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REVERSED-PHASE LIQUID CHROMATOGRAPHIC ANALYSIS OF Dns-SUGARS

OPTIMIZATION OF DERIVATIZATION AND CHROMATOGRAPHIC PROCEDURES AND APPLICATIONS TO NATURAL SAMPLES

KENNETH MOPPER*
College of Marine Studies, University of Delaware, Lewes, DE 19958 (U.S.A.)
and
LARS JOHNSON
Department of Analytical and Marine Chemistry, Chalmers University of Technology and University of Göteborg, Göteborg (Sweden)
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SUMMARY

The potential of 5-dimethylaminonaphthalene-1-sulphonylhydrazine (Dns-hydrazine) as a precolumn fluorimetric labeling reagent for reducing sugars was examined in detail. The kinetics of the reaction (and therefore yields of sugar hydrazones) were found to be strongly affected by several interacting parameters. However, when appropriate precautions were observed, quantitative results with excellent precision were obtained (S.D. $\approx \pm 0.4\%$ at the 0.5 nmol level). The detection limit was determined to be 2–5 pmol per injected sugar. Analysis of a wood hydrolysate with the Dns-derivatization method and with a conventional sugar analyzer gave excellent agreement. Using reversed-phase high-performance liquid chromatography and a mobile phase of acetonitrile–aqueous acetic (or formic) acid, fourteen sugar derivatives were separated isocratically in about 20 min. On-line sample clean-up, using a precolumn in place of the injection loop, was found to be more rapid and effective than off-line techniques. Applications to physiological fluids, beverages and wood hydrolysates are illustrated.

INTRODUCTION

Numerous liquid chromatographic techniques can be found in the literature for the determination of sugars. The simplest and perhaps most useful of these techniques, especially for the analysis of aqueous samples with complex matrices (*e.g.*, physiological fluids, acid hydrolysates, beverages, etc.), are those which permit the direct injection of samples with little or no pretreatment. In this regard, borate complex anion-exchange chromatography is noteworthy[1–3]. Unfortunately, run times, depending on the complexity of the samples, can be fairly long (*e.g.*, 2–20 h) when

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compared to modern high-performance liquid chromatographic (HPLC) techniques. In addition, special columns and postcolumn reaction/detection systems are required, thereby necessitating the construction or purchase of a dedicated sugar analyzer system. Unless sugar analyses are performed on a routine basis, acquisition and maintenance of such a system is generally not warranted. For this reason, sugar separation techniques utilizing general purpose HPLC equipment and columns are highly desirable. The growing popularity of the HPLC method using "carbohydrate columns" (*i.e.*, alkylamine bonded, porous silica columns) attests to this. Unfortunately, since this method is based on the normal phase mode, inconvenient sample desalting and purification steps are often necessary prior to injection. A sugar separation technique based on reversed-phase chromatography is therefore preferable since large volumes of aqueous samples can often be injected with minimal sample clean-up and with insignificant band broadening of components[4]. However, the high polarity and chemical similarity of simple sugar isomers do not permit their separation on reversed-phase columns [*e.g.*, (ODS) octadecylsilyl columns[5]] unless their polarity can be markedly decreased by derivatization. Methylated sugars, for example, can be readily separated by reversed-phase HPLC[6].

Derivatization can serve an additional important function. If the derivative is fluorescent or UV absorbing, the detection of sugars is markedly enhanced and the need for a dedicated postcolumn reaction system is eliminated[4]. For aqueous samples the derivatization reaction should ideally be rapid, mild, have few transfer steps and, most importantly, proceed in aqueous media. Of the various derivatization reagents available for sugars[7] only 5-dimethylaminonaphthalene-1-sulphonylhydrazine (Dns-hydrazine) meets these criteria. Recently, Alpenfels[8] demonstrated the potential of this reagent for precolumn fluorescence derivatization and reversed-phase HPLC separation of monosaccharides. Unfortunately, this investigator did not present information regarding optimal derivatization conditions, and losses due to precolumn clean-up procedures. In addition, no applications of the method to natural samples or hydrolysates were presented.

