

Bound-Monolayer Cation Exchangers for the Analysis of Biochemically Significant Molecular Species

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Abstract

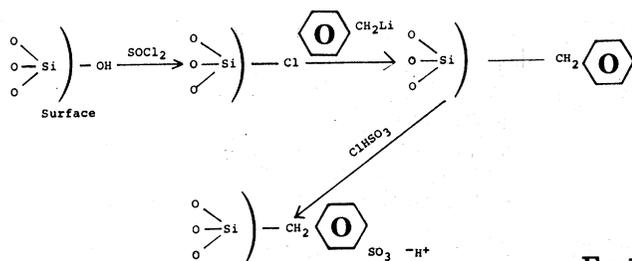
Silica chloride was prepared from porous silicas by reaction with SOCl_2 . Further reaction with benzyl lithium resulted in bound benzylsilicas which, upon sulfonation with chlorosulfonic acid, yielded cation exchangers in the H^+ form. Elemental analyses and titrations of the exchangeable protons indicated ion exchange capacities of up to 0.4 meq/g which reflected from 66 to 95% sulfonation of the bound benzyl moieties.

The capacities are intermediate to those for superficially-porous ion exchangers and ordinary polystyrene-divinylbenzene based phases. The latter tend to bleed UV-absorbing materials which interfere in the analysis of trace quantities of materials in natural products. Sulfobenzylsilica cation exchangers in the Na^+ form were used for the separation of proline from nitrosoproline by liquid chromatography. There was no evidence of bleed when a UV column monitor (254 nm) was set at 0.04 absorbance units full scale. This is significant since the presence of nitrosoproline is confirmed by mass spectroscopy and bleed from ordinary ion exchangers could be a significant source of interference in the analysis.

Particles of a sulfobenzylsilica in the Ag^+ form were used as a stationary phase in TLC. At approximately 2% loading of Ag^+ , cis and trans isomers of fatty acid methyl esters or mono-, di-, and trisaturated triglycerides were separated. Repetitive development on the same plate produced no significant change in R_f . The plates are stable to solvents as well as light and require only one-tenth the amount of Ag^+ utilized in AgNO_3 impregnated plates.

Introduction

Recent activity in high performance liquid chromatography has stressed the use of small particles of support materials to which thin films of stationary phase have been affixed. This report describes the use of surface modified, porous, inexpensive silicas in liquid and thin-layer chromatography. Most of the surface hydroxyls were replaced as in Equation 1. Readily available



ionic forms could thereby be provided and both ion ex-

change and sorption characteristics were utilized in order to develop separations by column or thin-layer chromatography.

Experimental

Liquid Chromatography

A detailed discussion of the syntheses, analyses, and column packing procedures has been reported (1). The small particle ($\sim 12 \mu$) Syloid 74 (W. R. Grace and Company)* had an ion exchange capacity of 0.39 meq/g and the ($\sim 150 \mu$) Porasil C (Waters Assoc.) had a capacity of 0.25 meq/g, after reaction. Analytical-scale separations were carried out with the aid of a DuPont 820 liquid chromatograph into which a 250 mm x 2.1 mm i.d., s. s. column had been incorporated. The sulfobenzylated Syloid was slurry packed in this column.

The sulfobenzylated Porasil C was dry packed in a 240 mm x 11 mm i.d. glass column (Fisher-Porter Co.) for preparative work. In this application, a mini-pump (Milton-Roy Co.) provided a constant flowing mobile phase. A pressure-limiting switch (Barksdale Corp.) and gauge (Matheson Inc.), which were placed in line for safety reasons, also provided sufficient pulse dampening. A Beckman DB recording spectrophotometer fitted with a 5 mm flow cell was used as a column monitor (240 nm). A Fractomat fraction collector (Buchler Instruments) was utilized where appropriate. All connections between modules were made with Cheminert fittings (Chromatronix Inc.). Standard solutions of ninhydrin were used to detect proline and other amino acids in collected fractions.

