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SEPARATION AND DETERMINATION OF PHENYL ISOCYANATE-DERIVATIZED CARBOHYDRATES AND SUGAR ALCOHOLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

The free hydroxyl groups of saccharides and sugar alcohols react with phenyl isocyanate to yield very stable and strongly UV-absorbing derivatives, which possess good chromatographic properties in a reversed-phase system. UV monitoring at *ca.* 240 nm allows detection down to the 0.5–10 ng level.

Reducing sugars yield a peak for each enantiomer, while non-reducing sugars and sugar alcohols yield a single peak. The chromatograms resemble those obtained by gas–liquid chromatography after silylation.

Oligomers containing eight glucose units have been separated with good resolution by this method. Applications to the separation and determination of sugar alcohols and wood hydrolysates are described.

INTRODUCTION

Underivatized carbohydrates are usually separated and determined by high-performance liquid chromatography (HPLC) using bonded aminophases[1], amino-modifiers on silica[2] or ion exchangers[3] together with a refractive index (RI) detector. This allows detection down to the μg level.

Trimethylsilylation followed by gas chromatography (GC)[4] results in a detection limit of *ca.* 10 ng of the carbohydrate. A peak for each anomeric form (if present) is obtained. Nachtmann and Budna[5,6] have described the use of 4-nitrobenzoyl chloride as derivatizing agent for carbohydrates and related compounds. The derivatives are chromatographed in a straight phase system and monitored by UV detection at 260 nm. The anomers are well separated and the detection limit is at the nanogram level for the appropriate carbohydrate.

The use of phenyl isocyanate (PHI) as derivatizing agent for compounds containing active hydrogen atoms, such as alcohols, water and amines, has been described in our previous papers[7–9]. PHI also reacts with the free hydroxyl groups of carbohydrates and sugar alcohols. The resulting derivatives are very stable and show excellent chromatographic properties in a reversed-phase system. UV-monitoring at 240 nm permits detection down to the nanogram level.

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The corresponding chromatograms resemble those of GC following silylation, *i.e*, reducing sugars give a peak for each enantiomer while non-reducing sugars and sugar alcohols give a single peak. Thus five peaks of derivatized L(+)-arabinose and one peak of derivatized sucrose are obtained. Oligomers containing up to eight glucose units have been separated with good resolution by this method.

EXPERIMENTAL

Apparatus

A Varian 5020 gradient liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with either a Perkin-Elmer LC-55B or LC-75 variable-wavelength UV–VIS detector (Perkin-Elmer, Oak Brook, IL, U.S.A.), a Goerz-Servogor 541 recorder (Goerz Electro, Vienna, Austria), a Valco loop injector and self-packed Spherisorb 5 ODS columns (125, 160 or 250 × 4.6 mm I.D.) (Phase Separations, Deeside Industrial Estate, Clwyd, Great Britain) was used for the chromatographic separation and detection of the derivatives. A Hewlett-Packard 3352 Lab Data System (Hewlett-Packard, Karlsruhe, G.F.R.) was used to calculate retention times and peak areas.

Reagents

The eluent was a mixture of acetonitrile (HPLC Grade S; Rathburn Chemicals, Walkerburn, Great Britain) and 0.01 *M* K_2HPO_4 adjusted to pH 7 with H_3PO_4 in twice distilled water. Phenyl isocyanate (PHI), methanol and pyridine (E. Merck, Darmstadt, G.F.R.) were of analytical grade.

The sugars and sugar alcohols (E. Merck, except for cellobiose, Koch-Light, and maltotriose, Sigma) were of biochemical grade. Commercial oligomers having more than three glucose units could not be found. Caution: use of a fume cupboard is recommended when handling PHI, because it is an eye and respiratory irritant.

Derivatization procedure

Tertiary nitrogen atoms seem to catalyze PHI reactions. Dimethylformamide (DMF) has proven to be an excellent solvent when derivatizing, *e.g*., aliphatic alcohols and amines[7,9], but in this case pyridine was chosen. It has the same catalytic properties but is a better solvent for sugars and sugar alcohols than DMF.