We have been using a Dns-hydrazine technique over the last 3 years for the reversed-phase HPLC analysis of mono- and disaccharides in various types of natural samples. We present here our findings regarding the advantages and limitations of this technique; in particular, detailed information is given pertaining to reaction conditions, methods of sample clean-up and effect of mobile phase composition on selectivity. Practical applications of the technique are presented.

EXPERIMENTAL

Apparatus

Two HPLC systems were employed: Varian Model 5020 and Beckman Model 324. The chromatographs were equipped with Valco (Model CV-6UHPa-N60) sampling valves with 10- or 100-μl sample loops. For some studies, a precolumn was used in place of the sampling loop. The precolumn was either a Brownlee cartridge (30 × 4.6 mm, Spherisorb ODS, 5 μm) or a short, home-made column (45 × 2 mm, Nucleosil ODS, 5 μm).

The fluorescence detectors used were a Kratos Model FS970 (excitation: 240 or 360 nm; emission: >470 nm, cut-off filter) and a Perkin-Elmer Model 650 S-LC (excitation: 370 nm; emission: 540 nm).

HPLC columns

Most separations were performed on reversed-phase columns. Commercial (Altex Ultrasphere ODS, 5 μm, 250 × 4.6 mm) and home-made columns (Nucleosil ODS, 5 μm, 200 × 4.6 mm) were employed in this study. Columns packed with porous silica (Nucleosil 50, 7 μm, 200 × 4.6 mm) and alkylamine bonded silica (Nucleosil, 5 μm, 200 × 4.6 mm) were also tested.

Chemicals

Reagent grade chemicals and double distilled deionized water were used. Organic solvents used in mobile phases and in the derivatization procedure were HPLC grade (Rathburn Chemicals; J. T. Baker). Dns-hydrazine was obtained from Pierce. HPLC Amine Modifier I was purchased from NATEC (Hamburg, G.F.R.). Individual and mixed saccharide standards (Sigma) were dissolved in water to give 10 m*M* solutions (100 m*M* for fructose). The standard solutions were stored frozen.

Derivatization procedure

The Dns derivatization method of Avigad[9] was optimized (see Results); the following procedure was found to give reproducible and quantitative results if samples and standards were treated identically.

Natural samples such as physiological fluids were deproteinated and diluted, when necessary, and filtered prior to derivatization. To 100 μl of aqueous sample (or sugar standard) were added 10 μl aqueous trichloroacetic acid (10%, w/v) and 50 μl Dns-hydrazine reagent (5%, w/v in acetonitrile; stored refrigerated). The reaction tube was sealed and placed in a water-bath at 65°C for 20 min. The reaction was stopped by immersion of the tube in an ice-bath. The mixture was diluted 1:1 in water and 10–300 μl were injected within 2 h. The reaction tube was stored in the ice-bath and in the dark prior to injection.

HPLC conditions

Separations were performed at ambient temperature and at flow-rates of 1.0 ml min^{-1} for the Nucleosil ODS column and 1.2 ml min^{-1} for the Ultrasphere ODS column. For gradient runs, the weaker mobile phase (A) was either 0.08 *M* acetic acid or 0.02 *M* formic acid. The stronger mobile phase (B) was acetonitrile. The gradient typically used for the Nucleosil ODS column was: isocratic at 21% B for 18 min; 21% to 70% B in 2 min; isocratic at 70% B for 2 min; 70% to 21% B in 2 min. The gradient generally used for the Ultrasphere column was: isocratic at 19% B for 2 min; 19% to 25% B in 16 min; 25% to 70% B in 2 min; isocratic at 70% B for 2 min; 70% to 19% B in 2 min.

The latter part of the gradients, which serve to elute strongly retained substances including unreacted reagent, can be omitted if a simple column switching technique is used. A precolumn is substituted for the sample loop on the injector. The precolumn is conditioned with acetonitrile, then water (4–5 ml of each solvent for the Brownlee precolumn; 1.5 ml for the Nucleosil packed precolumn). The sample is loaded on the precolumn which is then switched into the mobile phase stream. The precolumn is switched out after an appropriate period (30–90 sec for the Brownlee precolumn; 15–30 sec for the Nucleosil packed precolumn). The precolumn is washed with acetonitrile to remove excess of reagent and then conditioned with water prior to loading a new sample.