Thin-Layer Chromatography

Syloid, both unreacted and as the sulfobenzyl derivative, was used as the stationary phase in these studies. The ion exchanger was converted to a variety of ionic forms by slurrying with a large excess of the nitrate salt of the desired cation, followed by decan-

*Mention of commercial items is for reader convenience and does not constitute an endorsement by the U.S. Department of Agriculture of these products over others of a similar nature.

1. Saunders, D. H., Barford, R. A., Magidman, P., Olszewski, L. T. and Rothbart, H. L., *Anal. Chem.* **46**, 834 (1974).

tation and washing. Excess Ag^+ was precipitated as the chloride and the liberated H^+ was determined by titration with standard base. The data indicated that essentially all the H^+ was displaced by Ag^+ and that there was 0.35 meq/g of this ion in the stationary phase. TLC plates with the desired amount of silver were prepared by mixing the required amount of modified Syloid with the unreacted material. A 5.5 g sample of this mixture was stirred in 14 ml of H_2O for 90 sec and used to spread a 250μ thick layer on a 20×20 cm plate. Plates in the H^+ and Na^+ form were similarly prepared. Similar procedures were employed to formulate impregnated plates, except that AgNO_3 crystals were mixed with the unmodified Syloid (2). No binder was used in any of these cases. All plates were activated by heating at 105°C for 30 min.

Reagents

Nitrosoproline was prepared from A-grade L-proline (Calbiochem). Triolein was purchased from Applied Science Laboratories, Inc. All other lipids were prepared from the appropriate natural products by standard techniques, and their purity was determined to be $>95\%$ by GLC. All other chemicals were ACS reagent grade.

Particle Size Determination

The size distribution of Syloid 74 was determined after five sedimentations in which the fines and largest particles were removed. The particles were dried at 100°C at 0.1 mm Hg and samples were sprinkled onto a dilute collodion film which had been cast onto a microscope slide. After setting of the film, the particles were examined microscopically at 300X magnification through a calibrated eyepiece. Photomicrographs at the aforementioned magnification and 1000X were also utilized.

Results and Discussion

Liquid Chromatography

The particle size distribution after sedimentation is depicted in Figure 1. It was essentially unchanged after reaction to produce the bound ion exchanger. The photomicrograph depicts the irregular shape of the particles. The greatest disadvantage of the material is the large pressure drop encountered when the particles are packed into long, thin columns. Typical flow rates were 1.7 ml/min of hexane at 1500 psig and 0.16 ml/min of aqueous methanol at 1500 psig. Improved flow and other chromatographic properties could no doubt be attained through the use of size classified spherical particles. However, recent evidence indicates that irregular particles may have excellent properties in adsorption chromatography if they are specially packed at high pressure (3).

The stability of sulfobenzylated Syloid to organic solvents was particularly significant. For example, recent concern about the possible presence of nitrosamines in cooked bacon (4) and the related health aspects (5) has made it imperative that assay procedures useful in the parts per billion range be developed and that

contamination from the analytical tools be minimized. Methods for the assay of traces of nitrosoproline in cured meat samples required the separation of this compound from a large excess of proline and other components. The choice of eluents was restricted by the demands of subsequent analytical methodology, including tandem GLC-mass spectrometry (6). Methanol-water mixtures were acceptable, but this prescribed the use of ordinary ion exchangers (based on styrene-divinylbenzene copolymers) which bleed, although their high capacity would be desirable. The monolayer exchangers used in this study have a capacity intermediate to ordinary bulk-phase ion exchangers and most controlled surface-porosity exchangers designed for HPLC. In addition, they are more stable than either type in many organic mobile phases (7). The stationary phase was converted to the Na^+ form so that under the conditions of chromatography the nitrosoproline was essentially negatively charged and hence repelled by the benzenesulfonate groups of the stationary phase. It was thus eluted at the interstitial volume ($k' \cong 0$) although proline was present as the Zwitterion and eluted later. A plot of k' for proline as a function of volume fraction of methanol is depicted in Figure 2. The resolution passed through a maximum and then decreased due to broadening of the proline peak in solutions of high methanol concentrations. An analytical-scale separation of samples of

