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CONTRIBUTION TO THE ELUCIDATION OF THE MECHANISM OF SUGAR RETENTION ON AMINE-MODIFIED SILICA IN LIQUID CHROMATOGRAPHY

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SUMMARY

Liquid chromatography of reducing or non-reducing sugars results in single peaks on amine-modified silica with acetonitrile–water as eluent. In spite of the two anomeric forms of the reducing sugars, single peaks can be obtained because mutarotation is fast under these conditions. The bonded amine groups catalyse the mutarotation in such a way that triethylamine added to the eluent has no influence. The separation of the sugars is the result of their partition between two liquid phases, because the composition of the stationary liquid phase appears to be much richer in water than the eluent.

INTRODUCTION

Linden and Lawhead[1] and Palmer[2] were first to apply alkylamine-modified silicas for sugar analysis. A mixture of acetonitrile and water was used as eluent. Ever since, much has been written about the application of this system. Its popularity is related to the following facts:

(1) Silica is much more resistant to pressure than ion-exchangers (reviewed by Jandera and Churacek[3]).

(2) There is a quick exchange of mass between the mobile and the stationary phase, which enables high eluent flow-rates, without significant loss of resolution.

(3) No sample derivatization is needed.

Schwarzenbach[4] described the chemical modification of silica by reaction of aminopropyltriethoxysilane with the surface silanol groups. The separation process on this material was stated to be that of reversed-phase chromatography by Meagher and Furst[5], normal-phase chromatography (because increased polarity of the mobile phase leads to shorter elution times) by Rabel *et al.*[6] and competitive interaction of the water in the eluent and the sugar injected with the polar bonded phase by Hettinger and Majors[7]. Majors[8] reported that sugars are retained because their hydroxyl groups react with the bonded amine. Jones *et al.*[9] found that k' values were proportional to the amine loading becoming constant at higher loadings. Kahle and Tes-

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arik[10] separated the anomeric forms of mutarotating sugars on an alkylamine column (SO_4^{2-}). The retention time for the mixed peak on the free amine column was the average of those for the separate peaks on the column in the sulphate form.

Physical modification of silica by coating with amine has also been investigated[11–14]. Silica impregnated *in situ* should be considerably cheaper than chemically modified silica, and, according to Aitzetmüller[13], is also more stable. Chemically modified silica can lose amine by hydrolysis, resulting in decreased sugar retention. Using the physically modified carrier, unchanged retention times are obtained after prolonged use if some amine is added with the eluent. Wheals[12] and White[14] and their co-workers investigated the influence of different amines on sugar retention. Both retention[12] and separation efficiency[14] appeared to be influenced by the type of amine.

Other relevant research has concerned the composition of the mobile phase, that most often employed being a mixture of acetonitrile and water. Müller and Siepe[15] successfully applied an eluent comprising acetone, ethyl acetate and water, which has the advantage that there is no need for acetonitrile (which is poisonous to man). Rabel *et al.*[6] obtained poor separations using methanol–water eluents, which they attributed to the high polarity of methanol causing poor solvation of the bonded phase.

We now describe the influence of amine in chemically modified silica on the mutarotation rate of sugars, and also experiments carried out to determine the composition of the stationary phases at different acetonitrile–water ratios in the eluent. The results contribute to the elucidation of the mechanism of sugar separation, and to the understanding of such phenomena as column ageing, influence of the type of amine and the eluent composition.

EXPERIMENTAL

Influences of triethylamine (TEA) in the eluent on the separation of sugars using amine-modified silica

A sample containing fructose, glucose, sucrose and lactose was injected on a Waters μCarbohydrate column (300 × 3.9 mm) at a column temperature of 20°C and an eluent flow-rate of 2 ml/min. Other conditions were as described previously[16]. The eluent was acetonitrile–water (80:20), respectively without TEA, with 0.001 *M* TEA and with 0.003 *M* TEA.

Influence of different parts of the chromatographic system on the mutarotation rate

Freshly prepared solutions of 1 g glucose in

(a) 75 ml acetonitrile + 25 ml water + 1 g LiChrosorb NH_2 (E. Merck, Darmstadt, G.F.R.)

