose, GlcU, Glc-1-P and Rha, although the transient yellow colour formed upon addition of Schiff's reagent at higher concentrations of sugars. The sialic acids tested, Neu5Ac, Neu5Gc and Kdo, gave the highest response, followed by dGal, GalNAc and GlcNAc. GlcN and the neutral sugars Ara, Xyl, Gal, Glc and Man were the least responsive. Absorbance at 550 nm gave a good response for most sugars which formed the purple coloured complex in this assay.

Effect of pH during periodate oxidation on colour development

The pH of the periodate reaction medium is known to be important for the oxidation of carbohydrates [8]. Five carbohydrates (GalNAc, Neu5Gc (high responders), Gal (low responder), Lac and Fuc (no response)) were selected for assessment of the reaction medium pH using 50 mM sodium acetate, pH 4 and sodium phosphate, pH 7 compared to 7% acetic acid (pH 2.2 approximately) in the optimised PAS assay (as above). No response was noted from Fuc and Lac in any of the periodate reaction media, and a lower response was noted for the higher colour producing carbohydrates, GalNAc and Neu5Gc, at pH 4 compared to 7% acetic acid, with the response further lowered at pH 7 (Table 2). In agreement, Gal also produced less colour at pH 4, but there was a greater response at pH 7 compared to 7% acetic acid (Table 2). Of note, the absorbance spectrum of Gal at pH 7 (not shown) was more similar to Fig. 3A than Fig. 3B (i.e. the λ_{\max} was approximately 560 nm as opposed to 590 nm). Similar response trends in concentration of carbohydrates tested *versus* absorbance were observed across all reaction media (not shown, see Fig. 4A and B for example). As the purpose of this assay is to provide a means of detecting and analysing glycoconjugates that are low in neutral sugar content, the periodate reaction medium was maintained as 7% acetic acid for the optimised assay.

Linearity of concentration vs absorbance

All carbohydrates that reacted positively gave linear responses in the PAS assay, within a particular range (see Table 1). The absorbance of the highest responding sugars, including GlcN, plateaued at higher concentrations (Fig. 4A) while with the neutral sugars, including dGal, the absorbance peaked at less than 100 nmoles of carbohydrate, then decreased to a constant level at higher concentrations (Fig. 4A (dGal) and B). The response of fetuin, a glycoprotein which has both *N*- and *O*-linked oligosaccharides [21; 22; 23], ASF, PGM, PGM Sp, PGM Pel, aPGM Sp, aPGM Pel and RNase A was linear up to 150 μ g (125 μ g for aPGM Pel), the maximum concentration tested (Fig. 4C).

Discussion

It was apparent from our studies that the use of higher concentrations of periodic acid for this assay is not advisable as it may result in false positives. A sufficient volume of Schiff's reagent was also required to drive the reaction to completion. An insufficient amount of Schiff's reagent halted the reaction at a transition step or intermediate compound formation. In the presence of light, sodium metaperiodate is known to slowly spontaneously decompose and overoxidation of cellulose is known to occur, with only the theoretical one molar equivalent of periodic acid being taken up in the dark [8]. However, the effect of light is important only in long term reactions (i.e. in the order of days) [8], and therefore was not considered significant for this assay (just under 2.5 hours).

The low response of neutral monosaccharides Xyl, Ara, Glc, Man and Gal may be due to the aldehyde groups, generated after loss of formic acid, being in a non-optimal configuration for reaction with the Schiff's reagent [19]. This observation is supported by the

strong colour response of dGal, which follows the same concentration response trend as the other neutral sugars which gave lower absorbance (Fig. 4A). The lack of a hydroxyl group at the C-2 position of dGal (Fig. 5, structure 3) does not allow the loss of formic acid and therefore the reactive aldehyde groups may remain in closer proximity on a more rigid structure, thus favouring the formation of the coloured product. Similarly, the *N*-acetyl monosaccharides GalNAc and GlcNAc do not have a hydroxyl group at position C-2 (Fig. 5, structures 2 and 5, respectively) and may produce the structurally favoured oxidation product, which could be stabilised by the acetyl group on the electron-withdrawing nitrogen. This observation is supported by the low colour response of GlcN (Fig. 5, structure 6), which lacks the stabilising acetyl group but follows the same concentration response trend of GlcNAc and GalNAc. The sialic acids (Neu5Ac, Neu5Gc, Kdo and Kdn) gave a strong response in the PAS assay, which may be due to the more rapid oxidation with periodate of the exocyclic side chain which are known to be highly susceptible to even mild periodate oxidation conditions [24; 25].

