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TRIACETYLCELLULOSE AS A CHIRAL STATIONARY PHASE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A review of the structure of cellulose and its properties is given. Experimental data concerning the physical and chromatographic properties of microcrystalline triacetylcellulose (TAC) are reported. It is concluded that TAC columns can be used advantageously at high pressures. To improve resolution, high temperatures can be used. The TAC column is also compatible with many buffer systems (pH 5–10) and up to 70% of water can be used in the mobile phase.

INTRODUCTION

The use of triacetylcellulose (TAC) as a chiral stationary phase for separations of enantiomers on both a preparative and an analytical scale is well documented and several reviews have appeared[1–4]. Initially, fairly large particles of TAC were used in low- and medium-pressure chromatography, and it was sometimes erroneously stated that TAC was too soft to allow the use of smaller particles at higher pressures to produce efficient columns. Lindner and Mannschreck[5], however, have shown that TAC is also suitable for high-pressure applications. They successfully slurry-packed a steel column at *ca.* 200 bar with TAC of particle size 5–10 μm. The chromatographic experiments were then carried out at approximately 100 bar. TAC was both mechanically stable and maintained its discriminatory power against enantiomers at that pressure.

Analytical and preparative TAC columns for high-performance liquid chromatography (HPLC) are now available from several manufacturers. Despite the great generality shown in the area of preparative applications, purely analytical applications of TAC are still relatively rare. We believe that this lack is partly due to the

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above-mentioned misunderstanding that TAC columns for HPLC are not available or useful.

In this paper we report some studies of an analytical TAC column concerning pressure stability, efficiency, loadability and eluents, including pH buffers. Some of the results may be seen as complementary to previous reports of this kind. In order to compare TAC material from different sources, we have repeated some of the investigations earlier published[6,7]. In the first part of the paper a review of the structure and properties of cellulose is given.

STRUCTURE OF TAC AND CHIRAL RECOGNITION

The structure of cellulose has been extensively studied for a long time. A complete review of the subject is, however, beyond the scope of this paper. For detailed information we recommend some recent reviews and the references cited therein[8,9].

The cellulose molecule, 1,4-β-D-polyanhydroglucopyranose, is a linear polymer with D-glucose as the repeating unit (see Fig. 1). The β-D-glucose units exist in chair conformations with 2-OH, 3-OH and CH_2OH all at equatorial positions (see Fig. 2). The chain length of native cellulose or the degree of polymerization, *DP*, is not known for certain, as scission of the chains probably occurs when the molecule is isolated from its associated material in the cell. Values of *DP* ranging from 200 to 14 500 have been reported. So far as the chain length is concerned, the material is polydisperse.

Microcrystalline cellulose is obtained from purified α-cellulose, which is the portion of industrial cellulose pulp insoluble in cold (*ca.* 18%) sodium hydroxide. The α-cellulose is first hydrolysed with 2.5 *M* hydrochloric acid at *ca.* 100°C to remove amorphous material. The microcrystals are then freed from this material, so-called "level off degree of polymerization cellulose", by mechanical shearing in a water slurry. Microcrystalline cellulose of Avicel type has a *DP* of 200–300, corresponding to a molecular weight between 30 000 and 50 000 (ref. 10).

The microscopic subunit of cellulose, the fibril (microfibril, elementary fibril) consists of a number of parallel-packed cellulose molecules[9]. It has been suggested that there is a coincidence between the microfibrils and crystallites[9,11] and it has been shown that the microfibril has a continuous crystalline structure. The range of the crystalline structure is over several micrometres (> 1000 nm) without any amorphous domains larger than the resolution provided by the electron microscopic technique used (0.3–0.5 nm)[9]. Electron microscopy of cellulose from various sources has shown that the width of the elementary fibril is 35 Å[12]. The microfibrils are clustered to macrofibrils, which are embedded together with other polysaccharides and lignin in the cell wall of the plants (see Fig. 3).

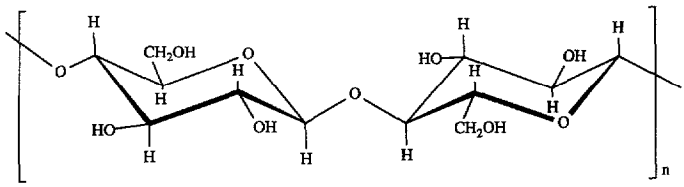


Fig. 1. Structure of cellulose.

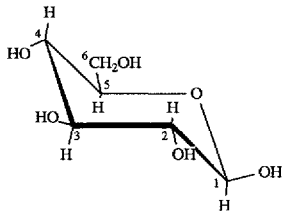


Fig. 2. β-D-Glucose, $^4C_1$.

Native cellulose, referred to as cellulose I, is a crystalline array of parallel chains, established from X-ray data of cellulose from the sea alga *Valonia ventricosa*[11]. The cellulose chains in this structure possess a two-fold screw axis of symmetry (helix axes, $P2_1$) along the chain axes. The ribbon-like chains are held together by intra- and inter-molecular hydrogen bonds in sheets with only Van der Waals interactions between successive sheets. Different conformations at the glycosidic bond have been reported[8], depending on the calculation method used. The chair, $^4C_1$, conformation[13] (see Fig. 2) of the glucose unit is, however, not questioned.

TAC, obtained by heterogeneous acetylation of native cellulose or microcrystalline cellulose, is believed to maintain the cellulose I structure[14]. Hesse and Hagel[15] showed that the morphology of cellulose plays an important role in the chiral

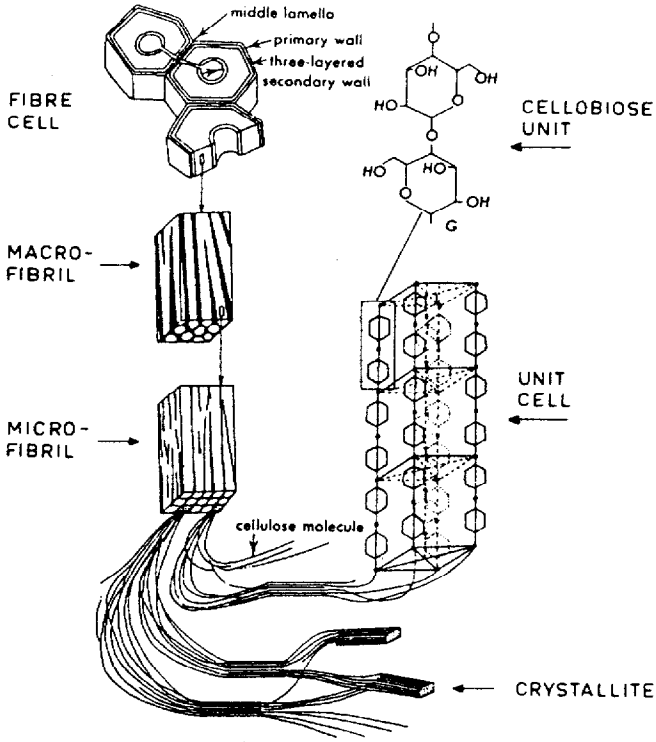


Fig. 3. Structure of cell wall. Reprinted with permission from T. Hattula, *Effect of Heat and Water on the Ultrastructure of Wood Cellulose (Thesis)*, Finnish Pulp and Paper Research Institute, Helsinki, 1985.

recognition mechanisms; the enantiomers intrude into certain kinds of chiral cavities between the laminae in the crystalline regions of cellulose. They also found that amorphous cellulose is not useful as a stationary phase owing to a supposed loss of these chiral cavities. It was not suggested, however, whether these cavities were located in the crystals, between the cellulose molecules, or between the elementary fibrils. Hesse and Hagel concluded that the inclusion of molecules in these cavities is mainly governed by the shape of the molecules and only to a minor extent by other factors such as electrostatic interactions involving the functional groups of the molecules. They denoted this kind of chromatography *inclusion chromatography*.

Francotte *et al.*[16] investigated the relationship between chiral recognition and the crystallinity of TAC. They found that an increase in the crystallinity of TAC by annealing at 240°C under vacuum afforded a material with low capacity and a reduced ability to discriminate between enantiomers. They ascribed this to a reduced mobility of the glucose units in the polymeric chains, thus making the intrusion of the molecules between the chains much more difficult. Also, Ichida and Shibata[3] determined, in agreement with Francotte *et al.*, that a perfect crystallite is too tightly packed to allow the inclusion of a molecule into the lattice. These findings are in accordance with the hypothesis that the cavities accessible to the analytes are found between the elementary fibrils of the swollen material (se below) and not between the cellulose molecules in the crystallites.

