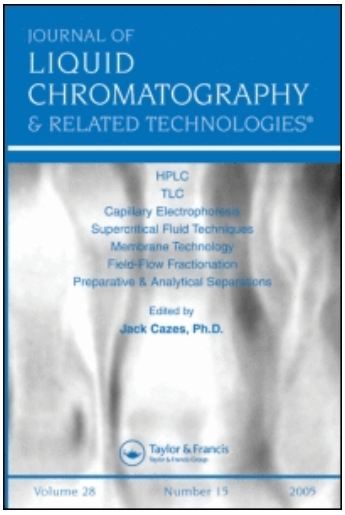

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L. Vaton-Chanvrier[a]; N. Bucaille[a]; Y. Combret[a]; J. -C. Combret[a]
[a] Université de Rouen, Mont Saint Aignan Cedex, France

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COMPARISON OF GRAFTING MODES FOR THE PREPARATION OF CHOLIC ACID-BASED STATIONARY PHASES. INFLUENCE ON ENANTIOMER SEPARATIONS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

L. Vaton-Chanvrier,* N. Bucaille, Y. Combret, J.-C. Combret

Laboratoire d'Hétérochimie Organique
UPRES EA 2659, IRCOF
Université de Rouen
F-76821 Mont Saint Aignan Cedex, France

ABSTRACT

An original chiral selector, namely the prop-2-enyl 3α-(N-phenylcarbamate)-7α,12α-dihydroxy-5β-cholan-24-oate, is bonded onto the silica surface by three different grafting modes. The influence of the total structure of the chiral stationary phases (CSPs) on enantioseparation is studied. These CSPs are prepared in two steps. In the first step, the silica surface is reacted with one of the following reagents: 1) (3-mercaptopropyl)trimethoxysilane, yielding a rather monomeric support, 2) chlorodimethylsilane, leading to a monomeric material, or 3) triethoxysilane, affording a polymeric packing. In the second step, the chiral selector is bonded onto the modified silica structures to provide the corresponding stationary phases **SH**, **CDS**, and **TES**, respectively. The CSP **SH** shows the poorest column performance. CSPs **CDS** and **TES** show similar mass transfer kinetics; this highlights the good behaviour of the polymeric packing. CSP **TES** displays globally the highest enantioselectivity and is found to be extremely stable despite its use over months. The silanization reaction with triethoxysilane provides a suitable support for chiral stationary phases.

INTRODUCTION

Two main strategies are employed for the covalent immobilization of a chiral selector onto the silica surface. The first approach involves the coupling of the chiral selector to a spacer arm (usually a chlorosilane or an alkoxysilane) followed by the "one–step" immobilization of this derivative onto the silica surface. The second approach includes two consecutive steps: the silica surface is first functionalized with a silane reagent and then allowed to react with the chiral selector. An aminopropyl silica gel is often used for this purpose. However, under normal-phase conditions, free amino groups function as sites for non enantioselective retention and thus reduce the enantioselectivity.[1,2]

For both strategies, monofunctional or polyfunctional silanes can be used. Monofunctional silanes lead to the formation of the so-called "brush-type" bonded phases, yielding a well defined and reproducible structure. Di- or trifunctional silanes produce a cross-linked polymeric structure in the presence of trace amounts of water. Compared to monomeric materials, polymeric bonded phases appear to be more stable to hydrolysis and better shield the unwanted residual silanols which are known to act as deleterious non enantioselective adsorption sites on the silica surface.[3-5] However, polymeric materials are often more difficult to reproduce and relatively thick stationary phases induce generally poorer stationary-phase mass-transfer kinetics.

An uncommon procedure of the silica surface organosilanization has been developed by Pesek et al.[6] In their "two-step" approach, the silanols are first converted to a hydride monolayer by reacting with triethoxysilane, the Si-H sites being then reacted with a terminal olefin to produce the final bonded material. This method presents the advantages of polymeric materials (high stability, masking the residual silanols) without the disadvantages of the abovementioned two-step procedure, since the residual Si-H groups create few interactions compared to polar amino groups.