The lack of colour evolution of cellobiose, trehalose, LacNAc, Lac, Fuc, GlcU, Glc-1-P and Rha may be due to unfavourable configuration of reactive aldehydes after oxidation, in certain cases by distortion of the oxidised structure by electron-withdrawing groups (e.g. GlcU and Glc-1-P (Fig. 5, structures 12 and 21, respectively)).

Periodate oxidation is very often carried out in water, acetate buffer at pH 4 – 4.5 or acetic acid for carbohydrate and histochemical applications, and it has been shown that the rate of oxidation in aqueous acetic acid is slower than that in water [8]. The pH of the periodate reaction medium is known to have an important effect on colour development, and for periodate oxidation of carbohydrates a lower pH range is preferable [8]. In agreement with literature [8], the higher pH of 7 tested resulted in less colour production for GalNAc and Neu5Gc, but Gal actually gave a greater response. This may be due to ‘overoxidation’,

which is known to be favoured at higher pH, or indeed, Gal may be most readily oxidisable at this pH in common with certain amino alcohols and partially methylated sugars [8].

The non-glycosylated protein RNase A had a very low response in this assay and ASF gave a lower response than fetuin, as expected after removal of the strongly responsive sialic acids. aPGM Sp gave only slightly lower response than PGM Sp, but remarkably aPGM Pel actually gave a greater response than PGM Pel, which supports the use of this assay for mucins.

The microtitre plate PAS assay optimised in this work did not suffer from problems of interference from protein (RNase A, Fig. 4C) or buffer (50 mM NaCl, 100 mM pyridinium acetate, not shown) under the conditions optimised in this work. The responses of individual sugars in the PAS assay were linear within a certain range of concentration, which varied slightly depending on the identity of the sugar, and a linear response was observed for glycoproteins and mucins up to 150 μ g. The simplicity of this method combined with the low sample consumption makes it an excellent choice for assaying a large number of chromatographic fractions containing carbohydrates, mucins and mucin-type glycoproteins, glycoproteins and proteoglycans for either screening or quantitation purposes.

Acknowledgements

This material is based upon works supported by the Science Foundation Ireland under grant no. 08/SRC/B1393, in support of the Alimentary Glycoscience Research Cluster (AGRC).

References

- [1] P. Wang, R.R. Granados, An intestinal mucin is the target substrate for a baculovirus enhancer, Proc. Natl. Acad. Sci. USA 94 (1997) 6977-6982.
- [2] T.H. Thanka Christlet, K. Veluraja, Database analysis of *O*-glycosylation sites in proteins, Biophys. J. 80 (2001) 952-960.
- [3] T. Masuko, A. Minami, N. Iwasaki, T. Majima, S.-I. Nishimura, Y.C. Lee, Carbohydrate analysis by a phenol-sulfuric acid method in microplate format, Anal. Biochem. 339 (2005) 69-72.
- [4] M. Monsigny, C. Petit, A.-C. Roche, Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod, Anal. Biochem. 175 (1988) 525-530.
- [5] J.L. Reissig, J.L. Strominger, L.F. Leloir, A modified colorimetric method for the estimation of *N*-acetylamino sugars, J. Biol. Chem. 217 (1955) 959-966.
- [6] V.P. Bhavanandan, M. Sheykhnazari, H. Devaraj, Colorimetric determination of *N*-acetylhexosamine-terminating *O*-glycosidically linked saccharides in mucins and glycoproteins, Anal. Biochem. 188 (1990) 142-148.
- [7] J.T. Park, M.J. Johnson, A submicrodetermination of glucose, J. Biol. Chem. 181 (1949) 149-151.
- [8] J.M. Bobbitt, Periodate oxidation of carbohydrates. Adv. Carb. Chem. 11 (1956) 1-41.
- [9] J. More, J. Fioramonti, L. Bueno, Changes in gastrointestinal mucins caused by attapulgate. Experimental study in rats, Gastroenterol. Clin. Biol. 16 (1992) 988-993.
- [10] U. Schumacher, M. Duku, M. Katoh, J. Jörns, W.J. Krause, Histochemical similarities of mucins produced by Brunner's glands and pyloric glands: A comparative study, Anat. Rec. A Discov. Mol. Cell. Evol. Biol. 278A (2004) 540-550.

