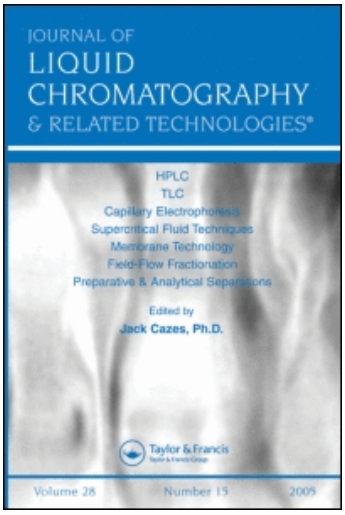

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LIQUID CHROMATOGRAPHY OF SUGARS ON DIOL COLUMNS. DETECTION WITH CUPRAMMONIUM REAGENT

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ABSTRACT

Post column reaction of sugars with cuprammonium reagent is discussed. Formation of the complex involves two molecules of carbohydrate and one molecule of $Cu(NH_3)_4(H_2O)_2^{2+}$ with the result of a shift towards longer wavelength in UV spectrum. 1 nanomole of fructose is detected, the reaction is simple, fast allowing very short reaction coil which does not increase band broadening it thus matches most of the requirements in the analysis of sugars in food products. Diol bonded silica is much less retentive than amino bonded silica and cannot compete in the separation of complex mixtures of sugars but is well suited for the analysis of one reducing sugar as important losses occur with NH_2 columns. Diol phase is well adapted to the separation of oligosaccharides.

The use of HPLC as a means of determining all types of sugars has greatly increased for the last years. Many types of packings (bare silica, ion exchange with Na^+ or Ca^{2+} , alkyl or amino bonded silica) are available and have been advocated (1). Classical detection can be performed with refractive index or UV detectors. With the former the sensitivity is rather low and gradient elution is impossible, the latter operated at 190 nm require high purity solvents.

Pre or post column derivatization techniques are more popular. Pre derivatization changes the elution scheme and may produce artefacts. A great deal of work has been performed on post column reactors and a lot of derivatization procedures have been published. A complete survey of the literature is far beyond the scope of this paper. Vratny et al (2) carried out a comparative study of post column derivatization reagents and selected the p amino benzoic hydrazide which exhibited the best sensitivity (10^{-8}g). Electrochemical detection may be used after derivatization ; 2 cyano acetamide derivatives exhibit oxidative properties as emphasized by Honda (3). The ability of Copper II to complex with sugars is well known and may be useful. Some interesting papers appeared such as the use of cuprammonium hydroxide (4) in post column reaction and UV detection, coating of a silicagel with copper ammonia solution (5) and noticeable LCEC detection of reducing sugars by copper phenanthroline reagent which is reduced by the solute (6). Claimed sensitivity are 450 ng, 12 ng and 0.2 ng respectively. The first technique looks simpler but is not widely used owing to the complexity of the reaction (2). With the advent of more sophisticated instrumentation we re investigated the applicability of cuprammonium reagent. Moreover this paper deals with some separations performed with diol packing which is less common than amino bonded silica in carbohydrate separations.

MATERIAL AND METHODS

Reagent

A stock solution is prepared from either copper sulfate or copper hydroxide. 25 g of $CuSO_4$, 2 H_2O are dissolved in 500 ml of distilled water and 500 ml of concentrated (26 %) ammonia are poured while thoroughly mixing. Working solution is prepared by adding 100 ml of stock solution to 250 ml ammonia and 650 ml of distilled water. The solution is filtered with 0.45 µm HA Millipore filter and degassed by Helium bubbling. The stock solution is stable for many weeks. To prevent any change stock solution and working solutions are stored in glass bottles wrapped in Aluminium foils.

Apparatus and Materials

The schematic of the apparatus is displayed on Fig 1. HPLC was carried out with a Hitachi 655 A. 11 Liquid Chromatograph

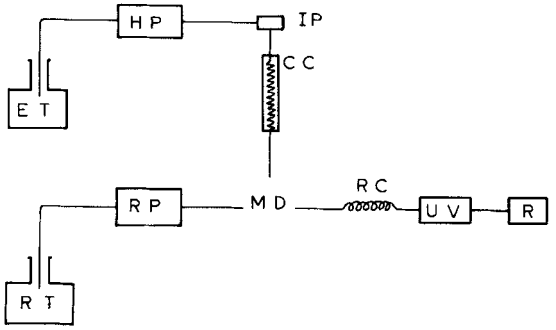

Fig 1 : Instrumental set up for postcolumn detection
ET : eluent tank ; HP : high pressure pump ; IP : injection port ; CC : chromatographic column ; RT : reagent tank ; RP : reagent pump ; MP : mixing device ; RC : reaction coil ; UV : UV detector ; R : recorder ;

equipped with a six port Rheodyne 7125 injection valve. Post column reactor is an Hitachi 655. A. 13.
Separations were carried out on Hibar columns (250 x 4 mm) packed with Lichrosorb NH_2 5 μm or Lichrospher Diol 5 μm. The whole equipment is available from Merck France (Nogent/Marne F).