Standards. A 1-ml volume of a standard solution containing about 2 mg sugar per ml of pyridine was pipetted into a 10-ml measuring flask equipped with a short magnetic rod. The flask was placed in a 323°K oil-bath on a heatable magnetic stirrer. A 2-ml volume of PHI was added and the mixture allowed to react for 1 h in the stoppered flask. A 1-ml volume of methanol was added to the cooled flask in order to destroy the excess of PHI. After 5 min the measuring flask was filled to the mark with either pyridine or DMF. Appropriate dilutions were made so that the injected solution usually contained <0.1 mg/ml of each sugar in the calibration solution.

Samples. Aqueous samples containing not more than 1 mg of each sugar to be determined were brought to dryness in a 10-ml test-tube in a rotary evaporator. A short magnetic rod, 1 ml of pyridine and 2 ml PHI were added, the tube was stoppered and treated in the same way as for the standards. The reaction mixture was then transferred into a 10-ml measuring flask and filled to the mark. Solid samples or dry samples in pyridine were directly weighed or pipetted into the measuring flask.

Blanks. It is advisable to prepare a blank in the same way as for the standards in order to check the "purity" of the chromatogram in the carbohydrate region.

Liquid chromatographic separation and quantitation

Derivatized samples and standards dissolved in pyridine or pyridine–DMF and free of particulate matter were injected (1–5 μl) into the chromatographic system via the Valco loop injector. To avoid tailing of the pyridine peak, it is important to turn the valve back to the load position after *ca.* 5 sec.

The Spherisorb 5 ODS column was at ambient temperature. The eluent, consisting of water and acetonitrile, was pumped (2 ml/min) either isocratically or using different gradients depending on the quality of the compounds to be determined. The peaks were monitored at 240 nm (Fig. 1). It is also important to employ low concentrations of the individual derivatives, usually <0.1 mg/ml as free sugar or sugar alcohol, so that they fall in the relatively narrow linear range.

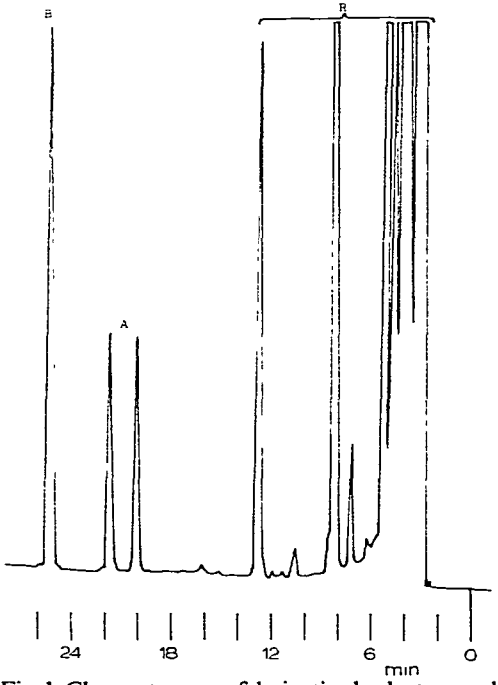

Fig. 1. Chromatogram of derivatized galactose and sorbitol. Column: Spherisorb 5 ODS, 120 × 4.6 mm I.D. UV detection at 240 nm, 0.05 a.u.f.s. Gradient: 45–80% acetonitrile at 1%/min. Peaks: R = reagents; A = α- and β-galactose; B = sorbitol.

Quantitation was carried out either in the usual way[8] using, *e.g.*, O-nonyl-N-phenylurethane or derivatized sorbitol as internal standard, or by the method of Nachtmann and Budna[6] which means that each reacted hydroxy group gives rise to a constant increase of the extinction coefficient. Thus a certain "response factor", f, for each known sugar can be calculated using eqns. 1 and 2:

$$C_{\text{sugar}} = \frac{A_{\text{sugar}}}{A_{\text{standard}}} \cdot \frac{C_{\text{standard}}}{f} \qquad (1)$$

$$f = \frac{MW_{standard}}{MW_{sugar}} \cdot \frac{n(OH)_{sugar}}{n(OH)_{standard}} \quad (2)$$

Where C = amount (concentration) reacted, A = peak area, MW = molecular weight and n(OH) = number of hydroxy groups reacted.