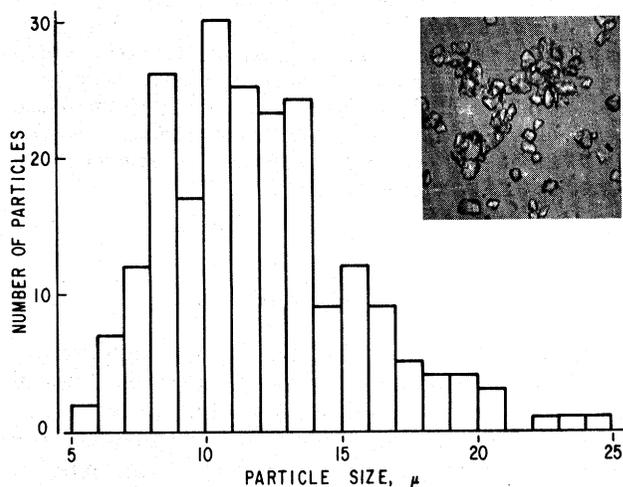


Figure 1. Syloid 74 particles after sedimentation. (a) Portion of photomicrograph at 1000X; (b) Size distribution determined at 300X.

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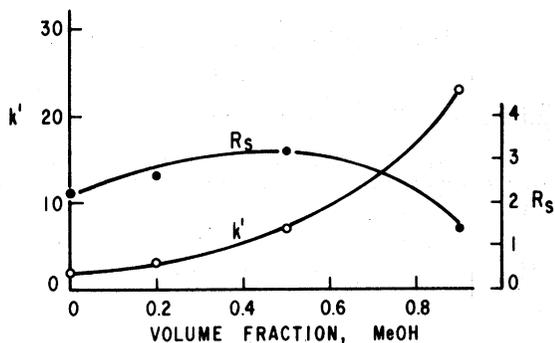


Figure 2. ○ Capacity factor, k' for proline on sulfobenzylated Syloid 74 in the Na^+ form as a function of volume fraction of aqueous methanol. ● Resolution, R_s , of the nitrosoproline-proline pair under these conditions.

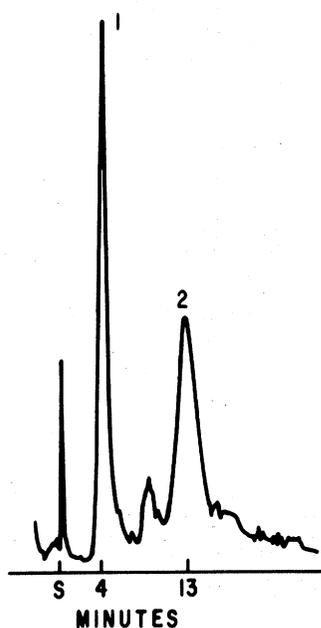


Figure 3. Separation of nitrosoproline (1) from proline (2) with sulfobenzylated Syloid 74 in the Na^+ form. (Column 250 mm x 2.1 mm, volume fraction aqueous methanol was 0.20, UV detector set at 0.08 A full scale, flow rate 0.15 ml/min). Sample size was 700 μg of proline and 0.7 μg of nitrosoproline in 10 μl of eluent.

nitrosoproline from proline is shown in Figure 3. In order to isolate the nitrosoproline-containing fraction for further study, a preparative separation was devised as is shown in Figure 4, utilizing a larger particle-size support. In this case, nitrosoproline was added to a bacon extract and the effluent was passed to a flow-through cell contained in a Beckman DB spectrophotometer. The low extinction coefficient of amino acids such as proline, and the relatively low sensitivity of this instrument necessitated the use of ninhydrin for the detection of the aforementioned species. Similar samples which did not contain added nitrosoproline resulted in elution profiles of much the same charac-