- [11] L. Janke, C.S. Carlson, C.A. St. Hill, The novel carbohydrate tumor antigen C2-O-sLex is upregulated in canine gastric carcinomas. *Vet. Pathol.* 47 (2010) 455-461.
- [12] P. Francesco, D. Silvana, T. Luca, F. Oliviero, Histochemical detection of the lectin-binding carbohydrates in the zona pellucida during oocyte growth in the wild boar (*Sus scrofa scrofa*), *Vet. Res.* 32 (2001) 581-590.
- [13] K. De Bock, W. Derave, M. Ramaekers, E.A. Richter, P. Hespel, Fiber type-specific muscle glycogen sparing due to carbohydrate intake before and during exercise, *J. Appl. Physiol.* 102 (2007) 183-188.
- [14] A. Speranza, G.L. Calzoni, E. Pacini, Occurrence of mono- or disaccharides and polysaccharide reserves in mature pollen grains, *Sex. Plant Reprod.* 10 (1997) 110-115.
- [15] F. Lahnsteiner, Characterization of seminal plasma proteins stabilizing the sperm viability in rainbow trout (*Oncorhynchus mykiss*), *Anim. Reprod. Sci.* 97 (2007) 151-164.
- [16] M. Kilcoyne, M. Shah, J.Q. Gerlach, V. Bhavanandan, V. Nagaraj, A.D. Smith, K. Fujiyama, U. Sommer, C.E. Costello, N. Olszewski, L. Joshi, *O*-glycosylation of protein subpopulations in alcohol-extracted rice proteins. *J. Plant Physiol.* 166 (2009) 219-232.
- [17] Y. Goso, K. Hotta, Dot blot analysis of rat gastric mucin using histochemical staining methods, *Anal. Biochem.* 223 (1994) 274-279.
- [18] M. Mantle, A. Allen, A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain, *Biochem. Soc. Trans.* 6 (1978) 607-609.
- [19] S. Kirkeby, D. Moe, T. Bøg-Hansen, C. Garbarsch, M.E. Matthiessen, Quantitative PAS assay of some carbohydrate compounds and detergents, *J. Biochem. Biophys. Methods* 24 (1992) 225-238.

- [20] R.A. Stanley, S.P. Lee, A.M. Robertson, Heterogeneity in gastrointestinal mucins, *Biochim. Biophys. Acta* 760 (1983) 262-269.
- [21] G. Zauner, C.A.M. Koeleman, A.M. Deelder, M. Wührer, Protein glycosylation analysis by HILIC-LC-MS of proteinase K-generated *N*- and *O*-glycopeptides, *J. Sep. Sci.* 33 (2010) 903-910.
- [22] E.D. Green, G. Adelt, J.U. Baenziger, S. Wilson, H. Van Halbeek, The asparagine-linked oligosaccharides on bovine fetuin. Structural analysis of *N*-glycanase-released oligosaccharides by 500-megahertz ¹H NMR spectroscopy, *J. Biol. Chem.* 263 (1988) 18253-18268.
- [23] R.G. Spiro, V.D. Bhoyroo, Structure of the *O*-glycosidically linked carbohydrate units of fetuin, *J. Biol. Chem.* 249 (1974) 5704-5717.
- [24] J.A. Kiernan, *Histological and Histochemical Methods*, Hodder Arnold, New York, 2005.
- [25] L. Van Lenten, G. Ashwell, Studies on the chemical and enzymatic modification of glycoproteins, *J. Biol. Chem.* 246 (1971) 1889-1894.

Footnotes

¹ *Abbreviations used:* PAS, Periodic acid-Schiff's reagent; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; GlcU, glucuronic acid; Lac, lactose; Xyl, xylose; Rha, L-rhamnose; Ara, arabinose; PGM, porcine gastric mucin type III; ASF, asialofetuin; CPC, cetylpyridinium chloride; dGal, 2-deoxy-D-galactose; Glc-1-P, glucose-1-phosphate; LacNAc, *N*-acetylglucosamine; Fuc, L-fucose; Neu5Ac, *N*-acetylneuraminic acid; Kdn, 3-Deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; Neu5Gc, *N*-glycolylneuraminic acid; PGM Sp, PGM supernatant; PGM Pel, PGM pellet; aPGM Sp, asialoPGM Sp; aPGM Pel, asialoPGM Pel.