Blackwell and Lee[17], however, obtained a complex between cellulose I (native cellulose) and ethylenediamine (EDA) of relatively high crystallinity. Elemental analysis of the complex revealed an approximate ratio of one EDA molecule per glucose unit. According to an X-ray study, the EDA molecules are hydrogen bonded between two cellulose chains in the unit cell. Furthermore, X-ray studies of that complex showed that some rearrangement of the cellulose chains compared with the corresponding structures in native cellulose had taken place. They were also able to recover the original cellulose structure by washing out the ethylenediamine with water. [Note: unmodified native cellulose (Avicel) has been used as a stationary phase for column chromatographic separations of, *e.g.*, amino acids into enantiomers[18] under relatively hydrophobic conditions.]

Shibata *et al.*[19] have shown that it is possible to use crystalline triacetylcellulose II, another type of crystalline TAC, as a chiral stationary phase. The capacity factor of benzene obtained on TAC II (0.46) was much smaller than that obtained on TAC I (10.3), indicating that benzene is almost unretained on TAC II. It appears as if the inclusion of benzene between the laminae, if any, of TAC II is prevented, perhaps because the distance between the laminae is too small to allow inclusion.

Okamoto *et al.*[20] prepared an efficient chiral phase by first dissolving TAC and then adsorbing it on macroporous silica. The morphologies of TAC in these composites were either crystalline (cellulose II) or amorphous. When compared with TAC, a reversal of the retention orders of the enantiomers of, *e.g.*, Troeger's base was obtained on the column packed with the TAC–silica composite. In the preparation of these materials it was found that the selectivity factors are sensitive to both the choice of silica matrix and the dissolving agent of TAC[3]. It is known that cellulose derivatives form liquid crystalline solutions in any solvent that dissolves sufficiently high concentrations of the polymer[21,22]. The cellulose derivative on the silica surface has presumably an ordered, not necessarily crystalline, structure, thus explaining the

relative similarity in chromatographic behaviour to the pure microcrystalline TAC material. It has been reported[23] that TAC I was obtained from TAC dissolved in an aqueous trifluoroacetic acid solution. The material was coated on a glass slide by precipitation with methanol or water and the morphology was determined by X-ray diffraction. No data concerning chromatographic experiments were reported.

Schulze and König[24] reported enantiomer separations on silica with covalently bonded, derivatized monosaccharides. This indicates that even the primary structure of cellulose may contribute to the chiral recognition. Kano *et al.*[25] obtained an enantioselective complexation of bilirubin (BR) and oligosaccharides. They found that a (1–4)-linked disaccharide is the minimum requirement for chiral recognition. They also concluded that the enantioselectivity is very sensitive to the conformation of the disaccharide. Further, it was found that the intensity of the circular dichroism signal of the oligosaccharide–BR complex increased in the order maltose < maltotriose < maltoheptaose owing to differences in binding constants, meaning that the enantioselectivity had increased. They also proposed that the hydrophobic environment provided by the saccharide is important in the formation of hydrogen bonds.

More detailed studies of the chiral recognition mechanisms based on theoretical calculations are now appearing. Wolf *et al.*[26] correlated the calculated molecular properties and retentions of a series of structurally related racemates. They found that the shape of the molecule together with the electronegativity of atoms close to the asymmetric centre contributed to the chiral recognition. The absolute configurations of the enantiomers were, however, not considered. We have used a chemometric approach to study chiral recognition on TAC[27]. A correct prediction of the retention orders of some chiral indole derivatives with known absolute configurations was obtained. In agreement with Francotte *et al.*, it was found that both steric and electrostatic factors contribute to the chiral recognition. However, to predict retention orders and even to predict if it is possible to resolve a racemate into enantiomers on the TAC column is at present based more on empirical experience than on a real knowledge of the mechanisms involved in the chiral recognition process.

Empirically it is found that there is a high probability of being successful with a separation of enantiomers if the compounds possess an aromatic or a non-aromatic ring close to the chiral centre, if the compounds have an asymmetric atom on a rigid ring structure, and/or if the compounds have one or several C_2 axes of symmetry[28]. A carboxylic, hydroxylic or amino group, not necessarily at the asymmetric centre, normally has an adverse effect on resolution. Generally, improvements in separations are obtained if these functionalities are converted to the corresponding esters, carbamates, amides, etc.

It is frequently found in separations of structurally related compounds that steric effects play an important role in chiral recognition[29]. Hesse and Hagel[30] concluded, as mentioned above, that the inclusion of a compound between the laminae is more sensitive to the shape than to other factors such as functional groups of the molecules. They suggested that the antipodes of a compound are different in the asymmetric milieu and thus only one can be bonded to the phase and retained.

To be able to predict the resolution and retention order of enantiomers on TAC it is necessary to carry out theoretical calculations to correlate structure and retention on series of structurally related compounds. At present the nature of swollen TAC is not sufficiently known to allow calculations that combine the structure of the analyte

and the structure of the chiral stationary phase, *e.g.*, molecular docking.

In the dissolution of cellulose or cellulose derivatives, the solvents generally first act as a swelling agent and then as a dispersing agent. Different kinds of swelling of microcrystalline TAC may occur, namely intercrystalline, intracrystalline and osmotically conditioned swelling[31]. When TAC is treated with acetone vapour or liquid, both an increase in amorphous X-ray scattering characteristic of intercrystalline swelling and an increase in the lateral interchain distances characteristic of intracrystalline swelling take place. The third type of swelling is a process in which a swelling agent is dissolved in the interior of a crystallite but is prevented from free diffusion into the bulk of swelling agent.

It has been proposed by Scallan[32] that swelling causes some cleavage in the radial planes of cellulose (compare with intercrystalline swelling above). An intrusion of solvent between the fibrils is thus possible. This behaviour of cellulose is sometimes denoted the honeycomb effect (see Fig. 4). The swelling and solubility of cellulose and its derivatives depend on the nature of cellulose, *e.g.*, crystallinity and *DP*, *i.e.*, the manner in which the cellulose has been isolated and treated after the isolation from its source. It has been shown, for instance, that a decrease in the *DP* of cellulose increases the amount of included solvent[33].

Compared with the role of crystallinity, little is known about the role of the swelling of TAC and its influence on chiral recognition mechanisms. It is well known, however, that non-swollen TAC shows only a weak chiral recognition ability.

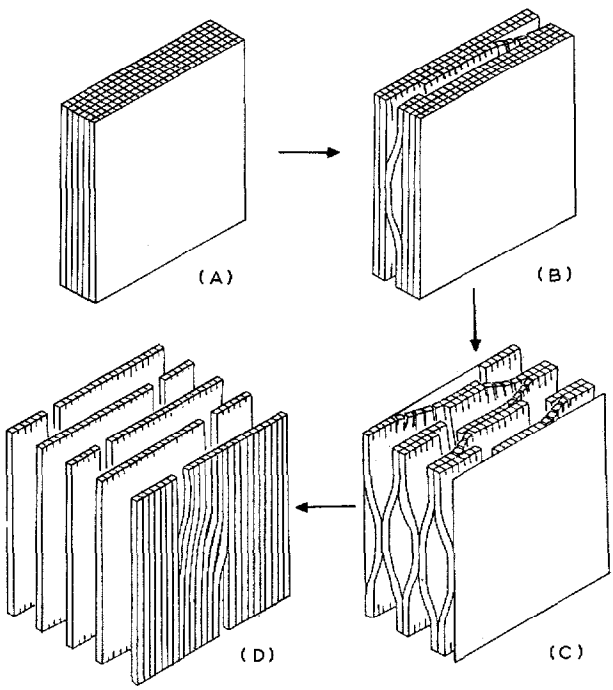


Fig. 4. Scallan's modification of the lamellar structure of delignified cell wall. On swelling (a–d) some radial cleavage of bonds occurs and gives the honeycomb effect (c). Reprinted with permission from ref. 32.