We have applied this procedure to the synthesis of new CSPs bearing original chiral selectors based on cholic acid derivatives. Successful enantioseparations of a variety of analytes were reported.[7]

Comparative chromatographic studies of CSPs prepared by different grafting modes have highlighted an important but not easily understood secondary effect of the hydride surface obtained with Pesek's method.[8, 9] Hence, further studies are needed to better understand the influence of this immobilization process on the chiral chromatographic performance of stationary phases.

For this purpose, the chromatographic results collected on cholic acid-based stationary phases prepared by three different grafting modes are compared. New phases are synthesized via a two-step procedure by bonding the

cholic acid derivative respectively to a 3-mercaptopropyl modified silica surface and to a chlorodimethylsilane-treated silica gel to provide the corresponding CSPs **SH** and **CDS**. The 3-mercaptopropyl silica has been widely employed for the preparation of numerous chiral stationary phases and was applied with success to the bonding of cholic acid derivatives onto silica gel.[10-13] As another support, the chlorodimethylsilane-treated material was also found suitable for the preparation of chiral stationary phases.[14] In this case, monomeric hydride groups cover at least partially the silica surface. The chromatographic performance of these CSPs **SH** and **CDS** is compared with that of CSP **TES**, a stationary phase prepared according to Pesek's method as described previously.[7] Prior work had shown that CSP **TES** was particularly enantioselective towards binaphthyl compounds.[7] In this paper, separations of new binaphthyl derivatives are investigated. Furthermore, a series of amino alcohols of pharmaceutical interest (β-blockers) are tested.

EXPERIMENTAL

Materials

Cholic acid (97% TLC) was purchased from Acros Organics (Acros Organics, Noisy le Grand, France). Chromatographic and chlorinated solvents (Carlo Erba Reactifs, Nanterre, France) were of HPLC grade. All CSPs were prepared from the same batch of silica gel (Hypersil, particule size 8 μm, pore diameter 10 nm, specific surface area 300 $m^2.g^{-1}$; Hypersil-ThermoQuest, Les Ulis, France). Other reagents were obtained commercially and used without further purification. The chiral test compounds (analytes) were provided by different sources, mainly Acros Organics and Sigma-Aldrich (Sigma-Aldrich, Saint Quentin Fallavier, France). When not deliverable by the prior mentioned companies, N-derivatized amines were synthesized according to standard derivatization procedures. Hydantoin samples and binaphthyl derivatives were obtained as gifts respectively from UC2M2 (Université de Rouen, France) and from URA CNRS 480 (ISMRA-Université de Caen, France).

Instrumentation

Elemental analyses were performed on a CE instrument model EA 1110 CHNS-O apparatus (ThermoQuest, Les Ulis, France) at the Laboratoire de Microanalyses (IRCOF, Rouen). The surface coverage of the stationary phases are calculated according to the Berendsen et al. method.[15]

Chromatographic experiments were achieved with a Varian (Varian, Les Ulis, France) system equipped with a 9010 pump, a UV 9050 detector and a computerized data acquisition system. The volume of sample injected was 20 μL. The flow rate was 0.8 $mL.min^{-1}$. The CSPs **CDS** and **SH** were slurry

packed into stainless steel tubes (250×4.6 mm i.d.). The hold-up time (t_0) was determined by the retention time of 1,3,5-tri-*tert.*-butyl benzene. The UV-detector was operated at 254 nm or at 220 nm for the β-blocker analytes.

Bonded Phase Synthesis

The chiral selector prop-2-enyl 3α-(N-phenylcarbamate)-7α,12α-dihydroxy-5β-cholan-24-oate was synthesized as previously reported[7] and characterized by IR, NMR, HPLC and mass spectrometry.

Chiral Stationary Phase CDS

The chlorodimethylsilane modified silica gel was prepared as reported by Yoshinaga et al.[16] Elemental analysis afforded C 2.46, H 0.35%. The calculated coverage was 1.089 mmol of dimethylsilane residues bonded per gram of silica gel (based on C). Bonding of the chiral selector was performed as follows. Prop-2-enyl 3α-(N-phenylcarbamate) -7α,12α-dihydroxy-5β-cholan-24-oate (2.74 g, 4.83 mmol) was dissolved in 20 mL toluene. After the addition of a 0.1 M solution of hexachloroplatinic acid in propan-2-ol (480 μL, $4.80.10^{-2}$ mmol), the mixture was heated and maintained at 90°C for about one hour. Then the silanized silica (4 g) was added and the suspension was refluxed for 96 hours under nitrogen atmosphere. The bonded phase was filtered and washed with toluene, dichloromethane, methanol, and diethyl ether. Elemental analysis afforded C 7.57, H 1.40, N 0.51%. The calculated coverage density of chiral selector was 0.135 $mmol.g^{-1}$ (based on C).