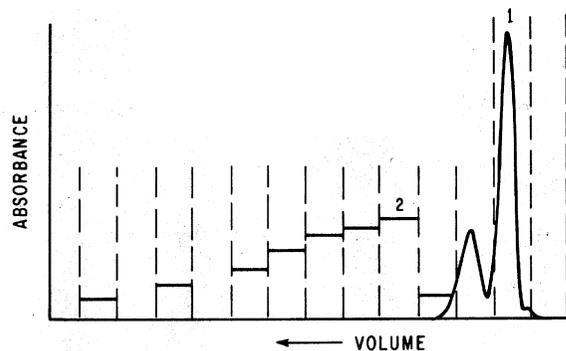


Figure 4. Preparative separation of 1 (nitrosoproline-rich fractions) from 2 (proline-rich fractions) with sulfobenzylated Porasil C in the Na^+ form. (Column 240 x 11 mm, volume fraction aqueous methanol 0.80. Beckman DB spectrophotometer set at 240 nm and 1.00 A full scale, flow rate 2.0 ml/min.) Vertical lines indicate fraction collection each 5 min period. Bars indicate absorbance at 450 nm of fractions treated with ninhydrin. Earlier fractions gave no detectable ninhydrin response. Sample size was 200 μl and contained a fat-free extract corresponding to 400 mg of bacon, 35 mg of L-proline, and 0.16 mg of nitrosoproline.

ter except in the region of the "interstitial volume" where the absorbance approached the baseline. Samples in this region were collected automatically, further derivatized and evaluated by GLC, utilizing an alkali flame ionization detector, and also by tandem GLC-mass spectroscopy. It became necessary to treat the column with 6M HNO_3 for 24 hr periods at 25°C occasionally, to remove strongly sorbed materials such as gelatin, which were present in the samples, followed by regeneration with NaNO_3 . Although this harsh treatment was required, the baseline remained stable and low throughout this study, and no appreciable loss of ion-exchange capacity was noted.

Thin-Layer Chromatography

The unreacted Syloid particles were easily cast onto glass plates for TLC. Class separations of fatty acids, their methyl esters and triglycerides, could be performed with petroleum ether-diethyl ether eluent mixtures on Syloid used as an adsorbent. In order to achieve more difficult separations, the Ag^+ -form ($\sim 12 \mu$) sulfobenzylsilica was used. A plate coated entirely with this support contains the same number of equivalents of Ag^+ as an ordinary silica plate impregnated with 6% AgNO_3 . The amount of Ag^+ on the ion-exchange plates was controlled by mixing the Ag^+ -form sulfobenzylsilica with unreacted Syloid. Chromatographic properties were then compared with plates prepared from unreacted Syloid impregnated with AgNO_3 . With the ion exchanger there is a trend towards retardation of unsaturated solutes, which form π -complexes with Ag^+ , which is directly related to the quantity of silver on the plate. The relative retention (R_f ratio of nearest neighboring spots) also increases directly with loading of Ag^+ . Typical plates are depicted in Figure 5.