Dissolved cellulose acetate has been studied in various solvents by ^1H NMR spectroscopy[34]. In these studies it was found that solvents with high dielectric constants interacted strongly with the acetoxy groups of the cellulose. It is therefore safe to assume that ethanol, the most commonly used swelling agent and eluent, is associated, *e.g.*, hydrogen bonded, to the acetoxy groups of TAC. The role of the swelling agent (ethanol) in the chiral recognition process has never been interpreted in detail but is to some extent probably comparable to the role of water layer(s) of normal-phase silica[35].

EXPERIMENTAL

Apparatus

Chromatography was performed with an LC-4A pump, SPD-2AS spectrophotometric detector and Chromatopac C-R4A integrator (Shimadzu, Kyoto, Japan) and a Valco (Houston, TX, U.S.A.) Model EC 6W sample injection valve. The temperatures of the mobile phase and the column were controlled by a 2219 Multitemo II water-bath (LKB, Bromma, Sweden). Mercury porosimetry was performed with a 9310 pore sizer (Micromeritics, Norcross, GA, U.S.A.) and the surface area was determined by use of a FlowSorb II 2300 (Micromeritics). For swelling experiments a Model 500S centrifuge (Wifug, Bradford, U.K.) was employed at 4000 rpm (2500 *g*).

Materials

The racemate and the pure enantiomers of nefopam were a gift from Riker Labs. (Loughborough, U.K.) and methaqualone a gift from Draco (Lund, Sweden). Benzoin and 1,3,5,-tri-*tert*.-butylbenzene (TTB) were purchased from Merck (Darmstadt, F.R.G.) and Fluka (Buchs, Switzerland), respectively. The racemate and the pure enantiomers of Troeger's base were obtained from EGA-Chemie (Steinheim, F.R.G.) and Fluka, respectively. The structures of the test solutes are shown in Fig. 5. Ethanol was obtained from Kemetyl (Stockholm, Sweden). Methanol and 2-propanol were of HPLC grade and ethanolamine, boric acid, tris(hydroxymethyl)aminomethane (Tris) and acetic acid were of analytical-reagent grade, purchased from Merck. The TAC material (ConBrio-TAC) from Perstorp Biolytica (Lund, Sweden) used had a particle size range of 15–25 μm. All chemicals were used as received.

Swelling

In a two-necked 500-ml round-bottomed flask were placed 20 g of TAC and 150 ml of swelling agent and the system was stirred with a magnetic bar to give a homogeneous slurry. Two reference samples were taken and centrifuged at 4000 rpm (2500 *g*) for 2 min in calibrated centrifuge tubes to obtain the volume of the non-swollen TAC (TAC is assumed to swell very slowly under these conditions). The flask was then immersed in a heated water-bath with stirring and the monitoring of time was begun. Duplicate samples were thereafter taken from the boiling slurry at intervals of 10 min until no further change in swelling was obtained. The samples were centrifuged and the volumes determined. The swelling was calculated according to the equation

$$\text{Swelling (\%)} = \left[\frac{\text{volume of swollen TAC}}{\text{volume of non-swollen TAC (reference)}} - 1\right] \cdot 100$$

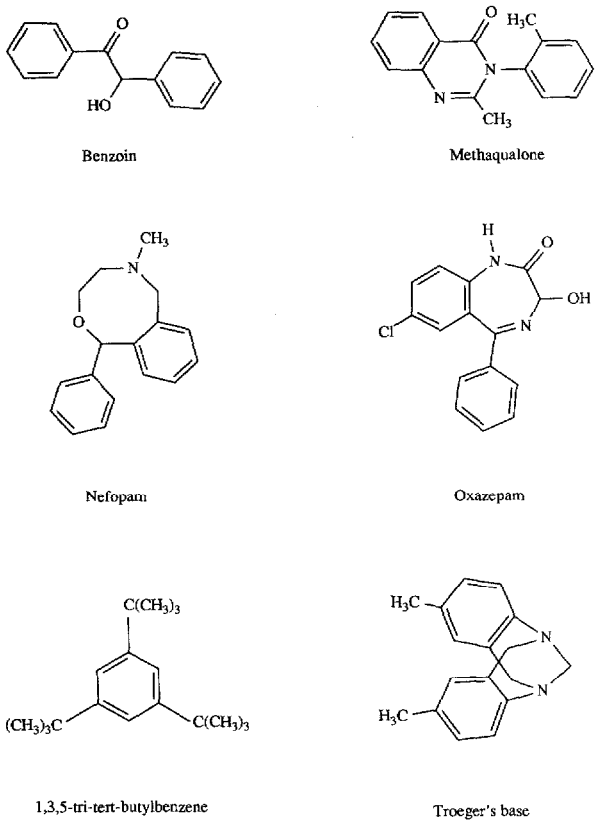


Fig. 5. Structures of test solutes.

In a separate experiment, 1-g samples of TAC were swollen for 30 min in toluene and in ethanol at *ca.* 80°C (the boiling point of ethanol) in calibrated centrifuge tubes. Centrifugation at 1500 rpm (350 *g*) for 5 min was performed and the volumes of the swollen material were compared.

Chromatography

The triacetylcellulose columns (ConBrio-TAC, particle size 15–25 μm, 250 mm × 5 mm I.D.) were obtained from Perstorp Biolytica (Lund, Sweden). All mobile phases containing buffers were filtered through a 0.5-μm HF PTFE filter (Millipore, Bedford, MA, U.S.A.) prior to use. The height of the column bed was checked visually by demounting the end-pieces of the column. The total porosity, ε_t (ref. 36), was determined according to a previously described method[7] by determining V_0 as the elution volume of TTB, assuming that TTB is not retarded; $\varepsilon_t = V_0/V$, where V is the volume of the empty column. Ethanol–water (95:5, v/v) was used as the mobile phase. Calculations of resolution, R_s, separation factor, α, capacity factor, k', and peak asymmetry factor, A_s, were carried out according to previously described procedures[37].

RESULTS AND DISCUSSION

Pore size and area

Mercury porosimetry was used for the determination of the pore size distribution and the pore volume of pores larger than approximately 60 Å. The total pore volumes of non-swollen Avicel and TAC were determined to be 0.04 and 0.09 cm^3/g, respectively. The experiment also showed that pores smaller than 300 Å contribute to the main part of the surface area. The nitrogen BET method permitted the determination of the surface area of smaller pores than obtained by mercury porosimetry. The surface areas of non-swollen Avicel and TAC obtained with the BET method were 1.1 and 6.1 m^2/g, respectively.

Nakai *et al.*[38] used the BET method and a "water vapour method" for measurements of the surface area of microcrystalline cellulose. The BET method gave a surface area of 1.0 m^2/g, in agreement with our result, whereas the water vapour method gave 150 m^2/g. The much higher value obtained by the water vapour method is due to the swelling of cellulose.

The result from mercury porosity and related methods is a measure of the permanent porosity of the material[39]. Swelling not only gives an increase in surface area but probably also gives rise to an extra porosity which is of great importance in the chromatographic process. The total porosity, ε_t (ref. 36), the sum of interstitial and internal porosity, of the ConBrio-TAC column was 0.63. A decrease in the pressure used for packing the column from 200 to 6–7 bar did not change the value of ε_t. Mannschreck and co-workers[1,6,7] have determined ε_t for several different TAC materials and obtained values from 0.5 to 0.78. It seems as if the total porosity depends on the way in which the TAC material is prepared. The internal porosity of TAC was not determined.

Chromatographic efficiency

The height equivalent to a theoretical plate, H, and the plate number, N, calculated from the peak width at half-height[37], were determined for the TAC column by use of a "non-retained" achiral compound, TTB, and by a chiral compound, Troeger's base. The results are given in Table I. The plate number generally decreases with increase in the capacity factor of the analytes, which also has been reported by Koller *et al.*[7]. The plate number is not a column constant but depends on the interaction between the stationary phase and the chiral analyte. Owing to difficulties in determining the size and shape of the TAC particles, no values for reduced plate height are reported.

TABLE I

CHROMATOGRAPHIC EFFICIENCY OF THE TAC COLUMN

Ethanol–water (95:5, v/v) was used as the eluent at a flow-rate of 0.25 ml/min; 10 μg of solute in 20 μl of eluent were injected onto the column.

Solute	N_1	N_2	H_1 (μm)	H_2 (μm)
TTB	2700		93	
Troeger's base	337	214	742	1168

Swelling and eluents

Ethanol–water (95:5, v/v) is the most frequently used eluent for TAC. Successful separations have also been achieved with lower alcohols, ethers, water–alcohol mixtures, hydrocarbons, aromatic hydrocarbons and mixtures of these eluents. Some common solvents such as acetone, THF, acetonitrile, chloroform and methylene chloride cannot be used as they dissolve the stationary phase more or less completely.