Effluent from the analytical column is led to a mixing device through which the reagent is added by the 655 A. 13 pump.
The reaction mixture is then led to a capillary coil of 0.25 mm i.d. made out of PTFE.
Detection is performed with a variable wavelength UV photometer
Absorbance spectra were recorded with a Beckman 2 A.

Chemicals

Acetonitrile is Lichrosolv grade. Water is distilled over potassium bichromate.

Sugar standards were available from Merck. (Darmstadt FRG)

RESULTS AND DISCUSSION

In aqueous solutions Copper II will complex with ammonia up to the formation of $\left[Cu\ (NH_3)_4\ (H_2O)_2\right]^{2+}$ as addition of the fifth

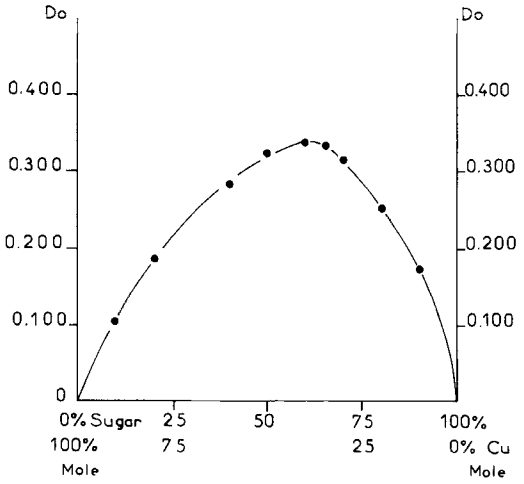

Fig 2 : Job's plot of recorded absorbance with the variation of relative proportions of reagents. Solvent acetonitrile/water ; 80/20 ; vol/vol.

and sixth molecule of NH_3 is difficult (7). The UV spectrum of this complex exhibits a rather broad peak with a maximum at 247 nm as previously described (4). when complexation with sugar occurs a shift towards longer wavelengths is noticed. λ max is slightly different with the involved sugar and Table I lists the observed data with some solutes. Interesting differences are noticed. Among the disaccharides Lactose will be more easily detected than saccharose as the absorbance is much higher.

It may be supposed that one or two molecules of carbohydrate will replace one or two ammonia moieties in the complex.

By variation of the relative proportions of sugar and reagent and checking at max of the sugar-cuprammonium complex according to the Job's method (8) it is possible to determine the sugar/reagent ratio. Data from Fig 2. clearly indicates that two molecules of carbohydrate are involved with glucose

$$2\ C_6H_{12}O_6 + \left[Cu(NH_3)_4(H_2O)_2\right]^{2+} \longrightarrow \left[Cu(NH_3)_2(H_2O)_2(Carb)_2\right]^{2+}$$

The rate of formation of the complex is very fast. We recorded the area of the peak when reagent and glucose are mixed in reaction coil

of variable length. The constancy of the observed area shows that the time of reaction is very short thus permitting a short reaction coil. To prevent any loss of efficiency a 20 cm capillary pipe was selected. In the same way an increase in temperature does not increase the area of the observed peak.
No drastic requirement is needed exept a high ammonia concentration to prevent microprecipitation of copper salts.

SELECTION OF CHROMATOGRAPHIC CONDITIONS

λ max listed in Table I could not be used in detection as cuprammonium reagent is absorbing and the difference $\lambda_{complex} - \lambda_{reagent}$ must be emphasized. At 315 nm the reagent has no absorption and this wavelength is suitable.

Addition of the reagent yields a dilution which contributes to band broadening. By keeping constant the total flow rate residence time in the capillary reaction coil will remain constant and peak area will be strictly related to the amount of detected solutes. Fig 3 shows variation of peak height and peak area versus the ratio reagent/mobile phase flow rate. Peak area remains constant indicating that the amount of formed and detected complex is constant whereas peak height decreases when reagent flow rate is increasing. For the sake of detection a maximum peak height is needed and minimum reagent flow rate of 0.25 $ml.min^{-1}$ was selected.