Legends to figures

Fig. 1. The effect of (A) increasing percentage of periodic acid in 7% acetic acid, (B) increasing volume of 0.06% percentage of periodic acid in 7% acetic acid, and (C) duration of incubation time at 37 °C post-periodic acid addition on the absorbance of the coloured product of GalNAc at 550 nm.

Fig. 2. (A) Influence of the volume of Schiff's reagent on colour development with water at 550 nm. (B) Influence of the volume of Schiff's reagent on colour development with GalNAc at 550 nm.

Fig. 3. (A) Absorption spectra of colour products from amino, *N*-acetylamino and deoxy sugars and sialic acids. (B) Absorption spectra of colour products from non-amino neutral sugars.

Fig. 4. Response of absorbance at 550 nm with varying concentration for (A) amino and deoxy sugars and sialic acids, (B) neutral monosaccharides, and (C) PGM ($R^2 = 0.9959$), PGM Sp ($R^2 = 0.9948$), PGM Pel ($R^2 = 0.991$), aPGM Sp ($R^2 = 0.9955$), aPGM Pel ($R^2 = 0.9990$), fetuin ($R^2 = 0.9988$), ASF ($R^2 = 0.9993$) and RNase A ($R^2 = 0.9743$).

Fig. 5. Structures of carbohydrates assayed. **1**, Gal, **2**, GalNAc, **3**, dGal, **4**, Glc, **5**, GlcNAc, **6**, GlcN, **7**, Man, **8**, Rha, **9**, Xyl, **10**, Fuc, **11**, Lac, **12**, GlcU, **13**, LacNAc, **14**, Ara, **15**, trehalose, **16**, cellobiose, **17**, Neu5Ac, **18**, Kdo, **19**, Kdn, **20**, Neu5Gc, **21**, Glc-1-P.