By using the column switching technique to eliminate excess of reagent, the sugar Dns-hydrazones can be separated by isocratic elution (21 % B for the Nucleosil ODS column; 24% B for the Ultrasphere ODS column).

RESULTS

Optimization of chromatographic conditions

Chromatographic mode. Underivatized sugars have been separated in the normal phase mode with HPLC columns packed with alkylamine bonded silica gel[7]. In a related technique, alkylamines added directly to the mobile phase have been used for the analysis of sugars with columns packed with bare silica gel[10]. We examined these techniques for the separation of Dns-sugars. The mobile phase was typically acetonitrile–water (80:20); HPLC Amine Modifier I was added to the mobile phase (0.03%, v/v) for the latter technique.

Poor selectivities were obtained for both techniques; only three or four Dns-sugars could be adequately separated. Furthermore, smearing of the main reagent

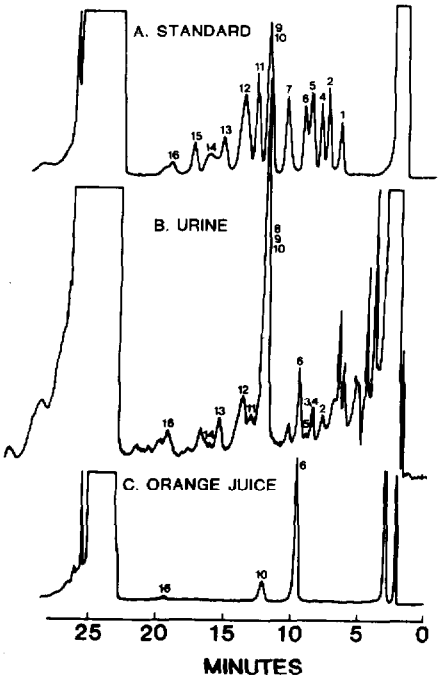

Fig. 1. Typical chromatograms of Dns-hydrazine sugar standards and samples; derivatization procedure as in the text. The large initial and late peaks are due to the reagent. Column, Nucleosil ODS, 250 × 4.6 mm, 5 μm, flow-rate 1 ml min^{-1}; mobile phase, 0.08 *M* acetic acid–acetonitrile (79:21); 10 μl reaction mixture injected. Wavelengths: 360 nm (excitation) and >470 nm (emission). A, Sugar standard: most peaks are 1 nmol (Fucose, 0.4 nmol; 2-deoxyribose, 0.2 nmol; fructose, 5 nmol). B, Injection of about 0.4 μl deproteinized urine. C, Injection of about 0.9 μl filtered, fresh orange juice. Peaks: 1 = gentiobiose; 2 = lactose; 3 = maltose; 4 = cellobiose; 5 = galactose; 6 = glucose; 7 = mannose; 8 = xylose; 9 = arabinose; 10 = fructose (1); 11 = 2-deoxyglucose; 12 = ribose; 13 = fucose; 14 = rhamnose; 15 = 2-deoxyribose; 16 = fructose (2). Fructose elutes as two peaks, see Discussion.

peak, which eluted just after the dead volume, resulted in an elevated baseline. In addition, a few minor reagent peaks coeluted with the sugars. Since the results were not encouraging, work with this chromatographic mode was discontinued.

The reversed-phase mode was then examined. In this mode, the sugar derivatives elute in a region between two groups of reagent peaks, as shown in Fig. 1. The first group is composed of hydrophilic degradation products of the excess of reagent; the second group is composed mainly of excess of reagent.

Mobile phase composition. Acetonitrile, methanol and tetrahydrofuran, used alone and in combination, were examined for their effect on resolution and selectivity of Dns-sugars. The weak eluent (A) was always 0.08 *M* acetic acid. In agreement with Alpenfels[8], we found that acetonitrile alone was superior to the other organic eluents.