SENSIBILITY LINEARITY

Four calibration curves are displayed on Fig 4. Peak height is plotted to evaluate the sensitivity as one could quickly have a glance on it on a recorder chart. Linearity is excellent through the range checked. The average limit of detection is 2 n moles which means about 300-400 ng detected.
The limit is much higher than the coated silica and the electrochemical detection quoted above (4,5). It must be pointed out that coating of silica is not very reproducible and no data on the life time is given. It is obvious that electrochemical detection is better and work is in progress in the laboratory with this device. However electrochemical detection needs very low noise and the pump used in the post column reactor device is not pulseless. Improvement in this area is needed. This detection limit lies within the range of that previously published by Grimble and thus represents what can be hoped

Table I

Carbohydrate	concentration	λ max. (nm)	Absorbance
Xylose	10^{-3}M	270	0.350
Ribose	10^{-3}M	270	0.350
Arabinose	10^{-3}M	265	0.450
Fructose	5.10^{-4}M	255	0.600
Sorbose	5.10^{-4}M	255	0.600
Glucose	2.10^{-3}M	258	0.700
Saccharose	5.10^{-3}M	273	0.410
Lactose	5.10^{-3}M	260	1.200

U.V. Absorbance Data of some cuprammonium sugar complexes. Solutions in Acetonitrile/water mixture 80/20 vol/vol.

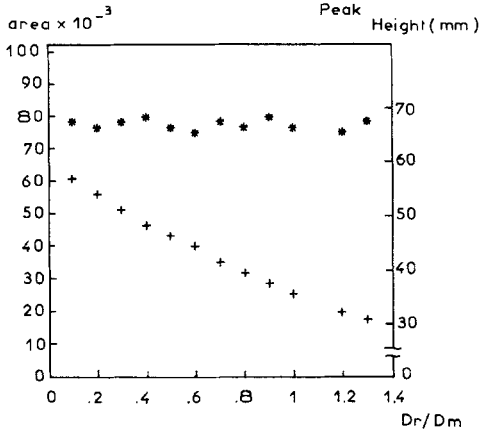

Fig 3 : Peak area and peak height versus the ratio of reagent and mobile phase flow rates.
D_r = reagent flow rate
D_m = mobile phase flow rate

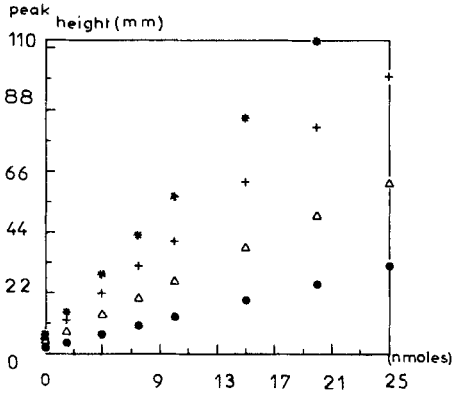

Fig 4 : Plot of peak height versus amount of injected solutes
✱: Fructose : + : Xylose ; △: Glucose ; ●: Sucrose

with commercially available equipment. It can match most of the analytical problems encountered.

COMPARISON OF NH_2 AND DIOL BONDED SILICA

Cuprammonium reagent is useful with either reducing or non reducing sugars. The amino bonded silica packing with a reversed phase type solvent Acetonitrile/water has gained wide acceptance in sugar analysis but this packing cannot be used successfully with reducing sugar owing to the formation of Schiff's base. Important losses of solute occur as demonstrated by Brons and Oliemann (9). Fig 5 displays the chromatograms of a standard carbohydrate mixture performed with amino and diol bonded silica with an acetonitrile water mixture as eluent. It is evidenced that the diol packing is much less retentive (k' are roughly divided by two) and less selective than the amino packing. An increase of the acetonitrile content does not improve the separation. It is will known that sugars are only slightly soluble in acetonitrile and many problems are encountered due to this solubility. Our data are consistent with the above quoted work (9) but it may be pointed out that the reproducibility of k' on diol phases is dependent on bonding procedure. Slight amount of polymeric material significantly changes retention. Nevertheless the diol bonded silica is particularly well