(b) 75 ml acetonitrile + 25 ml water

(c) 100 ml water

(d) 75 ml acetonitrile + 25 ml water + 1 g Merckosorb SI 60 (E. Merck)

(e) 75 ml acetonitrile + 25 ml water + 1 g LiChrosorb RP-8 (E. Merck) were allowed to mutarotate at 20°C while stirring. Samples of 10 μl were injected at different times on an Aminex A-5 (Ca^{2+}) column (250 × 4.6 mm) at 45°C. Distilled water was used as the eluent at a flow-rate of 0.5 ml/min. Under these conditions the mutarotation rate can be determined[16].

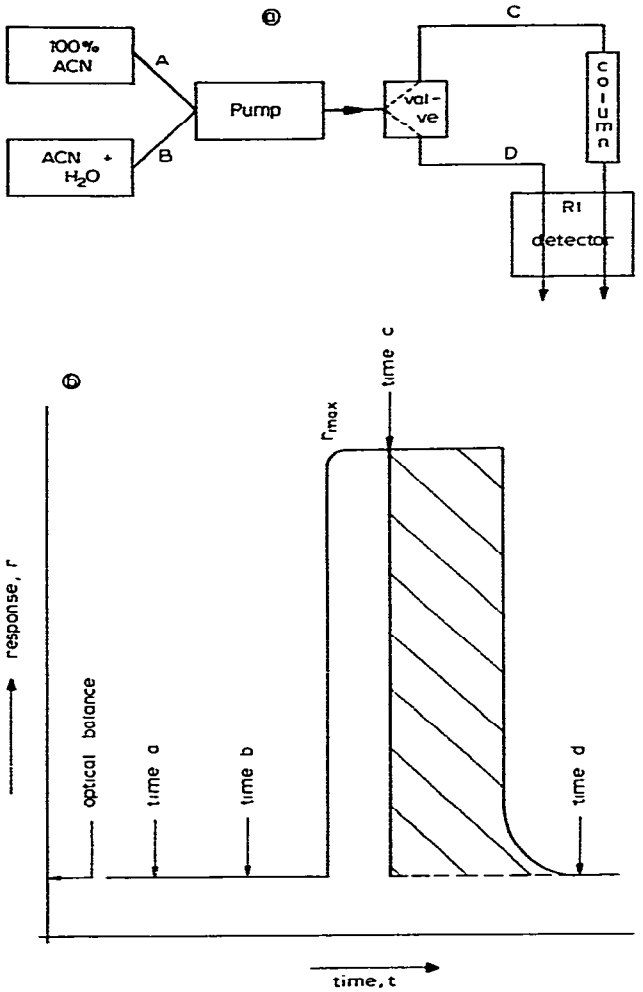

Fig. 1. a. Experimental set-up to determine the water hold-up in the column. b. Example of detector output from which the water hold-up could be calculated. ACN = Acetonitrile; RI = refractive index.

Carbon, hydrogen and nitrogen contents of LiChrosorb NH_2

These were determined with a Hewlett-Packard/F&M Model 185 CHN-analyzer. A known amount of sample was burned in an oxygen atmosphere. The nitrogen oxides formed were reduced, and the amounts were compared with those evolved from a reference sample.

Porosity of LiChrosorb NH_2

This was determined with a Carlo Erba Series 200 porosimeter. The amount of mercury which penetrated into the pores was determined as a function of the pressure applied.

Amount of water retained by LiChrosorb NH_2 in different acetonitrile–water eluents

A stainless-steel column (100 × 4.6 mm) was packed with LiChrosorb NH_2

and kept at 20°C. Eluent was pumped through this column at a flow-rate of 0.5 ml/min using a Waters 6000A pump. Between the pump and the column was placed a low-dead-volume valve to switch the eluent flow from the column to the reference channel of a Waters R 401 refractive index detector. The other channel of the detector was connected to the exit of the column, as shown in Fig. 1a. The delay in the attainment of equilibrium after changing from water-free eluent to eluents containing 10, 20, 30 and 40% (v/v) water was measured.

First, 100% acetonitrile was pumped via A and D, and after a constant detector signal was obtained, then via A and C, again up to a constant signal. By adjusting this constant signal to recorder zero with the optical zero of the detector, the situation reached was as depicted in Fig. 1b at time *a*. This corresponds to a completely water-free column and detector. At time *b* the eluent composition was changed by switching from A–C to B–D, and after some time a different but constant response was obtained, corresponding to a water-containing liquid in the reference and a water-free liquid in the measuring channel of the detector. Finally, at time *c*, the flow was changed from B–D tot B–C, and the water-containing eluent passed into the column. Some water is retained by the column and after some delay a new equilibrium in the detector is attained at time *d*.