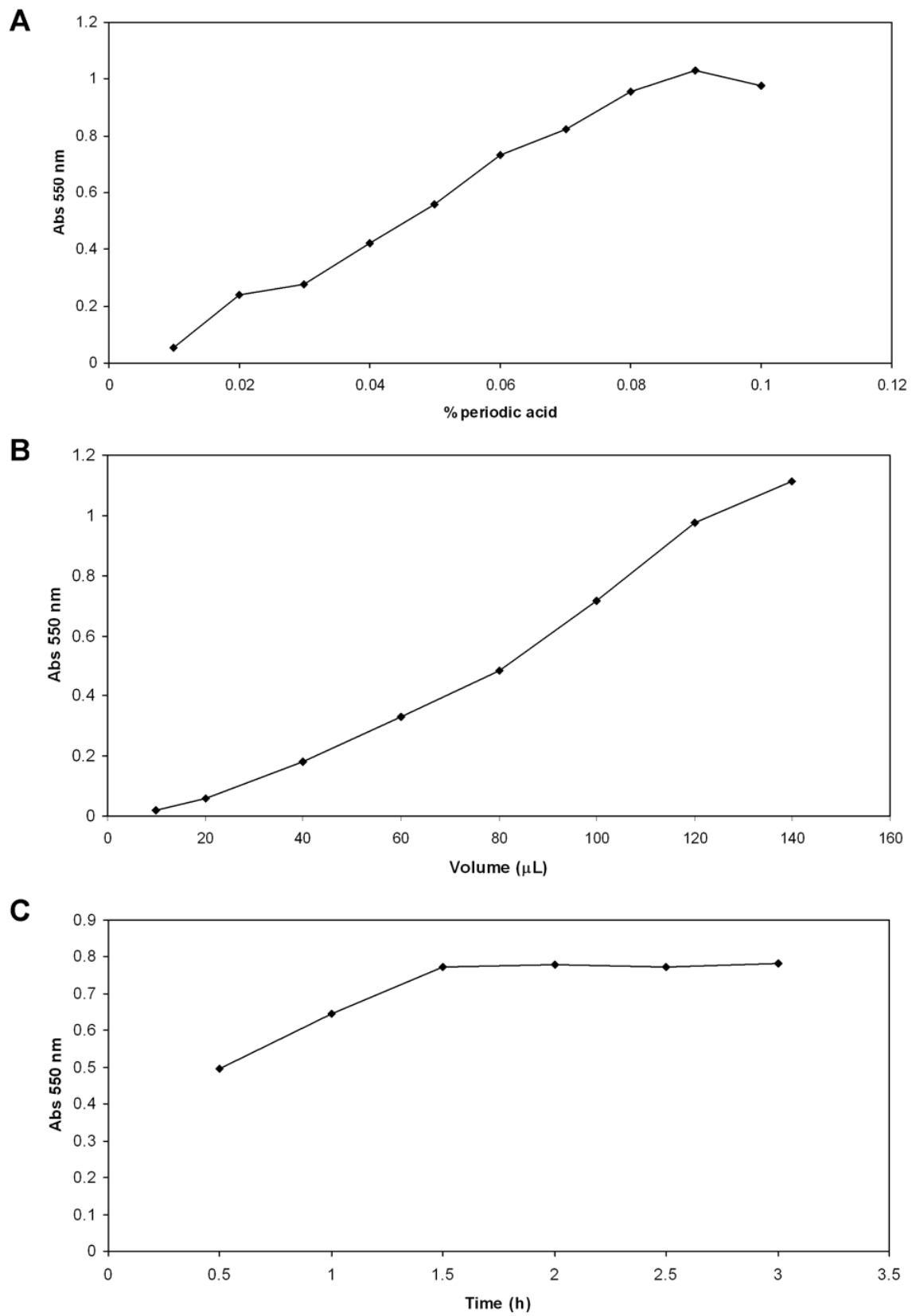


Figure 1

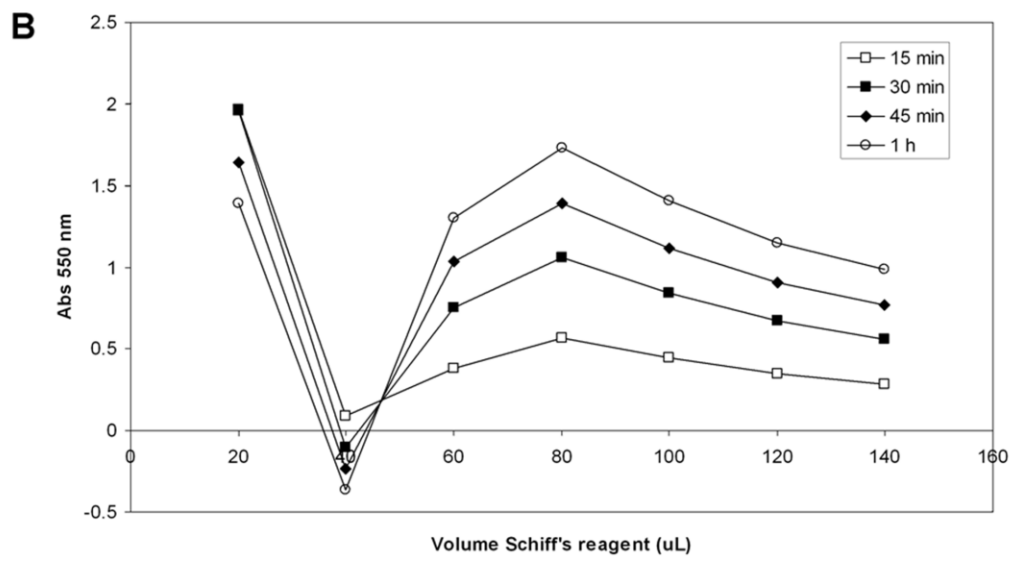
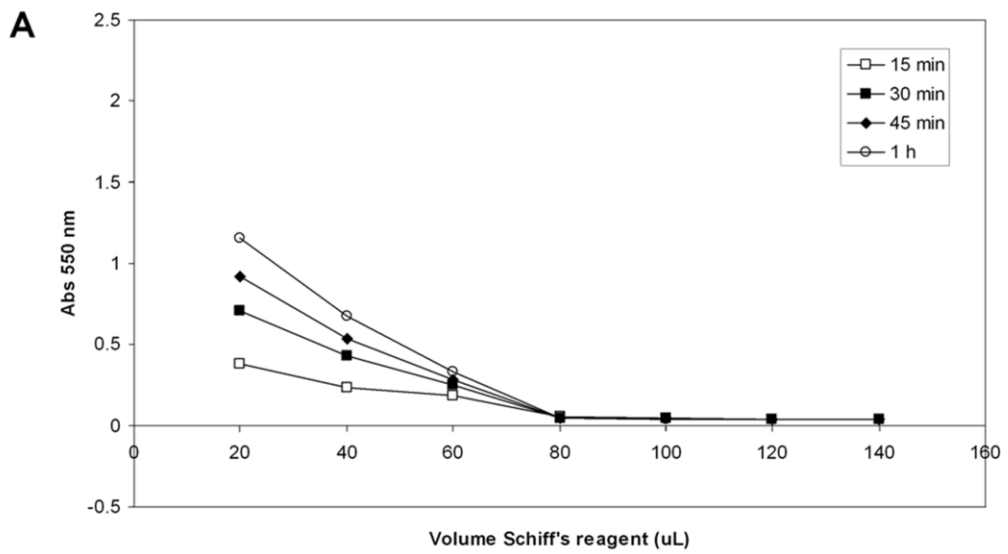