At 3% AgNO_3 impregnation no separations within

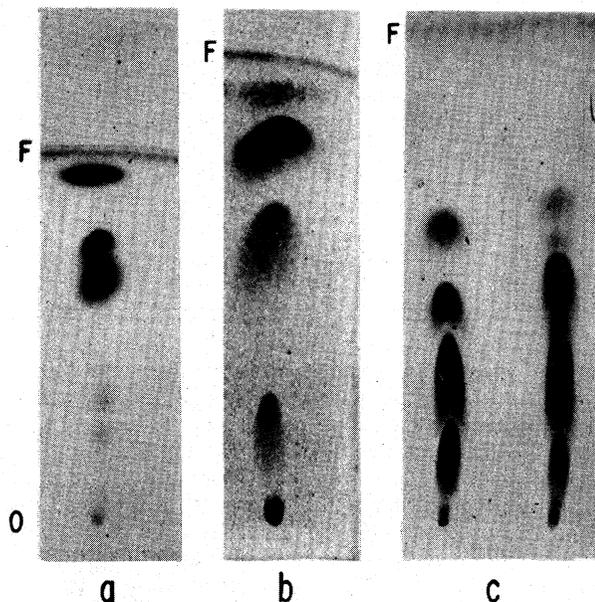


Figure 5. Separation of lipids by TLC on Syloid 74. Compounds (see Table I) are listed in decreasing order of R_f ; F and O denote front and origin respectively. (a) unreacted silica, solvent: 90/10/1 petroleum ether/diethyl ether/acetic acid. MeO, POP, oleic acid. (b) sulfobenzylated silica, Ag⁺ form (1%). Solvent: 80/20 Skellysolve F/diethyl ether. MeP, MeE, MeO, methyl linoleate. (c) sulfobenzylated silica, Ag⁺ form (1%). Solvent as in (b). Standard mixture on the left, PSS, POP, SOO, OOO; sample of beef tallow on the right.

the class of triglycerides or methyl esters were noted. The relative retentions at 20% loading on impregnated plates were usually not as good as those produced with 1% AgNO₃ equivalent on the ion exchange plates under otherwise identical conditions. Trends in retardation of solutes which form π -complexes as a function of AgNO₃ loading were not so evident on the impregnated plates. This could be expected since the silver salt crystallizes as the impregnated plate is dried and only the surface of the crystal is available for binding solutes. Variations in crystal size should lead to such variations in retention. Separations obtained with commercially-prepared 10 or 20% AgNO₃ impregnated plates were similar to those described in Table I for the same loadings.

The ion exchanger in the silver form was apparently much more stable to light than the impregnated material as noted by darkening of plates of the latter. The ion exchanger barely changed color over a time period of several days when exposed to both natural and fluorescent lighting and provided useful, but less effective separations, although similarly treated impregnated (10% loading) plates did not give useful separations after exposure to light. The ion exchanger formed a mechanically stable plate which was used repetitively after elution of the solutes to the top of the plate and drying, and no change in R_f was apparent after five such operations. This should make it of great utility in such operations as programmed multiple development (8). Reusable, permanently coated

Table I. R_f data for argentation TLC

% AgNO ₃	Impregnated: Solvent	Triglycerides			Methyl Esters		
		PSS	POP	SOO	MeP	MeE	MeO
30	A	.50	.36	.23			
20	A	.49	.36	.19	.90	.73	.61
10	A	.38	.27	.16	.79	.68	.54
30	B	.78	.61	.39			
20	B	.74	.54	.46	.94	.89	.76
10	B	.61	.49	.36	.80	.77	.67

Ion exchanger:

% AgNO ₃ equivalent		PSS	POP	SOO	MeP	MeE	MeO
3	A	.32	.07	.02	.73	.38	.22
2	A	.40	.13	.07	.83	.60	.40
1	A	.42	.26	.13	.79	.65	.52
0.5	A	.36	.27	.22	.74	.69	.61
—							
2	B	.50	.19	.08	.78	.58	.38
1	B	.56	.37	.20	.79	.67	.56
0.5	B	.50	.40	.29	.71	.71	.62

A = diethyl ether/Skellysolve F 10/90; B = diethyl ether/Skellysolve F 15/85; PSS = Glycerol-1-palmitate-2,3-distearate; POP = Glycerol-2-oleate-1,3-dipalmitate; SOO = Glycerol-1-stearate-2,3-dioleate; MeP = Methyl palmitate; MeE = Methyl elaidate; MeO = Methyl oleate. (Note: MeE is the trans isomer of MeO.)

plates are now sold commercially, and it would be a simple extension of the chemistry given here to make permanent ion-exchange plates when their use is warranted.

The Na⁺ form of the exchanger had similarly good mechanical stability although the H⁺ form resulted in poor, brittle plates. Separation of amino acids on the Na⁺ form plates demonstrated no advantage over use of ordinary Syloid as an adsorbent when H₂O was used as the eluent. The major utility of the bound ion-exchange silica in TLC should be evident when specific ionic interactions are desired or if ion-exchange TLC is to be performed.

Acknowledgment

Nitrosoproline was prepared by Mr. J. Pensabene and all triglycerides, other than triolein, were supplied by Professor R. Jensen (9). Micrographs were supplied by R. J. Carroll. ■

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