Precautions have to be taken when swollen materials such as TAC are used as stationary phases. A change from one eluent to another may change the swelling of the material. If, *e.g.*, shrinkage of the column bed occurs, it can cause undesirable front effects and a decrease in the efficiency of the column. In this study, TAC was swollen in organic solvents that were utilized as eluents in the chromatographic experiments (Table II). TAC swells more in lower alcohols than in ethers or in alkanes. In toluene TAC swells to almost the same volume as in ethanol. In binary or ternary systems the swelling is in general determined by the component in the solvent mixture that swells TAC most. For instance, in ethanol–hexane–water (67:3:30, v/v/v) the swelling was almost the same as in ethanol–water (95:5, v/v). The swelling in water was significantly less than in organic solvents. These results are in agreement with water sorption results published earlier[31]. It must be stressed, however, that the errors in measuring swelling are probably large[31].

Stability of the TAC column

The TAC column did not show any significant change in bed height on changing the eluent. It is often advantageous to elute strongly retained compounds at elevated temperatures or to use a pH buffer as the eluent to avoid decomposition of an analyte. Tests were carried out over extended times to find out if TAC is useful under such conditions. In these experiments the column was continuously fed for 6 days, (1) with ethanol–water (95:5, v/v) at 50°C (see Fig. 6) and (2) with ethanol–water (80:20, v/v) buffered to pH 8.0 with 50 m*M* Tris. The column did not show any loss of performance, within experimental error, or any notable decomposition of the TAC material after these extended tests.

TABLE II

SWELLING OF TAC

Solvent	*Composition (by volume)*	*Time*[a] *(min)*	*Swelling (%)*
Water		70[b]	5
Ethanol–water	95:5	30	42
Ethanol–2-propanol	80:20	30	38
Methanol		40	37
n-Hexane		60	30
n-Hexane–2-propanol–water	70:27:3	30	42
Diethyl ether		10	31
tert.-Butyl methyl ether		80	32
Ethanol–water–*n*-hexane	67:3:30	30	38

[a] Time used to obtain maximum swelling.

[b] Due to problems with wetting the TAC in water this time has to be considered as approximate.

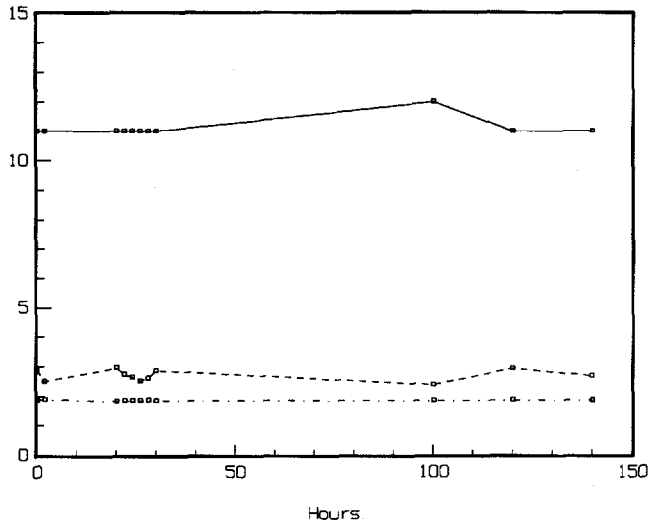


Fig. 6. Temperature stability of the TAC column at 50°C. Troeger's base was used as the test solute. Chromatographic conditions: flow-rate, 0.22 ml/min; mobile phase, ethanol–water (95:5, v/v); 4 μg in 20 μl of mobile phase were injected. –·–·–, α; – – –, R_s; ———, pressure (bar).

A pressure stability check of the TAC column was carried out. The flow-rate was continuously increased, far beyond the optimum working conditions, until a pressure drop over the column of approximately 200 bar was reached. The flow-rate was then continuously decreased to zero. The dependence of pressure on flow-rate is almost linear, only a small permanent increase in flow resistance being observed (see Fig. 7). No significant change was observed either in performance, verified by an analytical run with a test substance, or in the bed height of the packing material in the column. The results indicate that the material was not damaged by the rough treatment of the column.

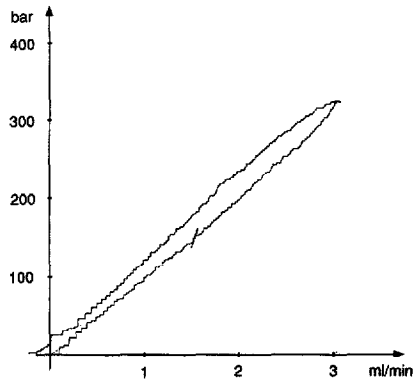


Fig. 7. Pressure stability of the TAC column. Column back-pressure as a function of flow-rate. Chromatographic conditions: temperature, 20°C; mobile phase, ethanol–water (95:5, v/v). The mobile phase was pumped through the column at a linearly increasing flow-rate from 0 to 3.0 ml/min in 10 min and back from 3.0 to 0 ml/min in 10 min.

Loadability

TAC has been successfully employed for preparative separations of enantiomers. Blaschke[4] separated more than 2 g of oxapadol in a single run on a column packed with 380 g of TAC, *i.e.*, more than 5 mg of analyte per gram of stationary phase.

In this study, the loadability of TAC was checked by the use of Troeger's base as the analyte. The sample injections were made under both "volume and mass overload" conditions. In each volume overload experiment (see Fig. 8), 20 μg of Troeger's base in various volumes was injected onto the column. The resolution was not affected until the injection volume exceeded 1000 μl, which corresponds to 40% of the volume of the first-eluted band.

In the mass overload experiment (see Fig. 9), a 500-μl loop was used, the amount of each enantiomer being varied from 0.0025 to 2.5 mg. Unexpectedly, the resolution increased with the amount of analyte injected and, perhaps most noteworthy, the capacity factor of the more retained enantiomer increased with the sample load. The asymmetry factor (A_s) (see Fig. 9) of the more retained enantiomer change from 1.7 to 0.5, *i.e.*, from a tailing to leading peak. The A_s of the other enantiomer showed a more normal behaviour, *i.e.*, the tailing increased with increased sample load. The same results were obtained when the corresponding amounts of racemate were injected.

The reason for this behaviour is not obvious to us. It appears as if some separation sites of the stationary phase are dynamically modified by the (+)-enantiomer, thus creating another stationary phase with more retentive properties for the (+)-enantiomer. Otherwise, if there had been different sites on the original TAC that would have attracted one of the enantiomers of Troeger's base, these sites that give the strongest interaction should have been occupied first by the (+)-enantiomer. Consequently, the retention at low concentration of the solute would be determined by

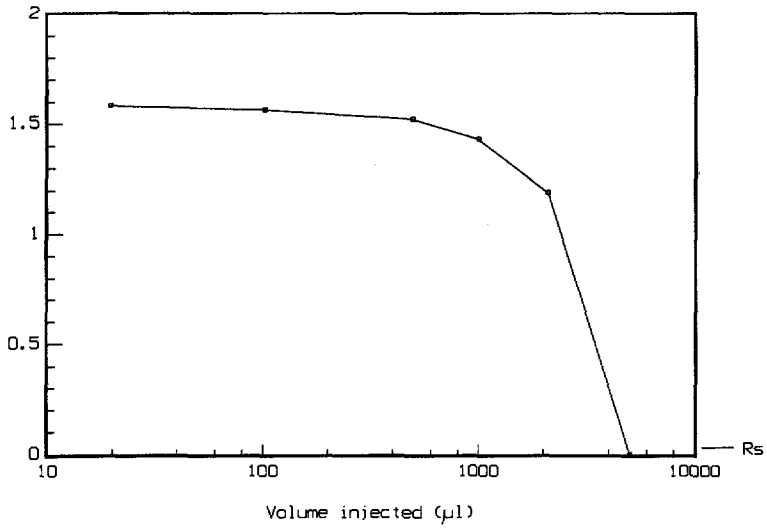


Fig. 8. Effect of injection volume on resolution; 20 μg of Troeger's base were injected each time. Chromatographic conditions: flow-rate, 0.25 ml/min; eluent, ethanol–water (95:5, v/v); UV detection at 235 nm.