The hold-up volume of water, v_w, on the column can be calculated as follows

$$v_w \text{ (ml)} = (q, r_{max} \int_c^d r dt - v_d) f_w$$

(for r, t, c and d see Fig. 1b), where q = eluent flow-rate; f_w = water fraction in the eluent, v_d = dead volume between valve and detector and $\int_c^d r dt$ = the surface underneath the response–time curve.

RESULTS AND DISCUSSION

With Aminex A-5 (Ca^{2+}) and water as eluent, the addition of TEA to the eluent causes a significant decrease in peak widths for mutarotating sugars[16]. However, in our experiments, there was no influence of amine addition on peak widths nor on retention times, for amine-modified silica with acetonitrile–water as eluent, not even at the highest (0.003 *M*) amine concentration. This may be caused by the α- and β-forms of the sugars having the same capacity factor (k') or by the fact that mutarotation proceeds rapidly in this system.

We thus determined the mutarotation rate under the conditions described under Experimental. The results are given in Fig. 2. Compared to the rate in pure water (c), a lower rate was found on addition of ACN (b). Further addition of *n*-octyl-modified SI 60 silica had hardly any effect (e), while unmodified silica had a slight positive effect (d). However, addition of LiChrosorb NH_2 greatly increased the mutarotation rate (a). It can be concluded that the amine groups are responsible for the much higher reaction rate. Under chromatographic conditions it is expected that the mutarotation rate will be even higher because the concentration of amine groups, as well as the ratio of these groups to the amount of glucose, is approximately 50 times higher.

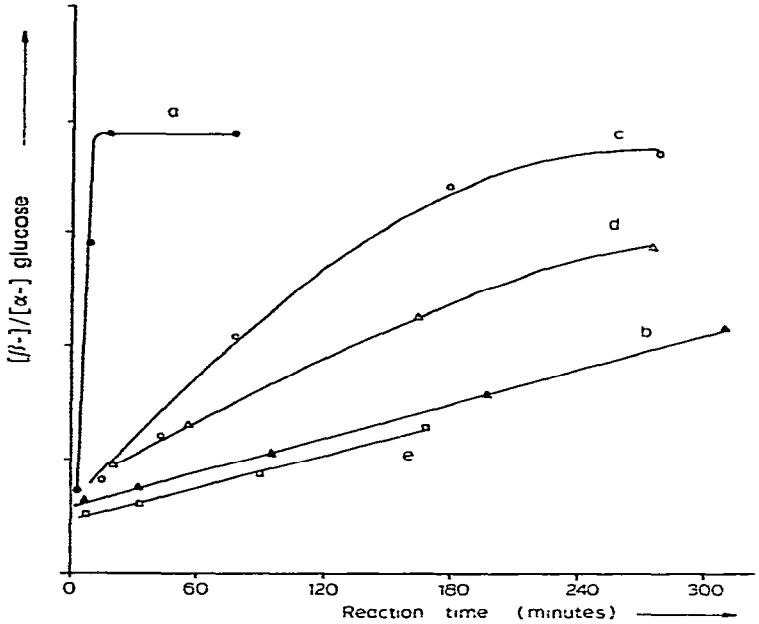

Fig. 2. Mutation rate of glucose under different conditions. (For conditions a–e, see the section *Influence of different parts of the chromatographic system on the mutarotation rate.*)

Kahle and Tesarik[10] separated anomeric α- and β-forms of sugars on an amine-modified column, neutralized with sulphate. The retention time, t_R, of the mixed peak on the unneutralized column corresponded to

$$t_{R(\alpha+\beta)} = t_0 [1 + k'_\alpha f_\alpha + k'_\beta (1 - f_\alpha)]$$

Where k' = capacity factor in the neutralized column, f_α = mol fraction of the α-anomer and t_0 is the elution time of an unretained compound. It can be concluded that the retention is not dependent on whether the amine is in the free or sulphate form, that the k' values for the α- and β-species are different and that when the amine is in the free form only one, mixed peak is observed, as a result of the high mutarotation rate.

