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A comprehensive HPLC analytical system for the identification and quantification of hexoses that employs 2-aminobenzamide coupling

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Kayo Hasehira[1], Shin-ichi Nakakita[1], Nobumitsu Miyanishi[2], Wataru Sumiyoshi[2], Shie Hayashi[1], Kaoru Takegawa[3] and Jun Hirabayashi[2,*]

[1]Department of Functional Glycomics, [2]Department of Glyco-Bioindustry, Life Science Research Center, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793 and [3]Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

*Jun Hirabayashi, Department of Glyco-Bioindustry, Life Science Research Center, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Tel: +81 87 891 2409, Fax: +81 87 891 2410; E-mail: glycofun@kms.ac.jp

Rare sugars are defined as monosaccharides with extremely low natural abundances. Their natural distributions and biological functions remain to be clarified. To establish a robust analytical system that can separate, identify and quantify rare sugars, 12 D-hexoses—including five rare aldohexoses and three rare ketohexoses—were labelled with 2-aminobenzamide (2-AB), and the fluorescently tagged monosaccharides were separated by high-performance liquid chromatography (HPLC). Because the ketohexoses were much less reactive than were the aldohexoses, the reaction conditions were optimized to achieve the maximum yields (>75%) for both aldohexoses and ketohexoses. The calibration curve determined for the rare ketohexose, D-psicose (Psi), was linear between 1 pmol and 1 μmol and had a correlation coefficient of 0.9999. Using the developed method, the psicose content in a leaf of *Itea virginica*, in which the presence of psicose has previously been reported, was found to be 2.7 mg psicose/g leaf. The result proved feasibility of the method even for natural products. Because the system is a comprehensive tool, it should help answer questions concerning the biosyntheses and functions of rare hexose sugars.

Keywords: aldohexose/2-aminobenzamide/fluorescence labelling/ketohexose/rare sugar.

Abbreviations: All, allose; Alt, altrose; 2-AB, 2-aminobenzamide; 2-AP, 2-aminopyridine; Fru, fructose; Gal, galactose; Glc, glucose; Gul, gulose; HPLC, high performance liquid chromatography; Ido, idose; Man, mannose; PA-, pyridylaminated; Psi, psicose; Sor, sorbose; Tag, tagtose; Tal, talose.

Rare sugars are defined as monosaccharides that exist in nature in extremely small amounts. They include the aldohexoses, D-/L-allose (All), D-/L-altrose (Alt), L-galactose (Gal), L-glucose (Glc), D-/L-gulose (Gul), D-/L-idose (Ido), L-mannose (Man) and D-/L-talose (Tal), and the ketohexoses, L-fructose (Fru), D-/L-psicose (Psi), D-/L-sorbose (Sor), and D-/L-tagatose (Tag). Izumori and colleagues have extensively studied the converting enzyme, D-tagatose 3-epimerase (*1*, *2*), which has been used to systematically produce rare sugars (*3*–*5*). Therefore, rare sugars can now be considered to be research reagents, as their biological and physiological properties have been extensively characterized (*6*–*10*). Rare sugars are of commercial value in the food, pharmaceutical, and nutritional industries because they can be used as low calorie sweeteners—they are difficult to digest (*6*, *7*)—as inhibitors of cancer cell proliferation (*8*), as inhibitors of microbial growth (*9*), and as memory enhancers (*10*). However, little is known about where and to what extent they are found in nature and in artificially prepared food products, primarily because there is no systematic method to detect and quantify them. Thus, this knowledge void inhibits understanding the biological significance(s) of rare sugars.

Rare sugars are essentially absent in nature in that their sugar nucleotides are not involved in glycan biosynthesis. In fact, there are only few reports of naturally occurring rare sugars (*11*–*13*). These include the finding of Psi in the plant *Itea virginica*. Conversely, rare sugars have been found in certain artificially prepared food products probably as a result of a chemical conversion, *i.e.* the Lobry de Bruyn-Alberda van Ekenstein transformation, from the more abundant common sugars, *e.g.* D-Glc, D-Fru (*14*–*16*). Therefore, it remains to be clarified if rare sugars are essentially absent in nature or if they exist more widely than has been recognized to date. There has been no reliable method that not only identifies, but also quantifies, the compositions of monosaccharide mixtures that include rare sugars. Various techniques have been applied to detect rare sugars, including an HPLC system connected to a refractive index detector (*4*), a biosensor that uses enzyme reactions (*17*), and a high pH anion-exchange chromatography system coupled to a pulsed amperometric detector (*16*). However, none of these systems has achieved the necessary sensitivity, *i.e.* picomole level detection, required to identify naturally occurring rare sugars. Nakakita and colleagues (*18*) recently reported a highly sensitive method for the separation and identification of D-aldoses including the six rare sugars, Lyx, Tal, Gul, Ido, All and Alt, by combining pyridylamination of the sugars with conventional HPLC separation. Unfortunately, the method cannot

be readily applied to ketoses, such as Psi, for which the natural occurrence has been of special interest (*12*, *13*). Ketoses are pyridylaminated much less readily than are aldoses (*19*, *20*).

Among the monoamine-coupling methods targeting oligosaccharides, two fluorescence-labelling reagents, 2-aminopyridine (2-AP) (*21*) and 2-aminobenzamide (2-AB) (*22*) have widely been used, because they are useful for both the separation and the detailed structural analysis of oligosaccharides derived from glycoproteins (*23*, *24*) and glycolipids (*25*, *26*), as well as the free oligosaccharides (*27*, *28*). However, such oligosaccharides are basically composed of aldoses (mostly aldohexoses), and thus, seldom contain ketoses. Therefore, the methods have only been occasionally attempted using ketoses (*19*, *20*). An important point to keep in mind when developing a detection/separation system for ketoses is that unlike aldoses, two stereoisomeric *R*/*S* (at the C2 carbon) reaction products will be formed (Fig. 1).