Initially, water was used as the weak eluent. This solvent was found to cause a smearing of the early eluting reagent peaks which, in turn, resulted in an undesirable elevation of the baseline under the sugar peaks, especially at high detector sensitivities. When 0.08 *M* acetic acid was used in place of water the smearing effect markedly decreased. A further decrease was observed when formic acid (0.02 *M*) was used. In addition, the size of early eluting reagent peaks decreased significantly when either acetic or formic acid was used in the mobile phase. Apparently, protonation of some of the Dns-hydrazine breakdown products caused them to elute much later (in the region of the excess of reagent).

In addition to the effect on the reagent peaks, the use of acetic or formic acid in the eluent A resulted in a marked decrease in the relative retentions of the Dns-sugars, as shown in Table I. The selectivity and resolution were as good or better than with

TABLE I

ELUTION OF SUGARS (CAPACITY FACTORS, k') FROM AN ODS COLUMN AS A FUNCTION OF MOBILE PHASE COMPOSITION

Chromatographic conditions as in Fig. 1.

Sugar	*A: 76% water B: 24% acetonitrile*	*76% 0.08 M acetic acid 24% acetonitrile*	*76% 0.02 M $CaCl_2$ 24% acetonitrile*	*76% 0.03% AM* 24% acetonitrile*
Gentiobiose	1.76	1.06	1.53	1.71
Lactose	2.08	1.13	1.77	1.90
Maltose	2.25	1.22	1.87	1.98
Cellobiose	2.29	1.24	1.91	1.98
Galactose	2.60	1.42	2.19	2.35
Glucose	2.65	1.46	2.35	2.49
Mannose	2.88	1.73	2.61	2.99
Xylose	3.71	2.00	3.19	3.24, 4.78
Fructose (1)	3.73	2.03	3.18, 3.21	1.73
Arabinose	3.78	2.04	3.27	3.46
2-Deoxyglucose	3.90	2.18	N.D.**	N.D.**
Ribose	4.08	2.31	3.62	1.94, 3.27
Fucose	4.59	2.54	4.11	4.40
Rhamnose	4.71	2.73	4.27	4.70
Fructose (2)	5.00	2.84	4.51, 4.59	4.96
2-Deoxyribose	5.31	2.94	4.73	1.70

* AM = HPLC Amine Modifier I.
** N.D. = not determined.

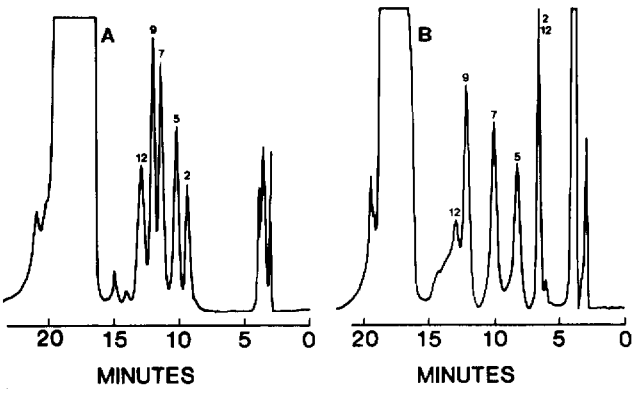

Fig. 2. Effect of alkylamines on the separation of Dns-sugars. Column, Nucleosil ODS, 250 × 4.6 mm, 5 μm; flow-rate 1 ml min^{-1}; gradient, 20% to 30% B in 10 min, 30% B to 70% B in 2 min, isocratic at 70% for 2 min, 70% B to 20% B in 2 min. A, Mobile phases: A = 0.08 *M* acetic acid, B = acetonitrile; B, A = 0.08 *M* acetic acid containing 0.03% HPLC Amine Modifier I, B = acetonitrile. Peaks as in Fig. 1.

water alone as the solvent A. With either eluent, fructose eluted as two peaks (Fig. 1) and the resolution between maltose and cellobiose, and between xylose, arabinose and the first fructose peak, was poor.