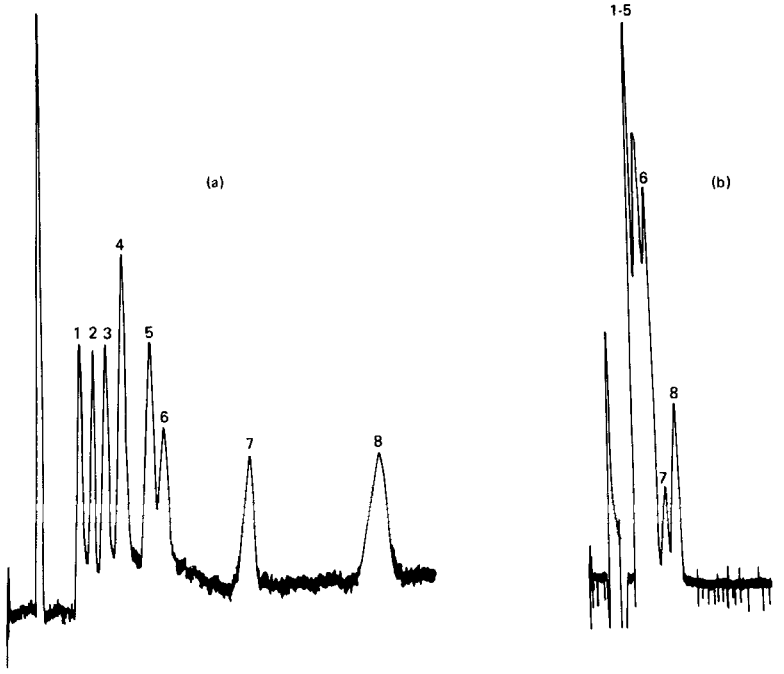

Fig 5 : Separation of a mixture of standard carbohydrates
a) column 250 x 4 mm packed with NH_2 bonded silica d_p = 5 µm
b) column 250 x 4 mm packed with Diol bonded silica d_p = 5 µm
Mobile phase acetonitrile/water 80/20
D_m : 1.0 ml/min ; D_r : 0.25 ml/min
Detection UV 315 nm 0.01 A U F S
1 : Ribose ; 2 : Xylose ; 3 : Arabinose : 4 : Fructose ;
5 : Glucose ; 6 : Galactose ; 7 : Sucrose ; 8 : Lactose.

suited for quantitation of one particular sugar - especially reducing - in a more or less complex matrix. Some examples are given below.

Applications

Quantitation of fructose in instant coffee or chicory is very easily performed by direct injection of a diluted solution as demonstrated in chromatogram of Fig 6.

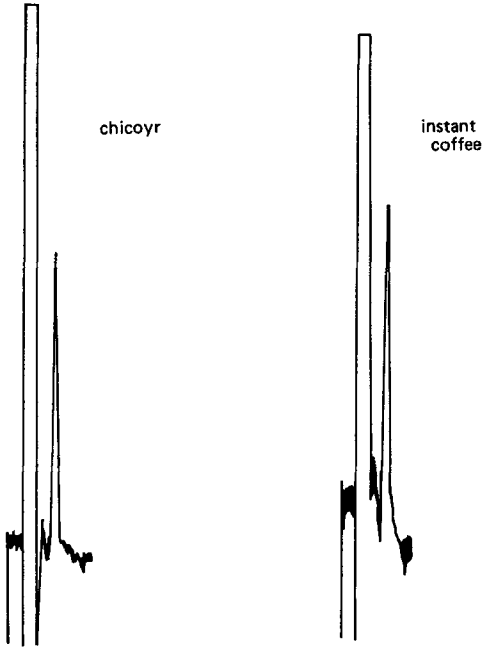

Fig 6 : Quantitation of fructose in coffee drinks
Column Diol bonded silica 250 x 4 mm d_p = 5 µm
Mobile phase acetonitrile/water 80/20
D_m = 1.5 ml/min
D_r = 0.25 ml/min
Detection 315 nm 0.02 A U F S

No clean up of the sample is necessary, provided a precolum is fitted to the analytical column, and the cuprammonium post column detection is well within the range of the amount of material.

Séparation of rhamnose, acetyl glucosamine, acetyl galactosamine, glucose and galactose in biological fluid is excellent on NH_2 column (Fig 7) but can only be used for qualitative purposes. The dosage of each solute performed on either NH_2 or Diol columns exhibits very significant differences particularly for glucose and galactose. It is clear that the loss of these sugars on NH_2 is important which does not occur with diol phase. To avoid misunderstanding the analysis were carried out with column of iden-