For the work reported herein, the D-aldohexoses, Glc, Man, All, Alt, Gul, Ido, Gal and Tal, and the ketohexoses, Fru, Psi, Tag and Sor, were coupled with 2-AB, but not 2-AP, and the 2-AB sugars were separated using various modes of liquid chromatography: gel filtration, reversed-phase chromatography, and normal-phase chromatography. To validate the reproducibility and sensitivity of the separation system, the types and the amounts of hexoses contained in a leaf of *I. virginica* were determined, and the rare sugar, Psi, was found. Although Psi has been found in the leaves of *I. virginica* previously (*12*, *13*), ours is the first study to quantify the amount of Psi present.

Materials and Methods

Materials

D-All, D-Tal and 2-AB were purchased from Tokyo Chemical Industry (Tokyo, Japan). D-Alt, D-Gul and D-Ido were obtained from Funakoshi (Tokyo, Japan). D-Fru, sodium cyanotrihydroborate ($NaBH_3CN$), acetic acid and dimethyl sulphoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan). D-Glc, D-Man, D-Gal and a Cosmosil 5C18-AR column (6 × 250 mm) were obtained from Nakarai Co., Ltd (Kyoto, Japan). D-Psi, D-Tag and D-Sor were obtained from Fushimi Pharmaceutical Co., Ltd (Kagawa, Japan). The 2-AB Glucose Homopolymer Standard was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). The TSKgel HW-40F resin was obtained from Tosoh (Tokyo, Japan). An Inertsil NH2 column (4.6 × 150 mm)

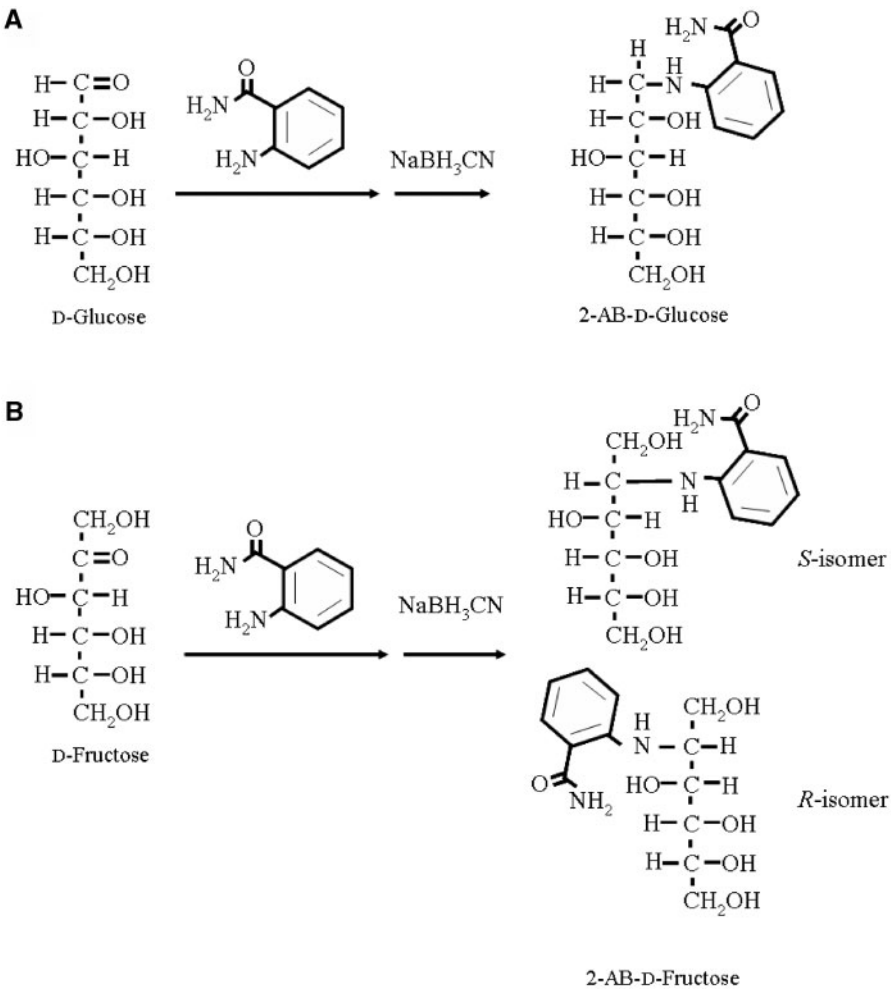

Fig. 1 The reaction of an aldohexose, (D-Glc; top) and of a ketohexose (D-Fru; bottom) with 2-AB. Note that when ketoses are subjected to monoamine-coupling and subsequent reduction, *R*/*S* isomers are generated at the C2 carbon (B). Whereas, only one product is produced for each of the aldohexoses (A).

was obtained from GL Science Inc. (Tokyo, Japan). An Ultrasphere ODS column (2.0 × 500 mm) was obtained from Beckman Coulter (Fullerton, CA, USA).

Fluorescent labelling

The previously established method that was used to derivatize oligosaccharides with 2-AB was modified to accommodate the lesser reactivities of the ketoses (*19*, *20*). Briefly, sugars (both aldoses and ketoses) were dissolved into 20 µl of a DMSO:acetic acid (7:3, v/v) solution containing various amounts (1–15 mg) of 2-AB and 1.0 M NaBH3CN. The mixtures were held at various temperatures (between 65°C and 90°C) for 3–36 h. After 20 µl of methanol and 40 µl of toluene were added to a reaction mixture, a stream of N_2 gas was blown over it, and the solution was heated to 60°C for 10 min *in vacuo* in a Palstation model 4000 apparatus (Takara Biomedicals, Kyoto, Japan). By repeating this procedure three times, excess 2-AB was removed almost completely. Residual amounts of unwanted materials were removed by gel filtration over a column of TSK gel HW-40F (7 × 55 mm) equilibrated with 10 mM ammonium acetate (pH 6.0). The fluorescence intensity of each chromatographic fraction was measured using excitation and emission wavelengths of 320 nm and 420 nm, respectively. Fractions containing fluorescent substances were pooled, and were further analysed by high performance liquid chromatography (HPLC) (see below).