Figure 2

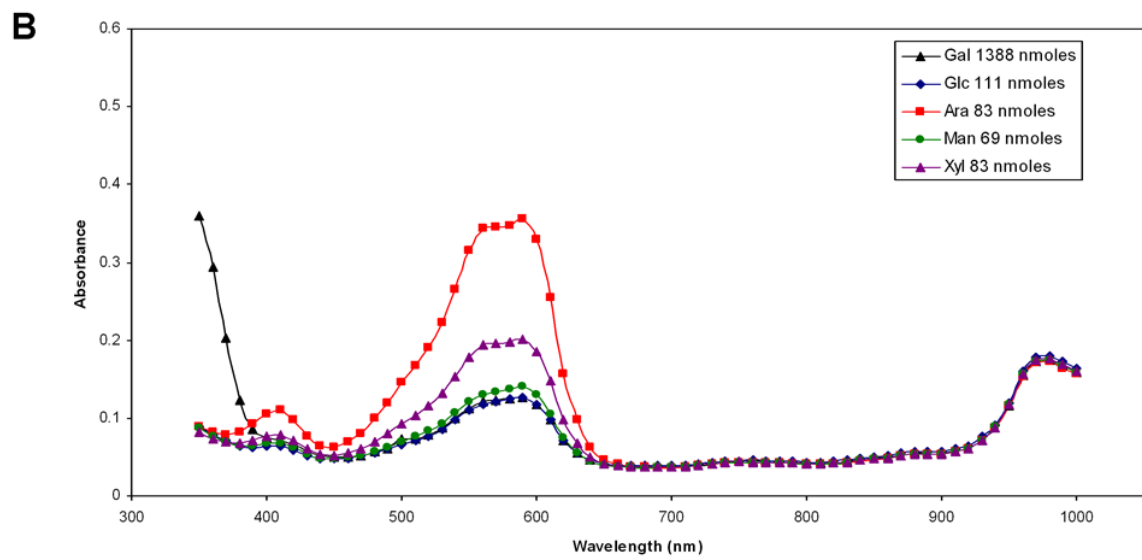
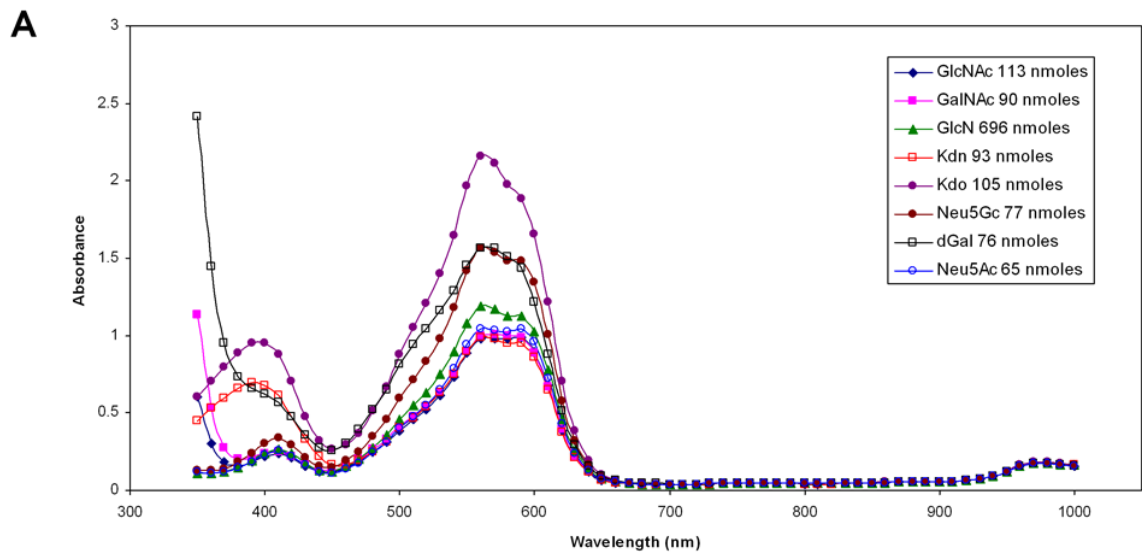