By use of a CHN-analysis, the quantity and quality of the amine modification was determined. The results are in accordance with the manufacturer's statement that propylamine modification had been used. We found 3.51 % C, 1.10 % H and 1.40 % N which corresponds to an atomic ratio of 3:11:1. The hydrogen content is somewhat high due to loss of desorbed or dehydrated water. We calculated that the amount of propylamine per gram of LiChrosorb NH_2 was 1.0 mmol; or 0.6 mol/l in a packed chromatographic column.

The pore volume of LiChrosorb NH_2 was approximately 0.7 ml/g with an average pore diameter of 60 Å.

There is a remarkable similarity in elution patterns with the systems amine-

modified silica, acetonitrile–water and anion exchanger/ethanol–water as described by Samuelson[17]:

increased retention with increasing molecular size of the sugar
higher retention for aldoses than for ketoses
higher retention for β- than for α-anomers[10,18]

Based on measurements by Rückert and Samuelson[19], which showed that the stationary liquid in the anion exchanger/ethanol–water system contained more water than the mobile liquid, and, therefore, that the sugars preferred the stationary phase[20], Samuelson[17] concluded that the separation mechanism is one of liquid–liquid or partition chromatography.

TABLE I

WATER HOLD-UP AND DISTRIBUTION OF WATER OVER MOBILE AND STATIONARY LIQUID PHASES FOR DIFFERENT ELUENT COMPOSITIONS

In a column with a volume of 1.662 ml containing 1.1 g LiChrosorb NH_2.

Eluent composition, acetonitrile–water (%, v/v)		*90:10*	*80:20*	*70:30*	*60:40*
Eluent needed for water saturation (ml)		2.05	1.964	1.670	1.525
(% of the empty column volume)		123	118	100	92
Water hold-up (mg)		205	393	501	610
Water content in mobile phase (mg)	*	35	70	105	140
	**	66	133	199	266
Water content in stationary liquid phase (mg)	*	170	323	396	470
	**	139	260	302	344
(%, v/v)	*	22	42	52	61
	**	30	56	65	74

* At a mobile phase fraction of 0.21.
** At a mobile phase fraction of 0.40.

To attempt to explain the similarities in these two chromatographic systems, we determined the water hold-up of an amine-modified silica column (see Experimental). The results are presented in Table I and indicate a water enrichment of the stationary phase. In order to quantify this water enrichment the following estimates of the volume fractions for the different phase, were made:

the stationary phase (silica + propylamine): based on a specific density of 2.3 for silica, the volume fraction of silica is 0.27 (weight of LiChrosorb NH_2 silica fraction)/(specific density of silica × empty column volume); using a specific density of 0.7 for propylamine, its volume fraction can be calculated in the same way as 0.06. So the total for the stationary phase is 0.33

the stationary phase pore volume fraction: this can be estimated as 0.46 from the pore volume per gram LiChrosorb NH_2 as determined

the mobile phase fraction is the residual part of the column volume, *i.e.*, 0.21.

A mobile phase fraction of 0.21 is rather low. A more common value is 0.40, which is used for the calculation of the residence time of an unretained component.

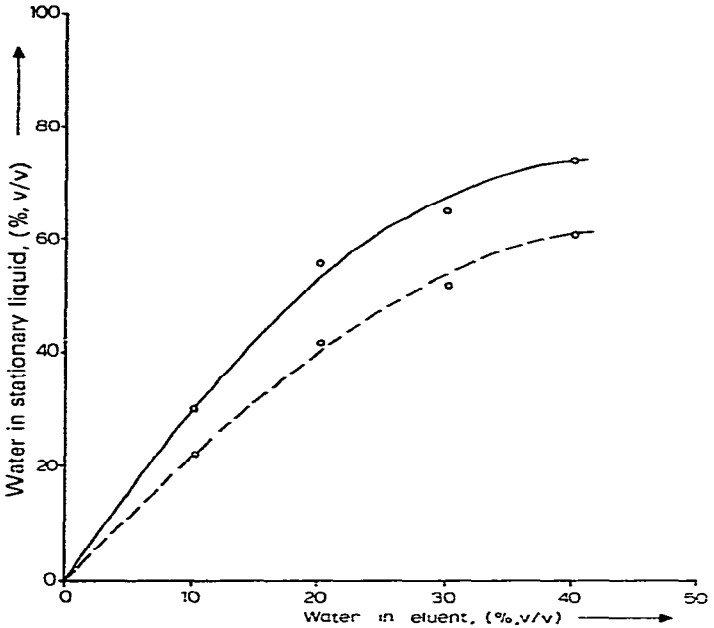

Fig. 3. Dependence of the water content of the stationary liquid on that of the eluent for different mobile phase values. — —, Low mobile phase value; ——, high mobile phase value.