High performance liquid chromatography

A Shimadzu Prominence HGE-Fluorescence system was used throughout this study. 2-AB monosaccharide fractions were first separated by size-fractionation HPLC over Inertsil NH2 using a 0.2% formic acid–acetonitrile gradient eluent at a flow rate of 0.6 ml/min at 30°C. After injection of a sample solution, the proportion of the eluent containing acetonitrile, 0.2% formic acid was changed linearly from 0% to 20% (v) in 1 min, to 27.5% (v) in 10 min and to 55% (v) in 43 min. The fluorescence of the 2-AB monosaccharides was detected using an excitation wavelength of 320 nm and an emission wavelength of 420 nm. A second separation followed that was performed using Cosmosil 5C18-AR reversed-phase HPLC with the isocratic solvent system, *a*: 50 mM acetic acid, 0.2% (v/v) acetonitrile. When necessary, an additional separation of 2-AB-aldohexoses was performed using Cosmosil 5C18-AR and the isocratic solvent system, *b*: 0.1 M citric acid, 0.1% (v/v) acetonitrile, Additional separations of 2-AB-ketohexoses were preformed using Cosmosil 5C18-AR and the isocratic solvent system, *c*: water, 0.1% (v/v) trifluoroacetic acid, 0.5% (v/v) acetonitrile. For the Cosmosil 5C18-AR chromatographies, the flow rates and the column temperatures were always 1.0 ml/min and 40°C, respectively. The elution positions of the 2-AB monosaccharides were detected fluorescently as described above.

Quantification of 2-AB monosaccharides

Each of the 2-AB monosaccharides was quantified on the basis of its peak area in comparison with that of the authentic standard (2-AB Glucose Homopolymer) that had been chromatographed under the same HPLC conditions over Inertsil NH2. The 2-AB monosaccharides thus quantified were also used as standards for reversed-phase HPLC analysis.

Determination of hexose contents in natural products

Itea virginica was a generous gift from Dr Takeshita (Fushimi Pharmaceutical Co., Ltd, Takamatsu, Japan). The leaf (25 mg, fresh weight) was cut into small pieces and ground in a mortar with a pestle under distilled water (0.2 ml). After the insoluble material was removed by centrifugation, methanol–chloroform (1:1, v/v) treatment removed the lipids. The extract was dried, and reacted with 2-AB. Each 2-AB hexose contained in the leaf extract was quantified on the basis of its peak area in comparison with that of the corresponding standard separated under the same reversed-phase HPLC conditions.

Results

Fluorescent labelling of ketohexoses with 2-AP

Bonn and colleagues have previously attempted to label Fru and Sor with *p*-aminobenzoic acid (2-AA). They found that the yields of the 2-AA ketoses were considerably lower than that of D-Glc labeled using the same conditions (*19*, *20*). Therefore, it was obvious that the possible means of labeling the ketoses and the reaction conditions should first be studied in detail and then optimized. At first, we tried to use 2-AP as the fluorescent reagent. Its reaction with the four D-ketohexoses, Fru, Psi, Tag, Sor, was carried according to the general procedure used previously for oligosaccharides (*21*). Unexpectedly, considerable amounts of side-reaction products were obtained. Figure 2 shows a typical Ultrasphere ODS chromatogram obtained for the reaction products of D-Sor. The last two eluting peaks (3 and 4) contained PA-Ido and PA-Gul, respectively; whereas, the first two were considered to correspond to the C2 carbon *R*/*S*-isomers of pyridylaminated D-Sor (Fig. 1). Fluorescence intensities of the four peaks indicated that the reaction yield for PA-Sor was about 45%. The low yield can be attributed to a base-catalyzed, ketose–aldose transformation, *i.e.* the Lobry de Bruyn-Alberda van Ekenstein transformation (*29–31*). In general, this transformation proceeds *via* an equilibrium between ketoses and aldoses, and thus, product yields depend on the relative thermodynamic stabilities of the individual PA-saccharides. Since pyridine behaves as both a solvent and a catalyst (*32–34*), it is quite possible that 2-AP catalyzed the transformation of Sor to the corresponding aldoses, *i.e.* Gul and Ido, before the monoamine-coupling reaction proceeded. Similar results were found for other three ketoses, *i.e.* Fru, Psi and Tag. If this is the case, it would be difficult to produce PA-ketohexoses quantitatively.

Fluorescent labelling of ketohexoses with 2-AB

We then attempted the monoamine coupling using another common reagent, 2-AB, which lacks a base-catalyst moiety. According to the reaction conditions