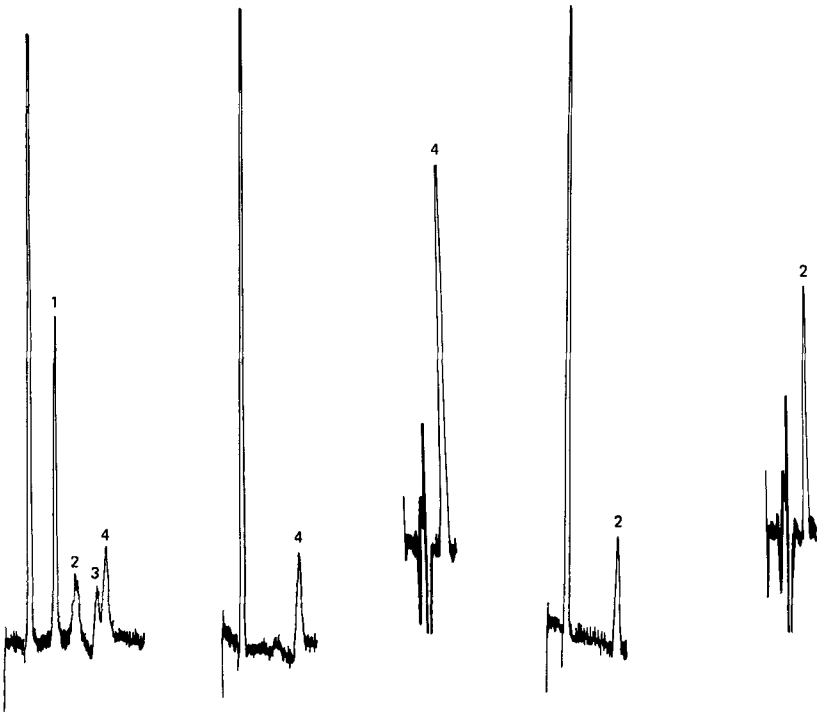

Fig 7 : Separation of mixture of sugars and related compounds in biological fluid.
Column NH_2 bonded silica 250 x 4 mm d_p = 5 µm
Mobile phase acetonitrile/water 80/20
D_m = 1.0 ml/min ; D_r = 0.25 ml/min
Detection 315 nm 0.01 A U F S
1 : Rhamnose : 2 : Acetylglucosamine ; 3 : Acetylgalactosamine ;
4 : Glucose ; 5 : Galactose.
b : Glucose on NH_2 column
c : Glucose on Diol column (250 x 4 mm : d_p = 5 m)
d : Acetylglucosamine on NH_2
e : Acetylglucosamine on Diol.

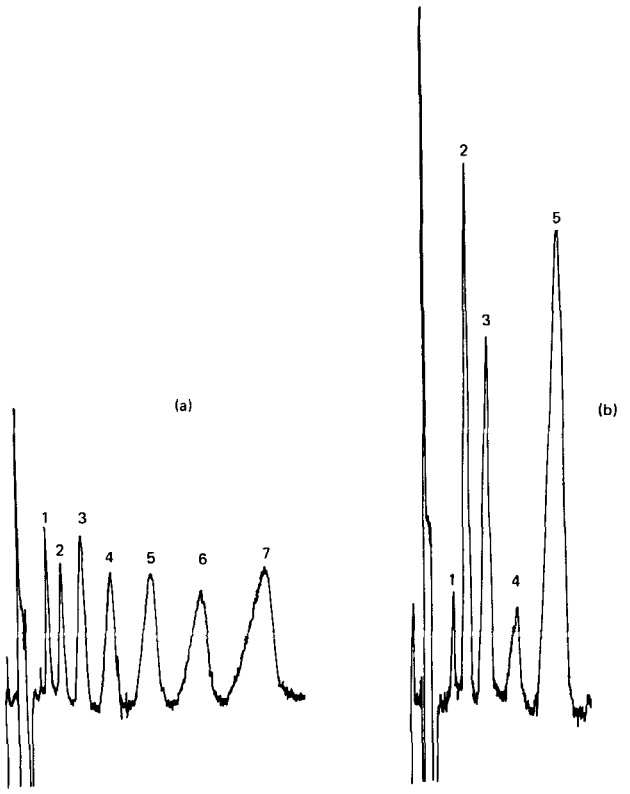

Fig 8 : Separation of Malto oligosaccharides
Column : Diol bonded silica 250 x 4 mm d_p = 5 µm
Detection UV 315 nm 0.02 A U F S
1 : Glucose ; 2 : Maltose : 3 : Maltotriose ; 4 : Maltotetrose ;
5 : Maltopentose ; 6 : Maltohexose ; 7 : Maltoheptose.
a) standard mixture
b) reaction with amylase.

tical performances and the retention was kept rather similar according to the work of Martin and Guichon (10).

In order to check the ability of diol phase to separate some carbohydrates we carried out the separation of malto oligo saccharides. The chromatogram of Fig 8a shows the excellent separation of such compounds. Peak tailing increases with the molecular weight but the selectivity is excellent and permits to use h.p.l.c. to check the kinetics of enzymatic reaction of α amylase with malto pentose (Fig 8b).

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