Attempts were made to alter selectivities by adding metal ions and alkylamines to the solvent A (water). The results of some of these attempts in terms of relative retentions are given in Table I. Metal ions tested were Ca^{2+}, Ba^{2+} and Zn^{2+}. In all cases, the relative retentions were strongly decreased compared to water alone. In the presence of these metal ions, fructose eluted as two groups of fused peaks. The order of elution and selectivity for all sugars were generally unchanged compared to water (Table I), however, the resolution between glucose and mannose did improve somewhat (Table I). For most of the sugars, sharper peaks were obtained with Ca^{2+} than with Ba^{2+} or Zn^{2+}. On the whole, the results with added metal ions were not significantly better than those obtained with water alone (or with aqueous acetic or formic acid).

The presence of small amounts of alkylamines, HPLC Amine Modifier I and nonylamine, in the solvent A (0.03% in water or 0.08 *M* acetic acid) strongly affected the elution order and resolution of some sugars, as depicted in Fig. 2. Although the resolution was markedly improved for some sugars, other sugars were adversely affected (Table I). Ribose split into two peaks, one of which coeluted with lactose while the other became broad (Fig. 2). Xylose also split into two peaks; fucose and rhamnose were unresolved. The first fructose peak and 2-deoxyribose coeluted as broad peaks prior to lactose. Because of these complications, the use of alkylamines in the mobile phase is not recommended.

Sample pretreatment

Sample clean-up prior to injection is desirable from the point of view of eliminating unreacted reagent and strongly retained substances in natural samples. On-line and off-line techniques were examined.

The off-line technique involved the use of Sep-Pak C_{18} cartridges (Waters Associates). Initially the procedure of Alpenfels[8] was examined. We found that re-

coveries of some Dns-sugars, especially those eluting early, were low and variable. Recoveries of the more strongly retained sugars were higher and more quantitative, however, large amounts of unreacted reagent were coeluted.

We attempted to optimize this off-line technique. Recoveries of early eluting sugars were found to be inversely related to the per cent organic solvent present in the diluted reaction mixture and in the rinse solution (10% organic in the procedure of Alpenfels[8]) and the flow-rate through the cartridge. The following procedure gave reproducible results. The reaction mixture (see Experimental) is diluted in water to an organic solvent concentration of 5% or less. A 5-ml aliquot of this mixture is loaded onto a Sep-Pak (preconditioned with 3 ml acetonitrile and 5 ml water). The cartridge is rinsed with 5 ml of 5% acetonitrile (in water) and the derivatives are eluted with 6 ml of 20% acetonitrile. During these steps, the flow-rate should not exceed 2 ml min^{-1}. The Sep-Pak is regenerated with 1 ml acetonitrile and 5 ml water.

Recoveries were >90% for early as well as late eluting sugars, however a considerable amount of unreacted reagent also eluted from the cartridge. In addition, the original sample (100 μl) is diluted by a factor of 60 after Sep-Pak treatment. The sample may be concentrated in a rotary evaporator, however this step results in the formation of hydrophilic fluorescent compounds, which are probably decomposition products of the excess of reagent.

On-line sample clean-up, using a precolumn in place of the injection loop (see Experimental), was found to be superior to the Sep-Pak treatment. The on-line procedure is faster, large dilutions of the sample are avoided, recoveries are 100% for all sugars and sugar derivatives are cleanly separated from the excess of reagent. The early eluting breakdown products of the reagent (Fig. 1) can also be eliminated using this technique. After loading the sample, the precolumn is simply rinsed with 1–2 ml of 10% acetonitrile (in eluent A). However, since this step affects the switching time and the relative retentions of the sugars, its use is not recommended.