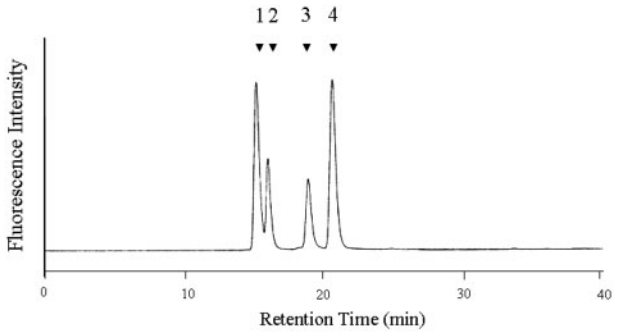

Fig. 2 Separation of pyridylaminated D-Sor and its derivatives by reversed-phase HPLC. D-Sor (100 nmol) was labelled with 2-AP, and an aliquot (1/10,000 volume of the reaction mixture) containing the PA-derivatives was eluted over Ultrasphere ODS (2.0 × 500 mm column) with 0.2 M sodium acetate (pH 4.0), 1% (v/v) acetonitrile at a flow rate of 1.0 ml/min. The PA-monosaccharides were detected using an excitation wavelength of 320 nm and an emission wavelength of 400 nm. Theoretically, a monoamine-coupling should produce the *S*/*R* stereoisomers at the C2 carbon. The corresponding two diasteriomers were found in peaks 1 and 2 of the chromatogram. Additionally, substantial amounts of by-products, produced possibly as a result of a base-catalysed transformation as described in the text, were generated. For reference, the elution positions of the two standard PA-aldohexoses, PA-Ido (*3*) and PA-Gul (*4*), are shown.

reported by Bigge and colleagues (*22*), each of the eight D-aldohexoses (Glc, Man, All, Alt, Gal, Tal, Ido and Gul; 10 nmol) and the four D-ketohexoses (Fru, Psi, Tag and Sor; 10 nmol) was dissolved in 20 µl of a DMSO: acetic acid (7:3, v/v) solution containing 1 mg 2-AB and 1.0 M $NaBH_3CN$. Each mixture was reacted at 65°C for 3 h. After the coupling reaction, excess reagents were removed as follows. Most of the 2-AB was removed using a stream of nitrogen gas, and then the remainder was removed by TSKgel HW-40F chromatography (*23*). The gel filtration procedure completely separated the 2-AB monosaccharides from the residual 2-AB and other low molecular weight by-products (Fig. 3). The 2-AB ketohexoses were eluted at fractions 20–24 (fraction volume, 1 ml), while the 2-AB aldohexoses were eluted at fractions 26–30. Notably, the peak areas of the 2-AB ketohexoses were smaller than those of the 2-AB aldohexoses (for D-Glc and D-Fru, see Fig. 3). To confirm these observations, the 2-AB monosaccharide fractions were subjected to size-fractionation HPLC using Inertsil NH2 (chromatogram not shown). As expected, the yields of the 2-AB ketohexoses were much lower (1–5%) than those of the 2-AB aldohexoses (80–90%). (For the latter, the less than complete yields may be attributed to the chromatography procedure itself). As no significant amounts of contaminating 2-AB aldohexoses were formed when the ketohexoses were reacted, it seemed worthwhile to further explore the 2-AB reaction.

To optimize the reaction conditions, the following parameters were examined: the 2-AB concentration, the reaction temperature, and the reaction period. As a solvent system for 2-AB coupling, 70% DMSO:30% acetic acid (v/v) was used throughout (*22*). The concentration of $NaBH_3CN$ was fixed at 1.0 M. The monosaccharides, D-Glc and D-Psi, were chosen as a representative aldohexose and ketohexose, respectively. The results of the surveys are summarized in Fig. 4A–C, and the following conclusions were drawn:

(1) At least 10 mg of 2-AB per reaction (reaction volume, 20 µl) was required to label Psi; whereas, 1 mg labelled >80% of the Glc present (Fig. 4A). Thus, the 2-AB concentration was set at 10 mg/reaction (500 mg/ml).
(2) The optimum temperature was 65°C for both D-Glc and D-Psi; although the yield of 2-AB Psi was more sensitive to temperature

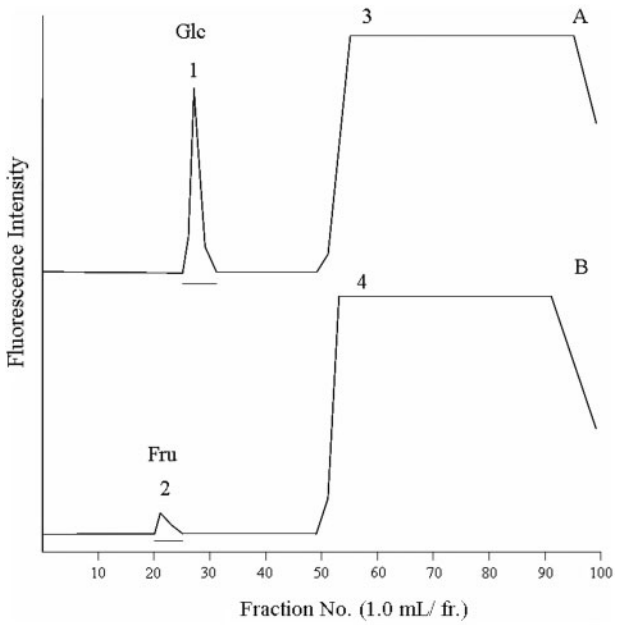

Fig. 3 Gel filtration chromatograms of D-Glc (A) and D-Fru (B) labelled with 2-AB using the conditions of Bigge and colleagues (*19*). The eluent for the TSK gel HW-40F chromatography (7 × 500 mm column) was 10 mM ammonium acetate (pH 6.0), and a flow rate of 0.1 ml/min was used. The fluorescence intensity of each 1 ml fraction was measured. The fractions marked by the bars were pooled. Peak 1 contains 2-AB Glc, and peak 2 contains 2-AB Fru.