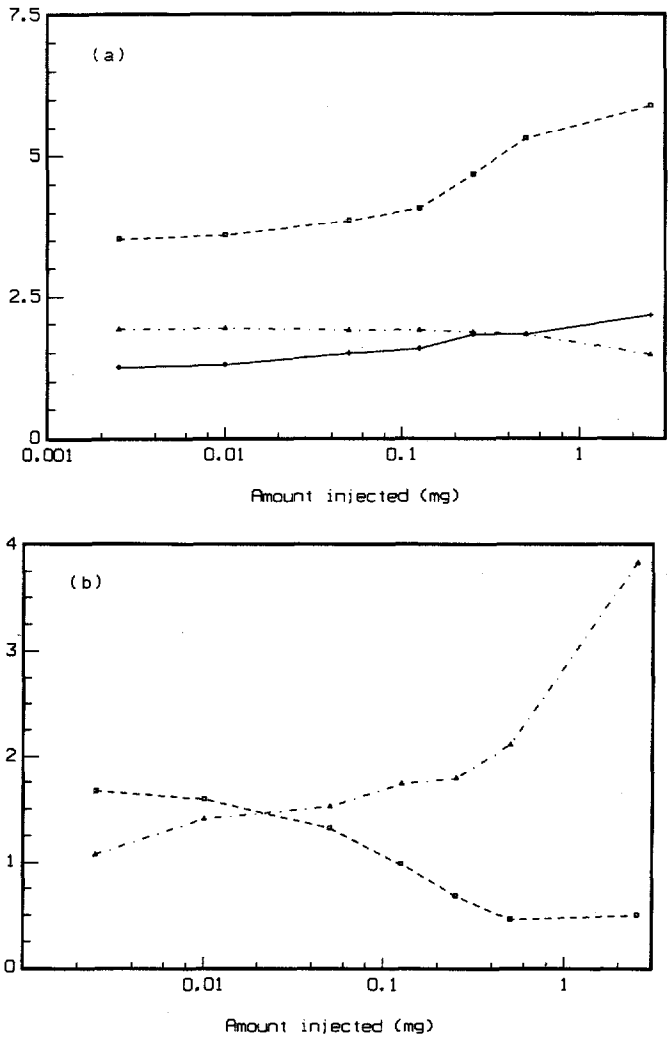


Fig. 9. Mass overload of the TAC column. The enantiomers of Troeger's base were injected separately. Chromatographic conditions: mobile phase, ethanol–water (95:5, v/v); flow-rate, 0.25 ml/min; injection volume, 500 μl. (a) ———, R_s; – – –, k' (+); — · – · —, k' (−). (b) – – –, A_s (+); – · – · –, A_s (−).

these sites. In our example, the retention time at low sample concentration should have been longer, not shorter as found in the experiment, than at high sample loads.

Roussel *et al.*[40] reported another example of unusual retention behaviour on TAC. They obtained an inversion of the retention order of a chiral atropisomer with an increase in the sample load. They rationalized this as an effect of different separation sites that are available on TAC.

The linear capacity θ_{10} (ref. 41) was calculated for the first-eluted enantiomer of Troeger's base and was 0.4 mg per gram of stationary phase (see Fig. 9). Rimböck *et al.*[6] reported that 0.5 mg of N,N,2,3,4,6-hexamethylthiobenzamide per gram of

stationary phase could be injected with hardly any changes in the capacity factor and plate height.

From experience, loss of resolution caused by volume overload in preparative separations on TAC is seldom any problem as the injection volumes are normally negligible in comparison with the volume of the eluted band.

The effect of injection conditions on the TAC column seems to be comparable to that found in other kinds of chromatography, *e.g.*, reversed-phase chromatography. A negative influence on resolution is obtained at injection volumes that exceed one third of the volume of the eluted band and at sample loads greater than 1 mg of analyte per gram of stationary phase. A mass overload of the columns is, of course, to be preferred to volume overload as far as Troeger's base is concerned.

Dependence of retention on temperature and eluent composition

The eluents used and the results obtained are given in Figs. 10–12. Both the capacity factors (k') and the selectivity factors (α) are influenced by changes in the eluent. It is often found, however, that a modification of an eluent has a much more pronounced effect on the capacity factors than on the selectivity factors. An example of this is the separation of Troeger's base using ethanol–water as eluent (see Fig. 11). The capacity factors k'_1 and k'_2 are 2.81 and 5.20, respectively, at a water content of 0.5%; they decrease to minimum values of 1.49 and 2.90 at a water content of 10% and increase to 6.86 and 15.36, respectively, at a water content of 50%. The corresponding selectivity factors change from 1.85 at 0.5% water to 2.23 at 50% water with a maximum of 2.45 at 40% water. Mannschreck *et al.*[1] reported only small differences in capacity factors and selectivity factors of methaqualone when ethanol was modified with water, *tert.*-butyl methyl ether–water or *n*-hexane–water. Significant changes were obtained, however, when ethanol was mixed with 2-propanol or completely replaced with either methanol or 2-propanol.

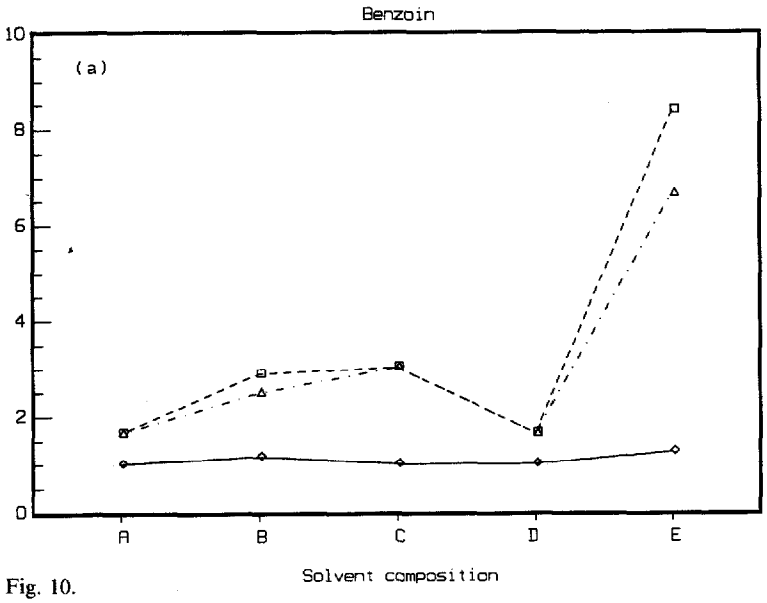
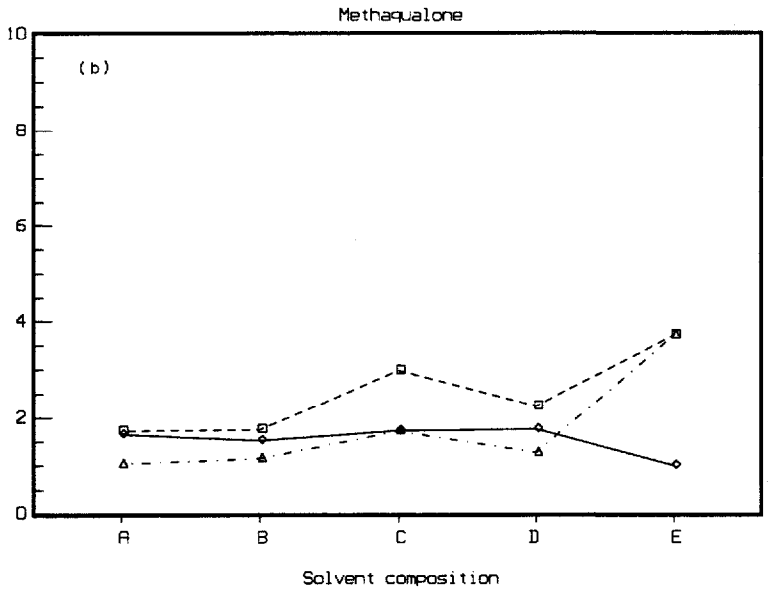


Fig. 10.