Optimization of derivatization conditions

Concentration of Dns-hydrazine: effect on quantitative analysis. Using the previously described derivatization procedure (see Experimental), the Dns-hydrazine

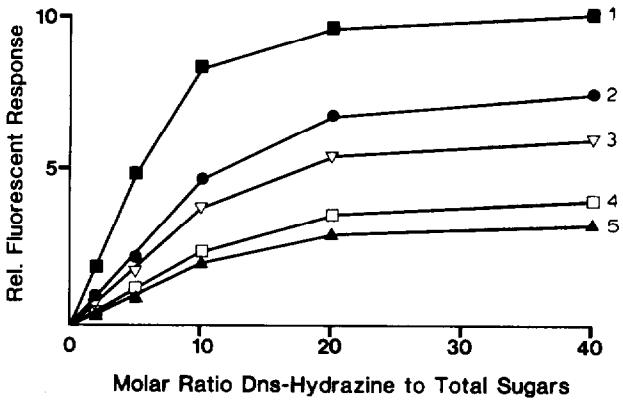

Fig. 3. Effevt of Dns-hydrazine concentration of the derivatization of reducing sugars. 1 = 2-Deoxyribose; 2 = arabinose; 3 = ribose; 4 = lactose; 5 = rhamnose.

concentration was varied by a factor of 20 in increments. The total amount of sugars present was always 0.7 μmol. The resulting molar ratio of reagent to total sugar was 2–40. From Fig. 3, it can be seen that a minimum twenty-fold excess of reagent is required for quantitative analysis. When the excess falls under ths minimum, calibration curves become non-linear. Therefore, samples containing high sugar concentrations should be diluted prior to derivatization in order to ensure that responses are linear. Linear responses were obtained over at least three orders of magnitude, as long as at least a twenty-fold reagent excess was maintained.

Concentration of trichloroacetic acid. The concentration of trichloroacetic acid in the final reaction mixture (see Experimental) was varied from 0.2% to 4.0% (w/v). Maximal fluorescent responses were obtained with 0.4–0.6% (w/v) in the reaction mixture [corresponding to about 10 μl of 10% (w/v) trichloroacetic acid in a final reaction mixture volume of 200 μl].

Effect of organic solvents on the reaction. Dns-hydrazine was dissolved to a concentration of 2% (w/v) in the following solvents: methanol, ethanol, isopropanol, acetonitrile and tetrahydrofuran. Appropriate amounts of different sugars were reacted as usual (see Experimental). The highest fluorescent responses (*i.e.*, hydrazone yields) were obtained with methanol. Normalizing to methanol, the average results for the other solvents were: isopropanol, 0.98; ethanol, 0.84; acetonitrile, 0.71; and tetrahydrofuran, 0.42. Although the reaction proceeds most favorably in methanol, acetonitrile is used in the derivatization procedure because of the markedly higher solubility of the reagent in this solvent.

Effect of water on the reaction. The percentage of water in the reaction mixture was varied from 20% to 80% (v/v). The organic solvent present was either acetonitrile or methanol (all other parameters being held constant). For either organic solvent, increasing the percentage of water in the reaction mixture adversely affected the reaction. For methanol, the fluorescent responses dropped about 60% as the percentage of water increased from 20 to 80%. For acetonitrile, the drop was only about 30%. Therefore, acetonitrile is preferred in the reaction mixture since, for this solvent, slight variations in the percentages of water and organic solvent in the reaction mixture will have only a minor effect on quantitation of the results.

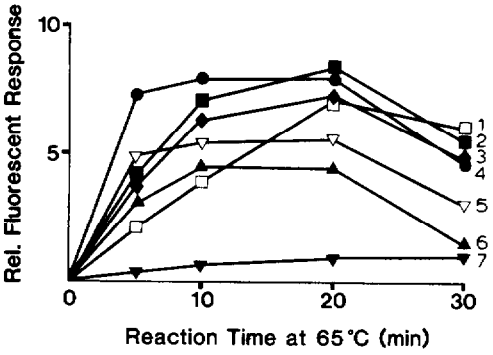

Fig. 4. Effect of reaction time at 65°C on Dns derivatization of reducing sugars; derivatization procedure as in the text. Approximately 1 nmol of each sugar (5 nmol fructose) was injected. 1 = Lactose; 2 = mannose; 3 = galactose; 4 = arabinose; 5 = ribose; 6 = fructose (1); 7 = fructose (2). Fructose elutes as two peaks, see Discussion.