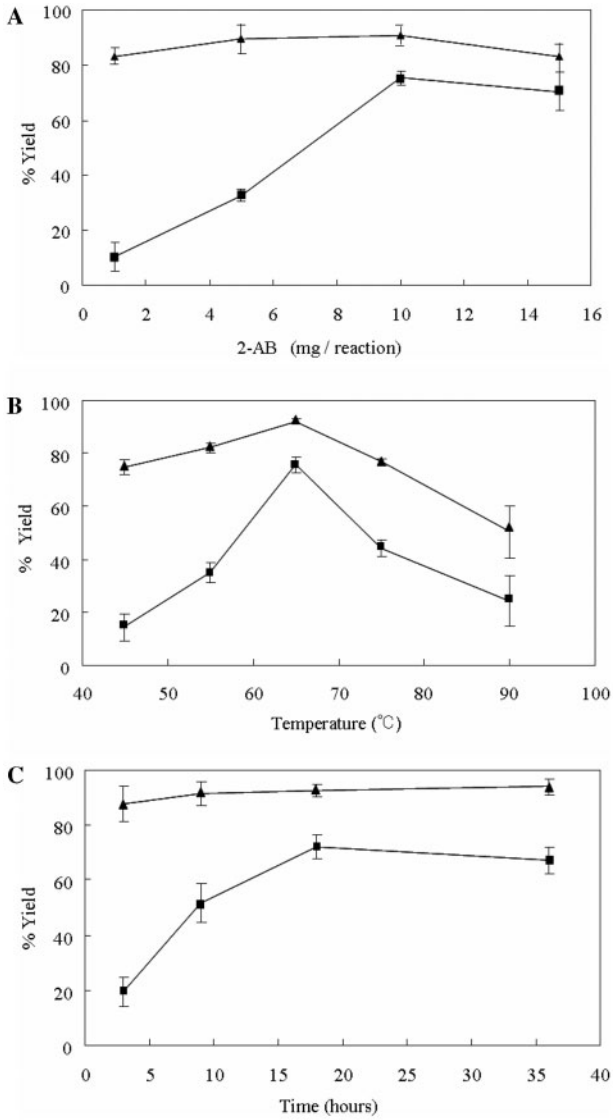

Fig. 4 Surveys of the experimental conditions for reaction of 2-AB with D-Glc (filled triangle) and with D-Psi (filled square). The amounts of the 2-AB monosaccharides were determined using the peak areas found for the Inertsil NH2 chromatographies. The yields were investigated as functions of (A) 2-AB concentration, (B) reaction temperature and (C) reaction period. Each data point is the average of three separate experiments and the bars show the standard errors.

(Fig. 4B). Thus, the reaction temperature was set at 65°C.

(3) At least 18 h were necessary to achieve the maximum yield of 2-AB Psi (>70%), while after 3 h >85% of the D-Glc had been labelled (Fig. 4C). Thus, the reaction time was set at 18 h.

Validation of the optimized conditions

Using the optimized conditions, the eight D-aldohexoses and the four D-ketohexoses were each labelled with 2-AB. Each sugar (10 nmol) was dissolved in 20 µl of the reaction solution (prepared by dissolving 75 mg 2-AB and 9 mg $NaBH_3CN$ in 70 µl DMSO/30 µl acetic acid to make up a 150 µl solution), and the solution was incubated at 65°C for 18 h. At the end of the reaction time, excess reagents were removed by a nitrogen flush and gel filtration, and the 2-AB sugars were chromatographed over Inertsil NH2. The yields of all of the 2-AB monosaccharides were referenced to the 2-AB Glucose Homopolymer Standard because authentic standards for 2-AB aldoses and 2-AB ketoses did not exist. Using the optimized reaction conditions, the apparent yields of the 2-AB monosaccharides were each at least 75% (Table I). However, the apparent yields of the 2-AB monosaccharides differed significantly. The apparent yields of 2-AB Alt and 2-AB Gul exceeded 100%, possibly because their relative fluorescent intensities are significantly greater than that of the standard. Conversely, the yield of 2-AB Psi was relatively low. It is known that the reducing terminals of monosaccharides significantly affect the fluorescent intensities of the label (*35*). Therefore, for each 2-AB monosaccharide, its absolute fluorescent intensity should be determined so that it can be precisely quantified.

Calibration curves were next obtained for D-Glc and D-Psi (Fig. 5). The D-Psi calibration curve was linear between 1 pmol and 1 µmol and had a correlation coefficient (R^2) of 0.9999; whereas, the D-Glc calibration curve was linear between 1 pmol and 10 µmol and had a correlation coefficient of 0.9999. For the Inertsil NH2 chromatographies, the detection limit was ~0.1 pmol, and the signal-to-noise ratio was approximately 3.

Table I. Yields of 2-AB monosaccharides under the optimized conditions[a].

2-AB aldose	Yield (%)	2-AB ketose	Yield (%)
2-AB Glc	87	2-AB Fru	90
2-AB Man	88	2-AB Psi	75
2-AB All	89	2-AB Sor	91
2-AB Alt	130	2-AB Tag	79
2-AB Ido	90		
2-AB Gul	120		
2-AB Gal	80		
2-AB Tal	93		

[a]Yield was determined from fluorescence intensity of each 2-AB monosaccharide in comparison with that of 2-AB Glc on the assumption that their fluorescence intensities are the same as that of 2-AB Glc.

Systematization of the HPLC separation of the 2-AB hexoses

Recently (*18*), we labeled a series of D-aldohexoses with 2-AP, and the PA-saccharides were separated by HPLC. For this report, D-ketohexoses, in addition to D-aldohexoses, were labelled with 2-AB, with the goal of constructing a universal separation system. We first used the separation conditions described by Chiba and colleagues (*36*) who separated 2-AB Glc, 2-AB Man, and 2-AB Gal over Cosmosil 5C18-AR. For this study, a mixture of the eight D-aldohexoses (Glc, Man, All, Alt, Gul, Ido, Gal, and Tal; 1 nmol each) or the four D-ketohexoses (Fru, Psi, Sor and Tag; 1 nmol each) was labelled with 2-AB, gel filtrated and eluted over Cosmosil 5C18-AR, equilibrated with solvent system *a* (Fig. 6A and B). The 2-AB aldohexoses eluted with retention times between 44.9 and 63.0 min (Fig. 6A). On the other hand, the 2-AB ketohexoses eluted much more rapidly, with retention times between 32.8 and 39.3 min (Fig. 6B). Because two diasteriomers are generated from each ketohexose (Figs 1 and 2), their chromatographic separations are more difficult. Using solvent system *a*, the following 2-AB monosaccharides migrated together: (i) Tal (*3*), All (*4*) and Ido (*5*) eluted at 49.6 min (Fig. 6A); (ii) Tag_2 (*10*) and Psi_1 (*11*) eluted at 35.1 min; (iii) Sor_2 (*13*) and Fru_1 (*14*) eluted at 37.6 min; and (iv) Fru_2 (*15*) and Psi_2 (*16*) eluted at 39.3 min (Fig. 6B). (The subscripts 1 and 2