In this work sorbitol ($n = 6$) was used as internal standard. The Hewlett-Packard Lab Data System was employed for peak area calculations.

RESULTS AND DISCUSSION

Amount of PHI, reaction time and temperature, destruction of excess of PHI

Alcohols, amines, carboxylic acids and water react with PHI. Therefore a large excess of the derivatizing agent should be used, at least with unknown samples. Water should be removed before derivatization[8,9]. In contrast to amines and aliphatic alcohols, carbohydrates and sugar alcohols do not react at room temperature. Various temperatures and reaction times were tested but the "smoothest" reaction conditions were found to be 1 h at 323°K; a time of 15 min at 353°K could also be used, at least for non-degradable sugars.

PHI reacts under these conditions with all free hydroxyl groups of the sugar or sugar alcohol. This was shown by infrared and ^{13}C nuclear magnetic resonance studies of the crystalline derivative of D(+)-glucose.

The excess of PHI must be destroyed before injection. Otherwise it reacts with water in the column. Methanol was found to be a suitable reagent for this purpose because the corresponding urethane is eluted before the sugar derivatives. It is advisable to cool the reaction vessel before adding methanol.

Standards

Standards were prepared from commercial sugars and sugar alcohols, when available, by the procedure mentioned above. Crystalline derivatives were not prepared except for D(+)-glucose, because they always tend to contain diphenylurea, the reaction product of water and PHI.

Oligomers were prepared from acid-hydrolyzed cellulose. No standards were available and peaks were identified from a comparison of the measured retention times with those of standard pentoses, hexoses and di- and trisaccharides (Figs. 2 and 3). A mass spectrometric study or exclusion chromatography would probably give further confirmation of these "unknown" peaks, but no such work has yet been done.

Note: Fructose decomposes during the derivatization procedure.

Stability of the derivatives

Derivatized standards and samples were stored in solution at room temperature for several days with no noticeable changes in the chromatograms. The urethane bond is not very pH-sensitive.

Liquid chromatography

As mentioned above, reducing sugars yield a peak for each enantiomeric form present. Thus, five peaks of derivatized L(+)-arabinose, three of D(+)-xylose, two of D(+)-glucose, etc., are obtained. This also leads to some degree of overlapping of

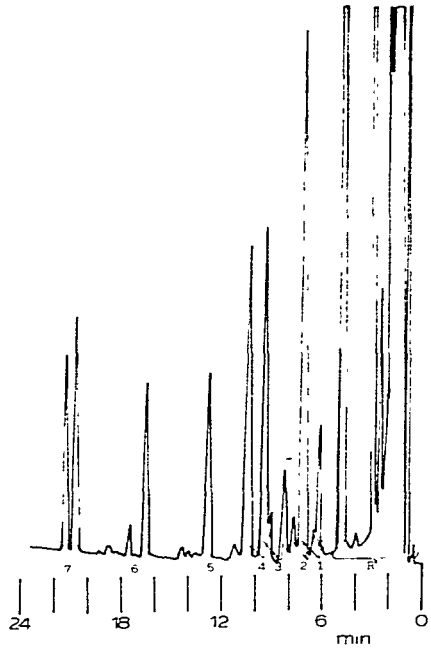

Fig. 2. Chromatogram of mono-, di- and trisaccharides. Column: Spherisorb 5 ODS, 150 × 4.6 mm I.D. Gradient elution: 55–95% acetonitrile in 25 min. UV detection at 240 nm, 0.08 a.u.f.s. Peaks: R = reagents; 1 = xylose; 2 = arabinose; 3 = glucose; 4 = mannose; 5 = sorbitol (0.03 mg/ml internal standard); 6 = cellobiose; 7 = maltotriose. Injection: 2 μl, *ca.* 0.06 mg/ml of each saccharide.