TABLE II

RESPONSE FACTORS NORMALIZED TO GLUCOSE DERIVATIVE

Gentiobiose	0.8	Fructose 1	0.2
Lactose	1.1	Fructose 2	0.05
Maltose	0.9	Arabinose	1.6
Cellobiose	0.9	2-Deoxyglucose	2.4
Galactose	1.7	Ribose	1.0
Glucose	1.0	Fucose	1.6
Mannose	1.6	Rhamnose	0.5
Xylose	1.6	2-Deoxyribose	2.6

Reaction time at 65°C. The reaction time was varied from 5 to 30 min, using the normal derivatization procedure (see Experimental). The results are shown in Fig. 4. The order of reactivity is: pentoses ≈ fructose > hexoses > disaccharides. Decreasing the water content of the reaction mixture adversely affects the stability of pentose derivatives. When the water content was reduced to 25%, maximal responses for pentoses were obtained by 8 min. After 20 min, the responses had dropped to about 65% of the maxima. Other sugars were only slightly affected.

Fluorescent responses

The fluorescent responses of fifteen sugars, normalized to glucose, are listed in Table II. These responses apply only to the standard derivatization procedure as given in the Experimental section. Changes in the composition of the reaction mixture or in the reaction time will affect the responses (*i.e.*, yields of hydrazones). Fructose was found to react poorly with the reagent, as observed previously by Avigad[9]. Attempts to optimize the reaction for fructose revealed that its optimal reaction conditions were nearly identical to those of other sugars.

Interferences

Under the reaction conditions used for sugars, Dns-hydrazine will react readily with all carbonyl compounds, in particular simple aldehydes and ketones[11] and keto steroids[12]. However, because of the high hydrophobicity of these derivatives, they elute at much greater relative retentions than the sugar derivatives. On the other hand, carbonyl compounds may interfere with the sugar derivatization in that they consume reagent. Therefore, samples containing high concentrations of carbonyls, in addition to reducing sugars, should either be appropriately diluted or derivatized with a more concentrated reagent solution.

Fluorescence wavelengths

By careful selection of the excitation wavelength, it was found that sugar Dns-hydrazones could be preferentially excited. Thus, excitation at 360–380 nm gave good fluorescent responses for the sugar derivatives, whereas the early eluting decomposition products of the reagent were only slightly excited at these wavelengths. Alpenfels[8] recommended an excitation wavelength of 240 nm, which is close to the maximum light output of the deuterium lamp in the Kratos detector. We found that not only the sugar derivatives but also the early eluting decomposition products were

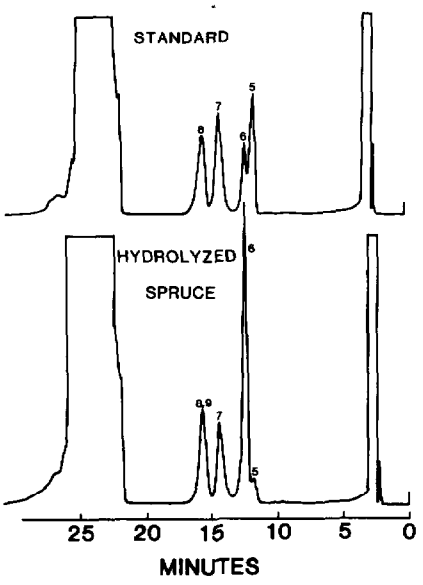

Fig. 5. Chromatograms used in intercalibration study. Conditions as in Fig. 1, except mobile phase was 0.08 *M* acetic acid–acetonitrile (81:19). Peak notations as in Fig. 1.

strongly excited at this wavelength. Therefore, we preferred the higher excitation wavelength.

Precision and accuracy

A series of eight identical isocratic runs were performed with a twelve-component sugar standard mixture. Equal aliquots of the mixture were derivatized by the normal procedure prior to each injection; a column switching technique was employed to remove excess of reagent. Peak areas were used as a measure of fluorescence response. The average standard deviation at the 0.5 nmol level was $\pm 0.4\%$. However, the standard deviation for lactose and maltose was about $\pm 0.7\%$. The latter sugars are somewhat more sensitive to variations in reaction time than other sugars (Fig. 4).