Figure 3

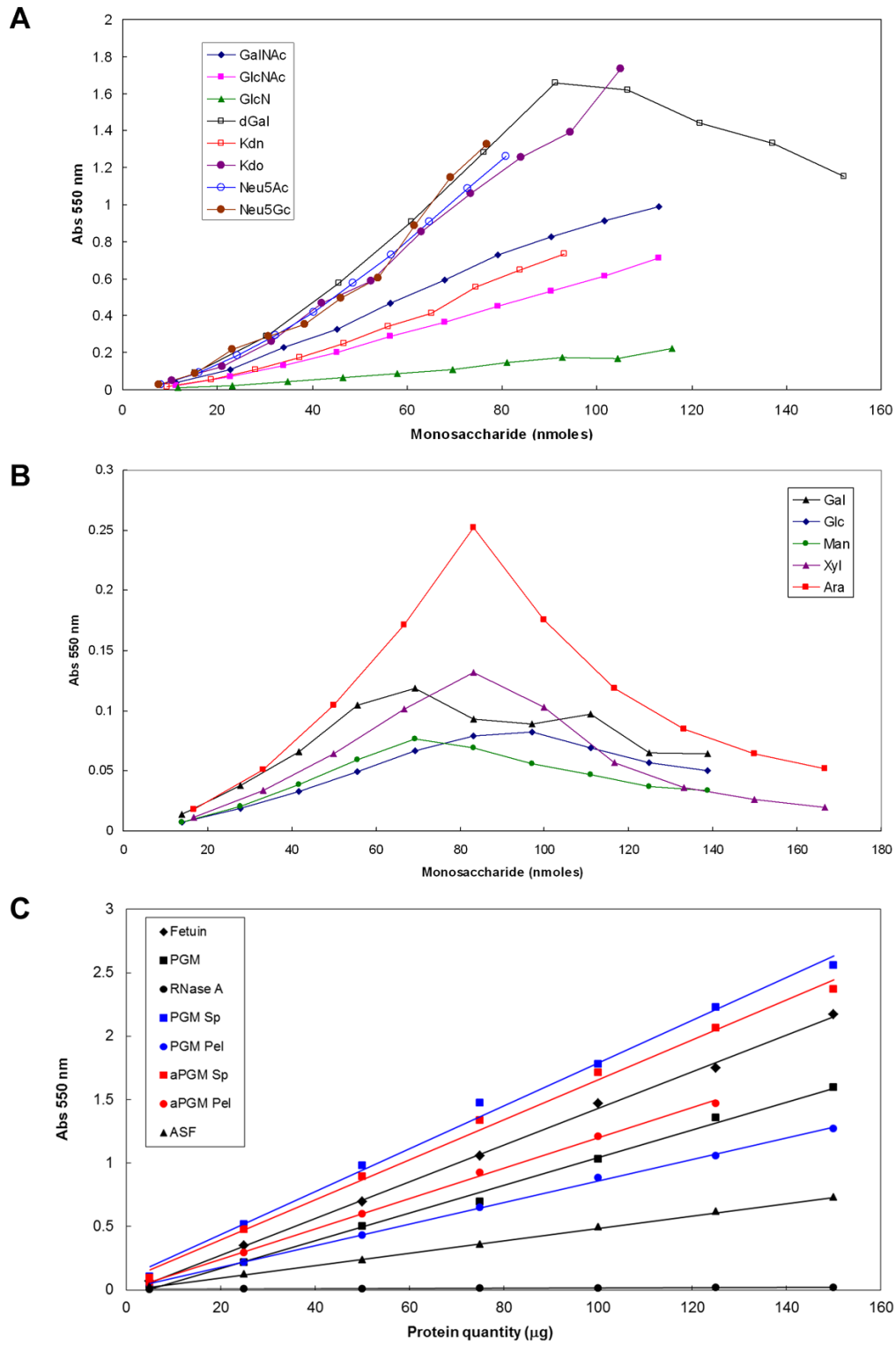