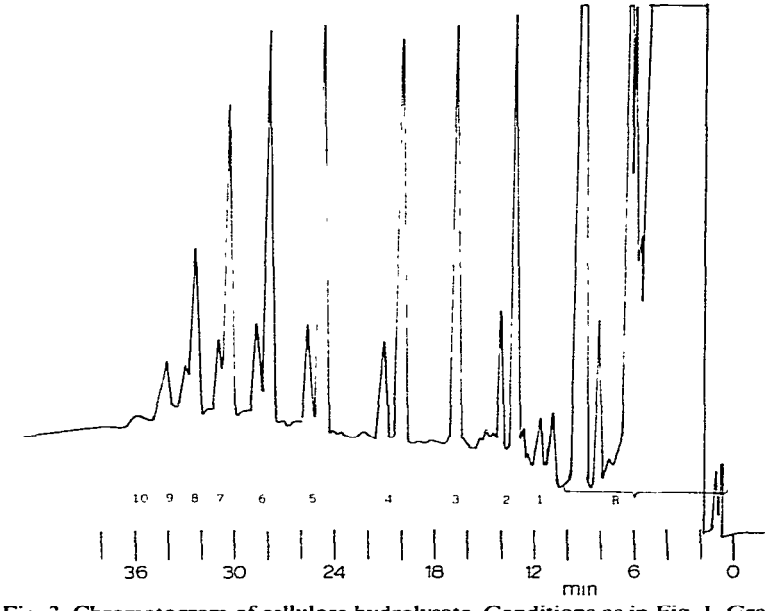

Fig. 3. Chromatogram of cellulose hydrolysate. Conditions as in Fig. 1. Gradient: 55–99% acetonitrile in 30 min. Peaks: R = reagents; 1 = pentoses; 2 = hexoses; 3 = sorbitol (internal standard); 4 = di-; 5 = tri-; 6 = tetra-; 7 = penta-; 8 = hexa-; 9 = hepta-; 10 = octasaccharide.

similar sugars (Fig. 2). The ratio between, *e.g.*, the α- and β-forms, varies depending on the origin of the sample and whether it is dissolved in pyridine before adding PHI. The sugars are eluted in the order of the number of free hydroxyl groups present in the original compound. Thus, pentoses are eluted before hexoses which are eluted before disaccharides, etc.

Non-reducing sugars and sugar alcohols (Fig. 4) yield a single peak as expected.

Linear gradients, starting from at least 45% acetonitrile, were usually employed to elute the compounds. To avoid tailing of pyridine the water was adjusted to *ca.* pH 7.2. Column lengths varied from 12.5 to 25 cm (0.46 cm I.D.), but *ca.* 15 cm packed with 5-μm ODS particles is sufficient for most purposes. Oligomers containing eight glucose units could be eluted by using this chromatographic system.