Possibly, a part of the (superficial) pores should be taken into account when estimating the mobile phase fraction. Therefore, in further calculations, a low and a high value for the mobile phase fraction (0.21 and 0.40) and thus a high and a low value for the stationary liquid phase (0.46 and 0.27) were used.

The results are presented in Table 1 and Fig. 3. It is clear that the volume fraction of water in the stationary liquid is much higher than that in the eluent. This water enrichment of LiChrosorb NH_2 is caused by the hydrophilic nature of the amine groups; these groups also catalyse the mutarotation.

The amount of water enrichment is expected to be dependent on the nature and the amount of bonded amine. An illustration of the importance of the amine is the variation of the capacity factors for sugars when different amines are added to the eluent, as reported by Wheals and White[12]. The relationship between k' and the amine concentration, as mentioned by Jones *et al.*[9], can be explained if the water enrichment of the stationary phase, initially dependent on the amine content, subsequently becomes more or less constant because of water saturation, so that further addition of amine has no effect. Column deterioration, which results in shortening of elution times, can be interpreted in terms of a decreased water enrichment due to loss of amine. The original elution pattern can be restored simply by reducing the water content of the eluent, because at low water concentrations the extent of water enrichment of the stationary phase is relatively high, as can be seen in Fig. 3. The constant elution times found for physically modified columns, where water enrichment will also occur, are caused by the constant amount of amine added with the eluent.

Rabel *et al.*[6] ascribed the poor separations with methanol–water as eluent to

the low extent of "solvation" of the amine phase. However, probably no water enrichment occurred because of the strong hydrophilic nature of methanol. The eluent, used by Müller and Siepe[15], water–acetone–ethyl acetate, apparently does allow water enrichment of the stationary phase. Generally, the eluent composition should be such that, in addition to a reasonable solubility of the sugars, the affinity of each component for the amine is different.

REFERENCES

1 J. C. Linden and C. L. Lawhead, *J. Chromatogr.*, 105 (1975) 125.
2 J. K. Palmer, *Anal Lett.*, 8 (1975) 215.
3 P. Jandera and J. Churáček, *J. Chromatogr.*, 98 (1974) 55.
4 R. Schwarzenbach, *J. Chromatogr.*, 117 (1976) 206.
5 R. B. Meagher and A. Furst, *J. Chromatogr.*, 117 (1976) 211.
6 F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatogr.*, 126 (1976) 731.
7 J. Hettinger and R. E. Majors, *Varian Instruments Appl.*, 10 (1976) 6.
8 R. E. Majors, *J. Chromatogr. Sci.*, 18 (1980) 488.
9 A. D. Jones, I. W. Burns, S. G. Sellings and J. A. Cox, *J. Chromatogr.*, 144 (1977) 169.
10 V. Kahle and K. Tesařik, *J. Chromatogr.*, 191 (1980) 121.
11 K. Aitzetmüller, M. Böhrs and E. Arzberger, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 589.
12 B. B. Wheals and P. C. White, *J. Chromatogr.*, 176 (1979) 421.
13 K. Aitzetmüller, *Chromatographia*, 13 (1980) 432.
14 C. A. White, P. H. Corran and J. F. Kennedy, *Carbohyd. Res.*, 87 (1980) 165.
15 H. Müller and V. Siepe, *Deut. Lebensm.-Rundsch.*, 76 (1980) 156.
16 L. A. Th. Verhaar and B. F. M. Kuster, *J.Chromatogr.*, 210 (1981) 279.
17 O. Samuelson, *Ion Exchange*, Vol. 2, Marcel Dekker, New York, 1969, Ch. 5.
18 O. Ramnäs and O. Samuelson, *Acta Chem. Scand., Ser. B*, 28 (1974) 955.
19 H. Rückert and O. Samuelson, *Acta Chem. Scand.*, 11 (1957) 303.
20 H. Rückert and O. Samuelson, *Acta Chem. Scand.*, 11 (1957) 315.