Figure 4

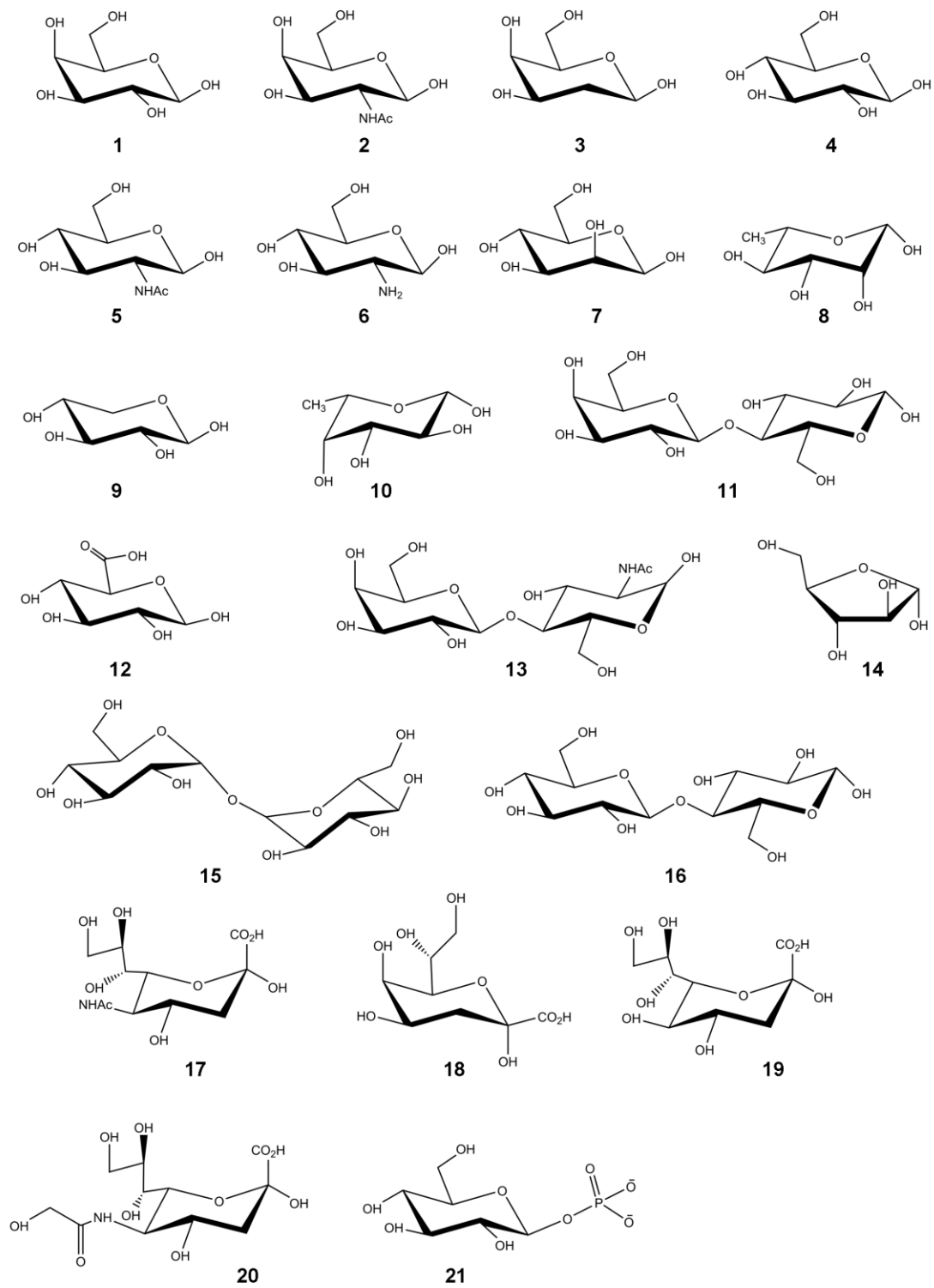


Figure 5