Detection and linearity

The derivatives show a very strong UV maximum at *ca.* 240 nm. This enables

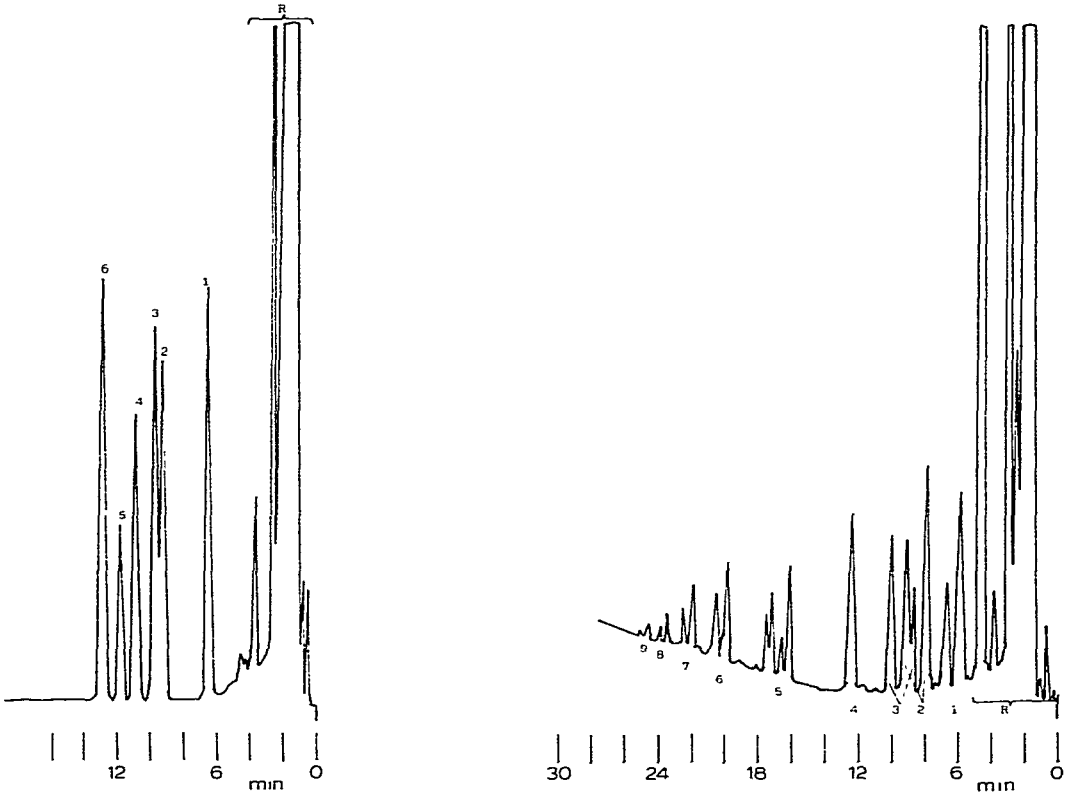

Fig. 4. Chromatogram of sugar alcohols. Conditions as in Fig. 2. Peaks: R = reagents; 1 = erythritol; 2 = arabitol; 3 = xylitol; 4 = rhamnitol; 5 = dulcitol; 6 = sorbitol + mannitol.

Fig. 5. Chromatogram of wood hydrolysate. Conditions as in Fig. 2. Peaks: R = reagents; 1 = xylose; 2 = glucose; 3 = mannose; 4 = sorbitol (internal standard); 5 = di-; 6 = tri-; 7 = tetra-; 8 = penta-; 9 = hexasaccharide.

detection down to the 0.5–10 ng level of the starting compound. A 1-ng amount of sorbitol gave a signal to noise ratio of 1:12.

The derivatization is quantitative, as shown by the reaction of various amounts of D(+)-glucose with PHI.

Derivatized D(−)-sorbitol was used as internal standard. Quantitation was by the usual internal standard method and by use of eqns. 1 and 2. The graphic plot of concentration *vs.* peak area was shown to be linear in the range 5–1000 ng counted as *e.g.* free glucose. If larger amounts are injected the curve becomes non-linear. This is due to peak broadening and poor resolution because of the relatively high molecular weights of the derivatives. For examples glucose (MW 180) has MW = 180 + 5 × 120 (PHI) = 780 when derivatized. Raising the column temperature to, *e.g.*, 323°K may extend the range of linearity.

Applications

This method has mainly been used to determine the content of mono, di-, tri- and oligomeric sugars in wood hydrolysates (Fig. 5). Since the anomer ratio was not of interest, no attempts were made to keep it constant, not even in the derivatization of standards. Sugar alcohols in various samples have also been determined by this method.

During the evaluation of the method the following sugars and sugar alcohols were derivatized with good results: D(−)-ribose, L(+)-arabinose, D(+)-xylose, L(+)-rhamnose, D(+)-mannose, D(+)-galactose, D(+)-glucose, maltose, lactose, sucrose, D-cellobiose, maltotriose, *meso*-erythritol, D(+)-arabitol, xylitol, dulcitol, rhamnitol, D(−)-sorbitol and mannitol.

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