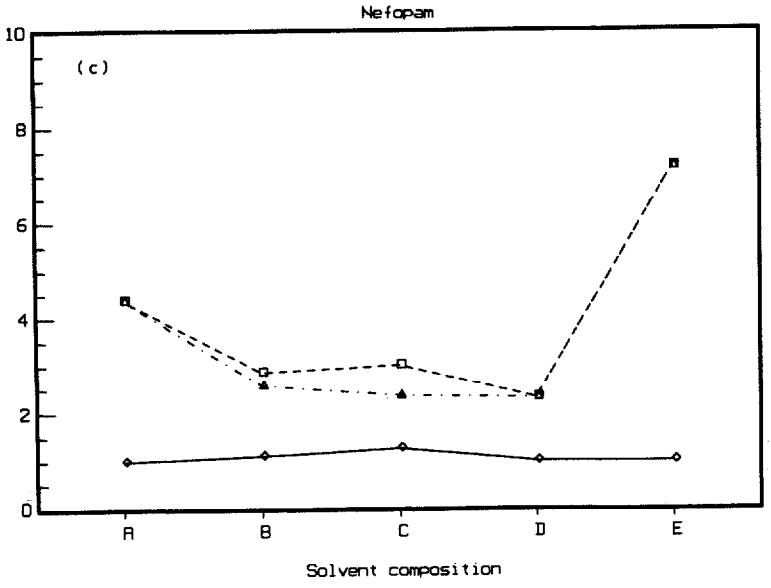


Fig. 10.

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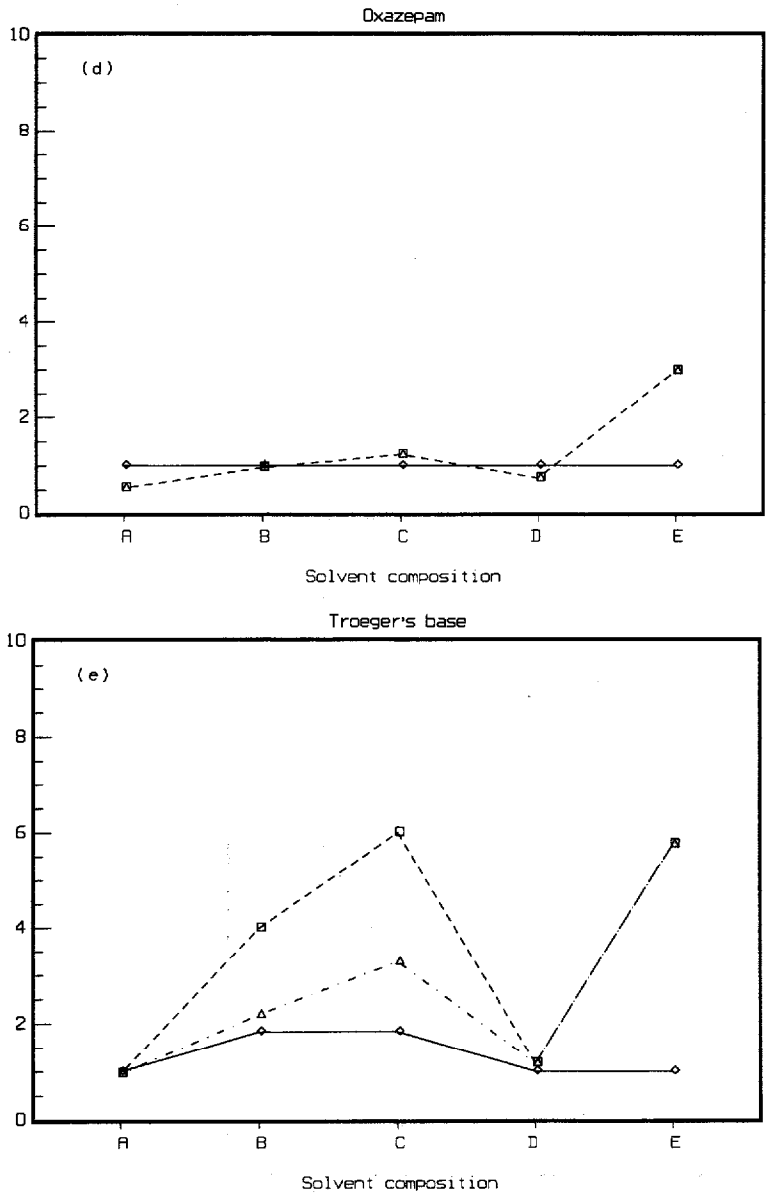

Fig. 10. Dependence of retention on eluent composition. Mobile phase: (a) methanol; (b) ethanol–water (95:5, v/v); (c) ethanol–water (99.5:0.5, v/v); (d) methanol–2-propanol (70:30, v/v); (e) 2-propanol. Chromatographic conditions: flow-rate, 0.5 ml/min; *ca.* 12 μg in 20 μl of mobile phase were injected. UV detection, benzoin 245 nm, methaqualone 254 nm, nefopam 230 nm, oxazepam 254 nm, Troeger's base 235 nm. ———, α; – · – · –, k'_1; – – –, k'_2.

It appears as if the eluent most strongly adsorbed on the surface of the cellulose has to be replaced in order to obtain a significant change in selectivity. This may help to explain the relatively large changes in the selectivity factor of Troeger's base when the

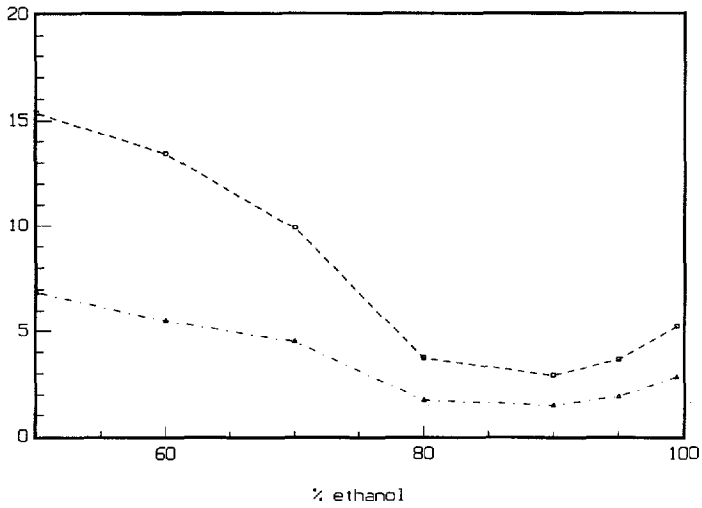

Fig. 11. Dependence of retention of Troeger's base on the eluent composition (ethanol–water). Chromatographic conditions: flow-rate, 0.2 ml/min; UV detection at 254 nm; 4 μg in 20 μl of mobile phase were injected. –·–·–, k'_1; ———, k'_2.

proportions of water and ethanol are altered in the mobile phase (see Fig. 11). The abilities of water and ethanol to hydrogen bond to TAC are probably comparable, indicating that the ethanol adsorbed on the surface can gradually be replaced with water. These findings also seem to be in accordance with the swelling experiments on TAC in binary and ternary swelling agents described earlier, which showed that the swelling was determined by that component in the swelling agent which gives the largest swelling. A modification of a mobile phase such as ethanol with an alkane has in general a smaller impact on selectivity than modification with another alcohol. Consequently, ethanol adsorbed on TAC is not exchanged to the same extent by an

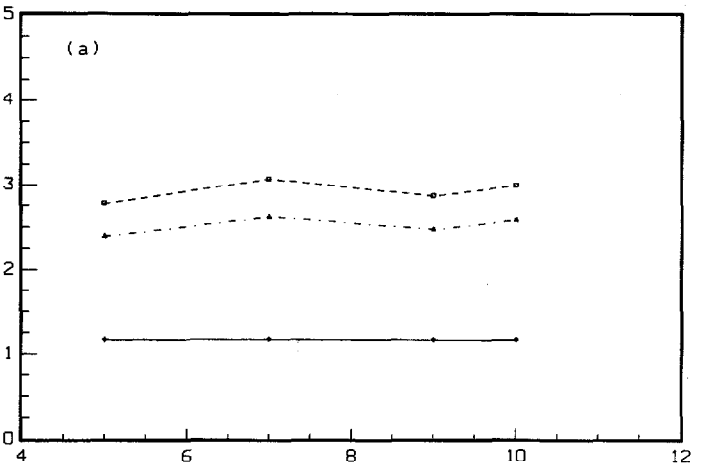

Fig. 12.