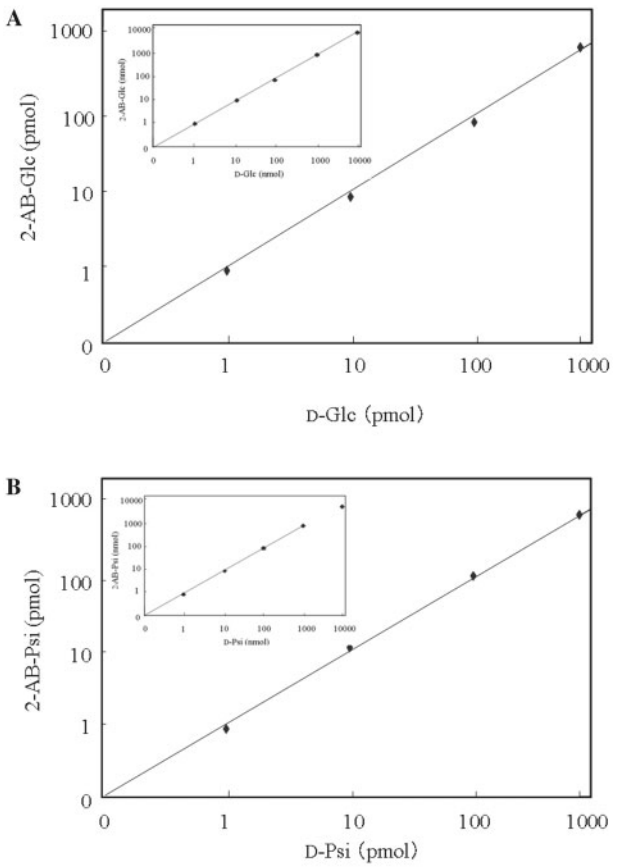

Fig. 5 Calibration curves obtained for (A) D-Glc and (B) D-Psi. Each data point is the average of three separate experiments and the bars show the standard errors.