To determine the accuracy of the method, an intercalibration was performed. Aliquots of a spruce wood meal hydrolysate[13] were analyzed with the present HPLC method (Fig. 5) and with the established low pressure partition chromatographic method of Martinsson and Samuelson[14]. The latter technique gave a mole per cent composition of: Glc, 77.0; Man, 19.1; and Gal, 3.9. Small amounts of arabinose and xylose were also present, but, since these sugars are poorly resolved by the HPLC technique (Fig. 5), the mole per cent composition was recalculated on an arabinose- and xylose-free basis. The present HPLC technique gave a mole per cent composition of: Glc, 79.3 $\pm$ 1.3; Man, 17.0 $\pm$ 0.8; and Gal, 3.7 $\pm$ 0.2. Therefore, the agreement between the two methods is quite good.

Detection limit

Using the normal derivatization procedure with sugar standards diluted 1:500, 5–15 pmol per injected sugar could be detected with a signal-to-noise ratio of about

10. This detection limit could only be realized with isocratic runs because severe baseline drift occurred during gradient elution at high detector sensitivities.

DISCUSSION

The kinetics of the sugar Dns derivatization reaction appear to be strongly influenced by several interacting parameters including: the concentration of Dns-hydrazine, the concentration of trichloroacetic acid, the percentage water present, the type of organic solvent present and the reaction time. Therefore, for quantitative analysis the above parameters must be held constant for both samples and calibration standards. In addition, at least a twenty-fold excess of reagent must be present in order to obtain linear sugar responses. Given that these precautions are observed, quantitative results with excellent precision can be obtained.

The method, however, does have a few noteworthy drawbacks. Non-reducing oligosaccharides, *e.g.*, sucrose and raffinose, do not react with Dns-hydrazine and, therefore, cannot be detected by this method. In addition, xylose, arabinose and the first fructose component are not resolved, at least with the columns tested in this study (Figs. 1 and 5; Table I). Fortunately, fructose can be quantified from its later eluting component (Table II). Columns with somewhat different selectivities, *e.g.*, C_8 or phenyl columns, may give better resolution of these components.

The retention mechanism was not studied. However, from the saccharide elution order (Table I), it is reasonable to conclude that the reversed-phase (or hydrophobic) effect is the primary mechanism[4]. Secondary retention mechanisms, such as adsorption due to interactions (*e.g.*, hydrogen bonding) between hydroxyl groups on the sugars and unreacted silanol groups on the silica gel, may also be active. Adsorption may cause some degree of band broadening or even peak splitting. If this is true, then reversed-phase columns with a high degree of alkyl coverage should provide the most efficient separations of Dns-sugars.

Of all the sugars examined, only fructose eluted as two components (Fig. 1; Table I). The large separation between these components, as well as their different reaction kinetics (Fig. 4), indicate that factors other than adsorption are responsible for this effect. One possibility is that a small fraction of the fructose becomes labeled with two Dns groups. Alternatively, anhydride formation may be occurring[15]. In any event, either or both fructose peaks can be used for calibration purposes.

Despite the drawbacks mentioned earlier, we have found this technique to be particularly well suited for the trace analysis of reducing sugars, especially for aqueous samples with complex matrices, *e.g.*, physiological fluids, beverages and wood acid hydrolysates. Samples can frequently be analyzed with little or no pretreatment. Furthermore, concentration and desiccation steps, which are required in other precolumn derivatization procedures for sugars[7], are avoided in the present method. This is particularly important because these steps may result in significant sugar losses from irreversible condensation reactions with other organics in the samples[16].

Figs. 1 and 5 show examples of the application of the method. Attempts are underway to adapt this method to the analysis of simple sugars in natural waters, where concentrations of these compounds are typically in the nanomolar range.

In our laboratories, this method has, for the most part, eliminated the need for dedicated sugar analyzer systems.

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