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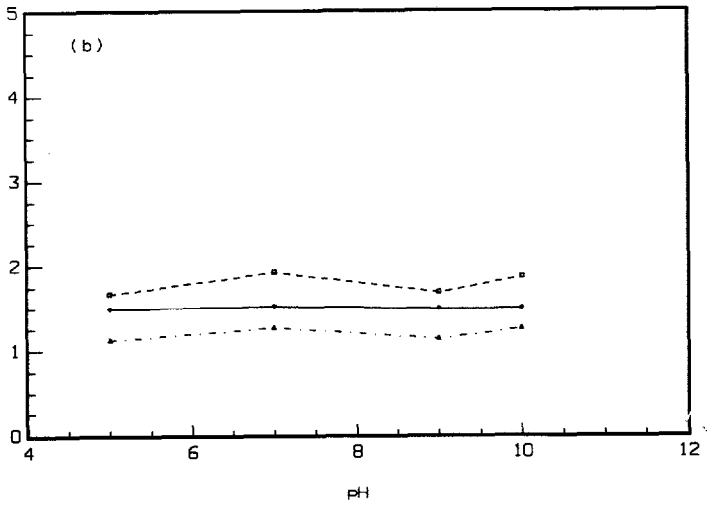

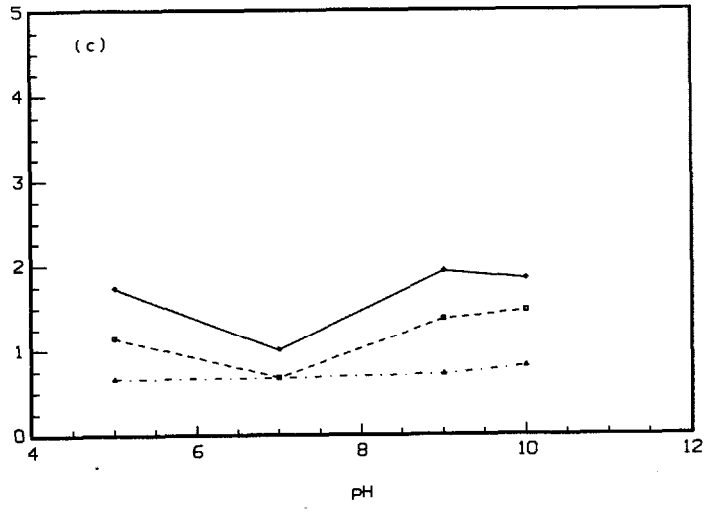

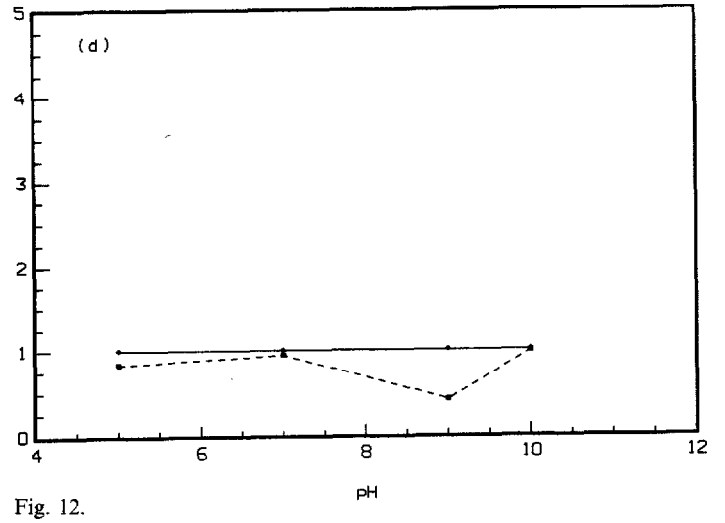

Fig. 12.

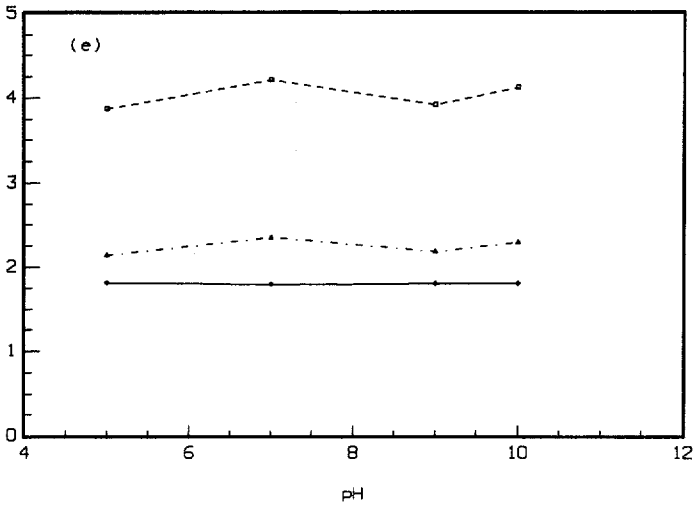

Fig. 12. Dependence of retention on pH of the mobile phase, ethanol–aqueous buffer (95:5, v/v). pH 5.0, 0.5 *M* acetic acid; pH 7.0, 0.5 *M* 2-aminoethanol; pH 9.0, 0.1 *M* boric acid; pH 10, 0.5 *M* 2-aminoethanol. Other conditions as in Fig. 10. ———, α; – · – · –, k'_1; – – –, k'_2. (a) Benzoin; (b) methaqualone; (c) nefopam; (d) oxazepam; (e) Troeger's base.

alkane. Similar effects are also found in chromatography on normal-phase silica[37]. In general, the weakly interacting solvent component in binary systems has a small influence on the selectivity factor.

From our experiments we draw the following general conclusions. The capacity factors are increased in the order methanol, ethanol, 2-propanol and, when methanol and 2-propanol are mixed, the capacity factors are similar to those obtained with methanol (except for nefopam). The highest selectivity factors were found using ethanol as mobile phase. Similar results were found by Mannschreck *et al.*[1]. The influence of the mobile phase on the capacity factors and selectivity factors is sometimes both complicated and unpredictable, as can be seen from the following examples. The capacity factors (α = 1) of nefopam in methanol and 2-propanol are 4.38 and 7.17, respectively, but only 2.32 in methanol–2-propanol (70:30, v/v). The α-values of methaqualone reported by Mannschreck *et al.*[1] are 2.4 in ethanol–water (96:4, v/v) and 1.5 in 2-propanol, but 3.0 in ethanol–2-propanol (80:20, v/v).

Some preliminary studies of the influence of pH on the selectivity factor and capacity factors were made (see Fig. 12). The pH of the eluent was adjusted with buffers to 10.0, 9.0, 7.0 and 5.0. The selectivity and capacity factors of the analytes in this study are relatively insensitive to changes in pH. The only exception is nefopam, for which the α-value is 1.83 at pH 10.0, 1.92 at pH 9.0 and 1.0 (no separation) at 7.0, but increases again to 1.73 at pH 5.0. The retention order was checked with the pure enantiomers and was found to be the same over the whole pH range. The sensitivity of nefopam to pH in the range 10.0–7.0 can probably be ascribed in part to differences in charge and conformational effects caused by the protonation/deprotonation of the nitrogen atom[42].

In addition to the nature of the eluent, one plausible reason for the significant

decrease in the capacity factor of oxazepam at pH 9.0 is the lower molarity of the buffer at that pH.

The retentive properties of the stationary phase, TAC, seem to be influenced only to a very small or no extent by changes of pH in the range 5–10. Changes in retention and stereoselectivity are therefore expected only when the analyte is affected by a change in pH, *e.g.*, by protonation/deprotonation or conformational effects. Buffered eluents can be used, for instance, in work with compounds that are unstable in weakly acidic media such as ethanol. The effects on selectivity and resolution are normally small and the performance of the column also is not influenced.