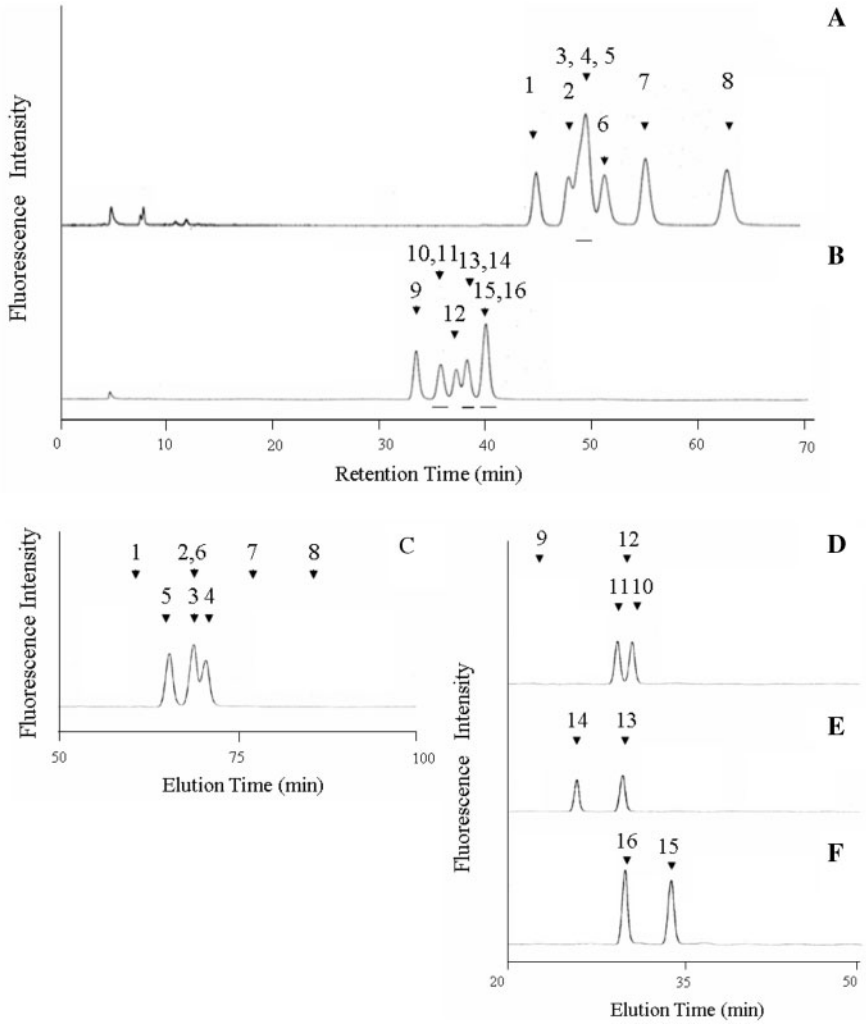

Fig. 6 Separation of (A, C) the 2-AB aldohexoses and (B, D–F) the 2-AB ketohexoses by Cosmosil 5C18-AR chromatography. For (A) and (B) the eluent was solvent system *a*; For (C), the eluent was solvent system *b*; For (D–F), the eluent was solvent system *c*. A mixture of (A) D-aldohexoses (Glc, Man, All, Alt, Gul, Ido, Gal, Tal; 1 nmol each) and a mixture (B) of D-ketohexoses (Fru, Psi, Sor, Tag; 1 nmol each) were each labeled with 2-AB, and the two 2-AB derivatives mixtures (50 pmol each) were individually eluted over Cosmosil 5C18-AR with solvent system *a*. The co-eluted 2-AB monosaccharide fractions (marked by bars) were pooled, and chromatographed over Cosmosil 5C18-AR using solvent system *b* (C, Tal/All/Ido) or solvent system *c* (D; Psi_1/Tag_2: E; Fru_1/Sor_2: F; Fru_2/Psi_2). For each chromatography, the 2-AB monosaccharides were separated isocratically at a flow rate of 1 ml/min. For reference, elution positions of all standard 2-AB hexoses are indicated with arrowheads; 1, 2-AB Gal; 2, 2-AB Man; 3, 2-AB Tal; 4, 2-AB All; 5, 2-AB Ido; 6, 2-AB Glc; 7, 2-AB Gul; 8, 2-AB Alt; 9, 2-AB Tag_1; 10, 2-AB Tag_2; 11, 2-AB Psi_1; 12, 2-AB Sor_1; 13, 2-AB Sor_2; 14, 2-AB Fru_1; 15, 2-AB Fru_2; 16, 2-AB Psi_2. For each ketohexose, two peaks corresponding to the two diasteriomers with *R*/*S* chirality at the C2 carbon are generated (also see Fig. 2). Because it is not known which peak contains the *S* or the *R* isomer, the individual isomers are labeled with the subscripts, 1 and 2, which identify their order of elution.

reference the diasteriomers of the 2-AB ketohexoses and their elution positions).

To separate completely the co-eluted 2-AB hexoses, we then tried Cosmosil 5C18-AR chromatography in conjunction with solvent system *b* (Fig. 6C). Each mixture containing the co-eluted 2-AB derivatives, was individually applied to the column and eluted. Using this system and the 2-AB aldohexoses (Fig. 6C), 2-AB Tal (*3*) and 2-AB All (*4*) were partially separated from each other (50% baseline resolution) with retention times of 68.9 min and 70.7 min, respectively; whereas, 2-AB Ido (*5*) was cleanly separated with a retention time of 65.6 min. Thus, the 2-AB D-aldohexoses were successfully separated after two chromatographic rounds.

However, for the three pairs of ketohexose derivatives (Psi_1/Tag_2, Fru_1/Sor_2 and Fru_2/Psi_2), solvent system *b* was unsatisfactory because Psi_1/Tag_2 still eluted together, even though Fru_1/Sor_2 and Fru_2/Psi_2 were separable (data not shown). Thus, the ability of solvent system *c*, to separate the 2-AB ketohexoses was assessed. Using this solvent system, each of the 2-AB ketohexose pairs were separated (Fig. 6D–F).

Taken together, the results clearly demonstrated that the 2-AB hexoses could be separated chromatographically by using gel filtration to separate the aldohexoses from the ketohexoses and then using reversed-phase HPLC to separate the individual aldohexoses and the individual ketohexoses. In both cases,

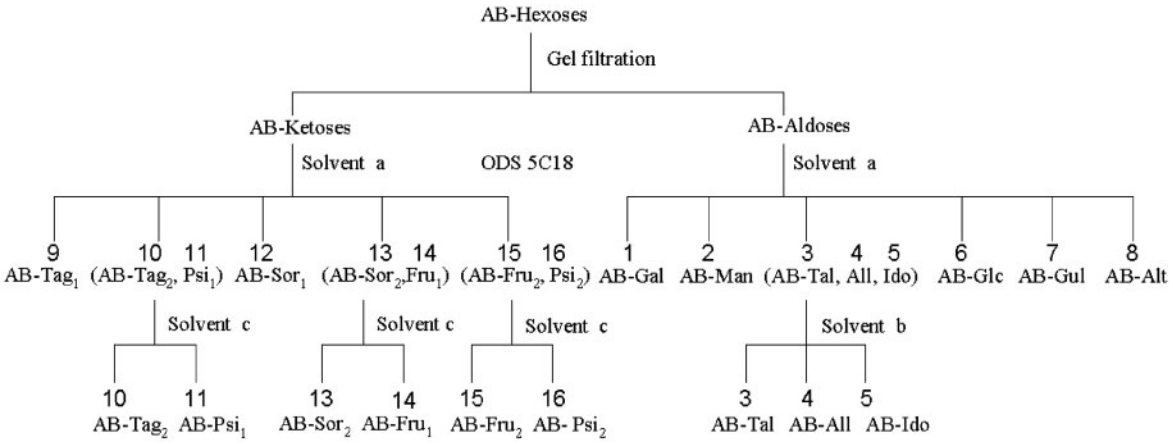

Fig. 7 Schematic flowchart for the separation of the 2-AB hexoses. The sugar numbers 1–16 correspond to those used in Fig. 6.

Table II. Elution Times of the 2-AB Hexoses Upon Reversed-phase HPLC, Cosmosil 5C18-AR Chromatography[a].