At elevated temperatures (see Fig. 13), an improvement in resolution was obtained for Troeger's base in spite of decreases in the selectivity factor and retention time. If the gain in efficiency compensates for the loss in selectivity, it could be utilized to optimize the throughput per unit time, for instance, in preparative work. An increase in temperature may also be useful in shortening the retention time of a more

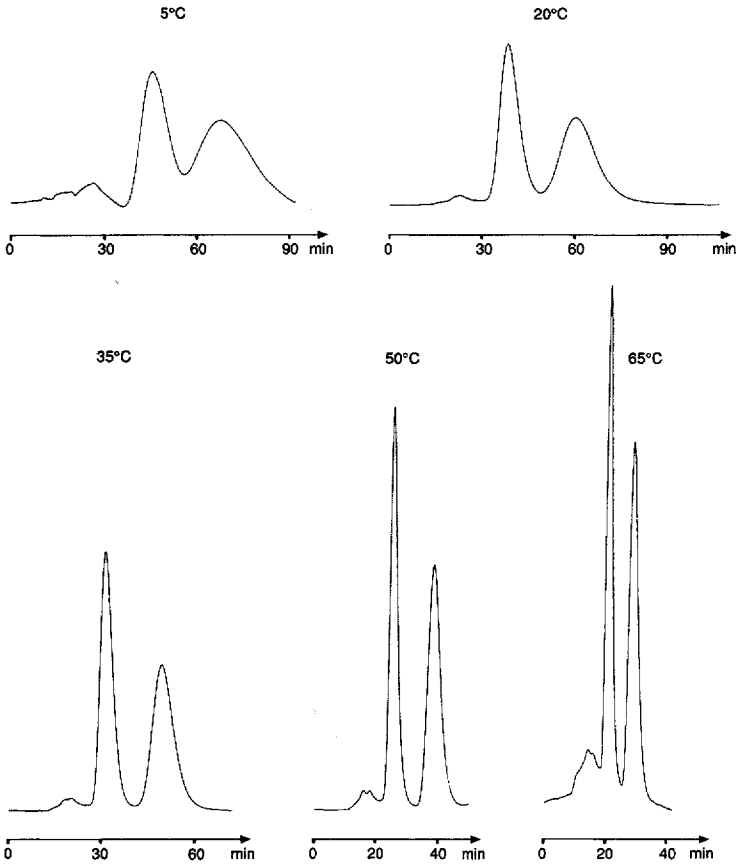

Fig. 13. Influence of temperature on the resolution of Troeger's base. Chromatographic conditions: mobile phase, ethanol–water (95:5, v/v); flow-rate, 0.25 ml/min; 10 μg in 20 μl of mobile phase were injected; UV detection at 254 nm.

strongly retained enantiomer and from an analytical point of view an increase in efficiency will increase the sensitivity of the method.

The atropisomers (enantiomers) of methaqualone were separated at different temperatures between 50 and 5°C (see Fig. 14). No racemization of methaqualone is expected under these conditions as the half-time ($t_{0.5}$) for racemization is 47 min[43] at 135°C, meaning a half-time of several days at ambient temperature. At 50 and 35°C the peaks overlap, whereas at 20 and 5°C there is virtually complete separation (see Fig. 14). At the lowest temperatures the viscosity increase of the eluent causes an increase in the pressure drop over the column and at the same time a decrease in efficiency.

Compounds that have short half-times of racemization at ambient temperature cannot, of course, be separated at high temperatures. An example of such a separation was reported by Mannschreck *et al.*[44]. On a TAC column they separated N,N-dimethylthiobenzamide, the half-time of racemization of which was *ca.* 2 h at ambient temperature, at different temperatures. Owing to the rapid racemization no separation was obtained at 60°C. At 25°C, however, a good baseline separation was obtained. To avoid racemization the isolation of the pure atropisomers of this compound from the eluent has to be carried out at low temperature.

The enantiomers of methaqualone were also separated at different flow-rates. The resolution, R_s, increased with decreasing flow-rate, indicating slow mass transfer (see Fig. 15).

During the course of this work the columns were exposed to a variety of different experimental conditions. Therefore, it was of interest to test the performance of each column after the completion of the experimental series. An analytical test with Troeger's base as solute was made and the chromatogram was compared with the original test chromatogram. In the pH range selected in this study (5–10), it appears as if TAC is not or is only very slowly hydrolysed. It must be emphasized, however, that eluents with high water contents at high or low pH have to be avoided as TAC is

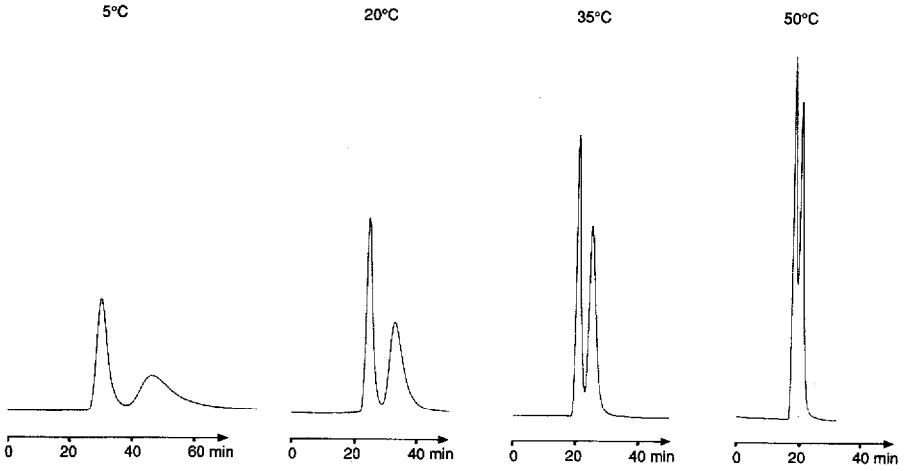

Fig. 14. Influence of temperature on the resolution of methaqualone. Chromatographic conditions: mobile phase, methanol; flow-rate, 0.25 ml/min; 20 μg in 20 μl of mobile phase were injected; UV detection at 254 nm.

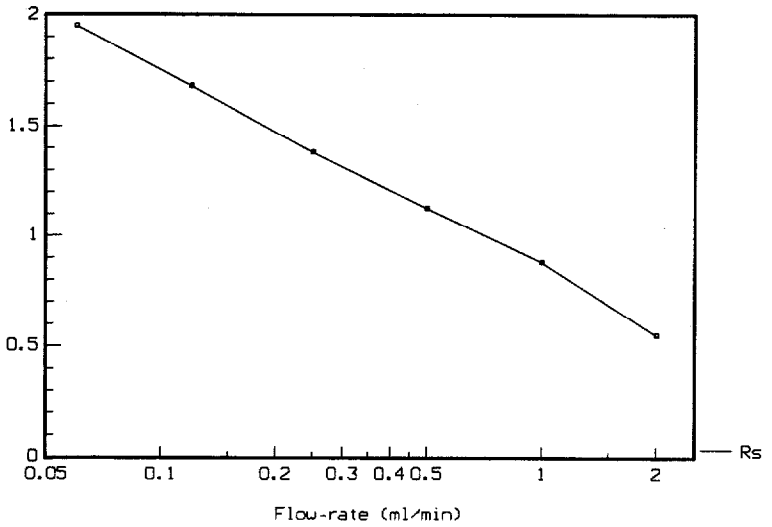

Fig. 15. Resolution (R_s) of methaqualone as a function of flow-rate. Chromatographic conditions: mobile phase, methanol; 20 μg in 20 μl of mobile phase were injected; UV detection at 254 nm.

hydrolysed under these conditions[45]. Except for slight increases in pressure drop over the columns, no significant changes in the performance of the columns had occurred, especially concerning the efficiency or the selectivity factor.

CONCLUSIONS

The results indicate that TAC can be used at relatively high pressures without any permanent compression of the bed, and can be used at both high and low temperatures. Further, TAC seems to withstand mobile phases with relatively high water contents (< 70%) and pH buffers in the range 5–10 for long periods, without any significant loss of chromatographic properties. The efficiency of the TAC column is not as good as that of a silica-based C_{18} column, but it is comparable to that of other chiral columns, *e.g.*, protein-based columns[46,47].

The retention behaviour of the analytes shows the dual character of the column, *i.e.*, a combination of normal- and reversed-phase chromatography. Both hydrophilic and hydrophobic interactions seem to play an important role in the retention mechanisms. The efficiency is strongly dependent on temperature and flow-rate of the mobile phase, indicating slow mass transfer.

The present knowledge of the mechanisms behind chiral recognition is too limited to permit predictions of separation or of retention orders of enantiomers. To be able to make such predictions, it is necessary to have a better knowledge of the structure of cellulose, especially the structure of swollen triacetylcellulose.

In work with a new compound it is advisable to start with ethanol–water (95:5, v/v) as the mobile phase and then proceed to other alcohols. If a separation is not achieved with alcohols, other eluents such as hydrocarbons or ethers may be advantageous. If a compound possesses an ionic group, pH buffers should be tried.

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