	Aldohexoses								Ketohexoses							
Solvent system[b]	Gal	Man	Tal	All	Ido	Glc	Gul	Alt	Tag_1	Tag_2	Psi_1	Sor_1	Sor_2	Fru_1	Fru_2	Psi_2
a	44.9	48.0	49.6	49.6	49.6	51.4	55.3	63.0	32.8	35.1	35.1	36.6	37.6	37.6	39.3	39.3
b	61.5	68.3	68.9	70.7	65.6	68.5	76.9	87.9	41.9	42.8	42.8	45.3	46.6	48.2	48.4	50.3
c	20.9	22.3	23.2	23.2	23.2	24.9	26.2	26.2	23.3	31.2	28.6	30.0	30.0	26.3	32.8	29.1

[a]Elution times in minutes. [b]*a*, 50 mM acetic acid, 0.2% (v/v) acetonitrile; *b*, 0.1 M citric acid, 0.1% (v/v) acetonitrile; *c*, water, 0.1% (v/v) trifluoroacetic acid, 0.5% (v/v) acetonitrile.

the first separation involves Cosmosil 5C18-AR chromatography with solvent system *a* and then is followed by Cosmosil 5C18-AR chromatography with solvent system *b* for the aldohexoses or solvent system *c* for the ketohexoses, respectively. A flowchart for the separation is shown in Fig. 7, and the HPLC elution times of the 2-AB hexoses are summarized in Table II.

Practical application: assessment of the hexose content in a leaf of I. virginica

It has been reported that *Itea* plants contain D-Psi in their leaves and flowers (*12*, *13*). Because Psi is thought to occur unmodified in nature, we measured the amount of free Psi contained in 25 mg of a fresh *I. virginica* leaf. Monosaccharides were extracted from the leaf, labelled with 2-AB, and the 2-AB hexoses were quantified using Cosmosil 5C18-AR reversed-phase HPLC (Fig. 8). The Psi content was determined to be 2.7 mg/g leaf (wet weight), and no other rare sugars were identified. To our surprise, the Glc content was much lower (0.6 mg/g) than that of Psi. Considering the natural abundance of these monosaccharides, the amount of Psi found in the leaf is extremely large. The common monosaccharides, Gal and Fru were also detected; although, their amounts were relatively low (0.03 mg/g and 0.1 mg/g, respectively). Our results are consistent with the previous report by Hough and colleagues (*12*), who detected Psi using paper chromatography. Notably, that method did not detect the less abundant Fru and Glc even when 6 g (fresh weight) of leaves was assayed. Thus, we are now able to measure a series of hexoses contained in both natural and artificial products. Although, its practical application regarding other rare sugars remains to be tested.

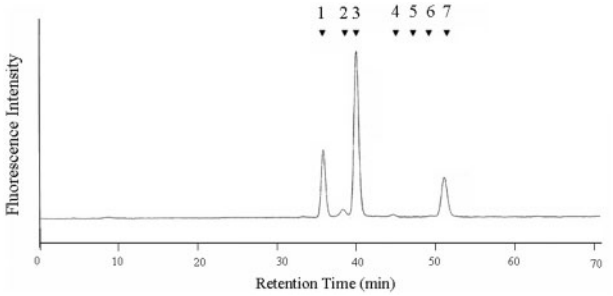

Fig. 8 Separation of 2-AB hexoses extracted from a leaf of *I. virginica*. The monosaccharides extracted and labelled with 2-AB were separated by Cosmosil 5C18-AR chromatography using solvent system *a*. The elution positions of standard 2AB monosaccharides are indicated with arrowheads: 1, 2-AB Psi_1; 2, 2-AB Fru_1; 3, 2-AB Psi_2, 2-AB Fru_2; 4, 2-AB Gal; 5, 2-AB Man; 6, 2-AB All; 7, 2-AB Glc.

Discussion

For this study, we developed a comprehensive analytical HPLC system for the separation, identification, and quantification of common and rare hexoses. With the exception of our recent report (*18*), no practical methods to detect and quantify naturally occurring rare monosaccharides exist. Moreover, although various monoamine-coupling methods have been taken employed for oligosaccharide analyses, they have never been used to detect and quantify ketoses.

Our first attempt, using 2-AP, was unsuccessful, not only because 2-AP reacted poorly with the keto-hexoses, but also because it catalyzed keto-aldose isomerization in accordance with the Lobry de Bruyn-Alberda van Ekenstein transformation. Conversely, reaction of the monoamine-coupling reagent, 2-AB, with the ketohexoses proceeded without the undesirable isomerization; although, it was necessary to optimize the reaction conditions to generate 2-AB ketoses in large yields. Our optimized reaction conditions for 2-AB labeling of hexoses are as follows: 10 mg 2-AB/20 µl reaction mixture, a reaction temperature of 65°C, and a reaction time of 18 hours. The detection limit was approximately 0.1 pmol for both aldohexoses and ketohexoses, while signal-to-noise ratio was approximately 3. Since the fluorescent intensity of a 2-AB monosaccharide significantly depends on the structure of the sugar, an accurate molar fluorescence coefficient needs to be determined for each sugar. Because the present HPLC system does not discriminate between the *R*/*S*-isomers of the 2-AB ketoses or between their D/L-enantiomers, other methods must be developed to solve these issues. We expect that NMR spectroscopy and bio-affinity techniques can be used to discriminate between the *R*/*S*-isomers and the D/L-enantiomers.

Our system should be capable of detecting and quantifying rare ketoses and aldoses in both natural and artificial products. As an application of this method, we could identify free Psi using a relatively small amount of a natural material, *i.e.* 25 mg of a fresh *I. virginica* leaf. The determined Psi content was 2.7 mg/g leaf (wet weight), while no other rare sugars were identified. Compared with the Glc content (0.6 mg/g), this value is apparently high. At the moment, however, it is not clear why the leaf should contain such a high amount of Psi as its biological function(s) is not known. Using this methodology, other types of monosaccharides, such as ketopentoses, could also be targeted. If the locations, quantities, and biosynthetic pathways, of rare sugars could be determined, it should be possible to begin to understand their biological functions. This would pioneer a novel, attractive field in glycoscience, especially considering the recent findings that some rare sugars, *e.g.* D-Psi, are biologically active (*6*–*10*). In this regard, rare sugar studies have just begun.

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Conflict of interest
None declared.

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