Chiral Stationary Phases SH1 and SH2

The 3-mercaptopropyl-silanized silica gel was prepared according to the method reported by Rosini et al.[17] Elemental analysis afforded C 3.84, H 0.78, S 2.4%. The above treated silica gel (4 g), prop-2-enyl 3α-(N-phenylcarbamate)-7α,12α-dihydroxy-5β-cholan-24-oate (2.78 g, 4.90 mmol), 2,2'-azobisisobutyronitrile (AIBN, 236 mg, 1.44 mmol) and chloroform (40 mL) were heated to reflux under nitrogen for 12 hours. Elemental analysis indicated C 9.60, H 1.36, N 0.3, S 1.66%. The calculated coverage of chiral selectors was 0.153 $mmol.g^{-1}$ (based on C). This slightly bonded CSP is designated **SH1**. Another reaction was performed as described above except that the reflux was maintained for 40 hours. Elemental analysis afforded C 13.42, H 1.86, N 0.2, S 2.02%. The calculated coverage density of chiral selector was 0.271 $mmol.g^{-1}$ (based on C). This highly bonded CSP is designated **SH2**.

Reaction of Accessible Thiol Groups with Hex-1-ene

This "end-capping" treatment was performed *in situ* as described by Veigl et al.[18] To calculate the coverage of the "end-capping" evaluated by elemental analysis, 0.5 g of each stationary phase was treated in the same conditions in a post-column coupled in series with the analytical column during the treatment. The elemental analysis showed lower surface coverage on both CSPs after the "end-capping" reaction (CSP **SH1**: C 8.98, H 0.96, N 0.1, S 2.23 %; CSP **SH2**: C 12.28, H 1.58, N 0.1, S 2.96%). This unexpected data may result from the usual deviations of C, N, S analysis of modified silica gels or may be due to a partial loss of chiral selectors.

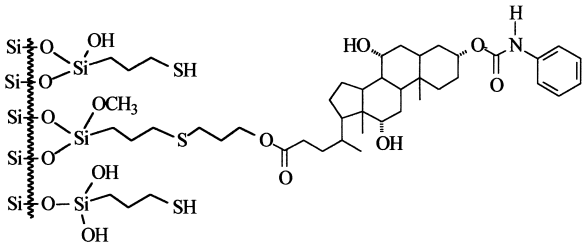
RESULTS AND DISCUSSION

Stationary Phases Synthesis and Characterization

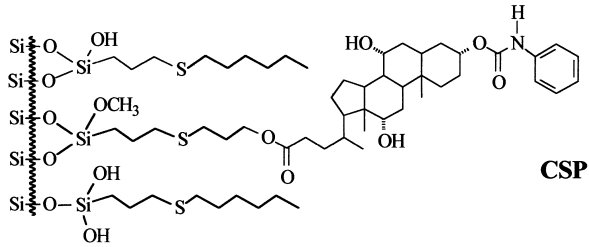
The four chiral stationary phases are outlined in Figure 1. The cholic acid derivative is bonded to the hydride silica surface by a hydrosilylation reaction in the presence of hexachloroplatinic acid catalyst to produce the CSPs **CDS** and **TES**. The CSPs **SH** are obtained by the radical anti-Markownikow addition of a terminal thiol group on the 3-mercaptopropylsilica to an allyl group on the chiral selector. The success of the bonding process is controlled by DRIFT experiments, as previously reported.[7] Characteristic bands of the chiral selector are identified for each stationary phase. Dissimilar stretching vibrations of residual Si-H groups (2250 cm^{-1} for CSP **TES** and 2150 cm^{-1} for CSP **CDS**) confirm the different environment of the hydride groups in the polymeric and monomeric supports. CSPs **SH1**, **CDS**, and **TES** present equivalent surface densities of chiral selector (respectively 0.153, 0.135, and 0.133 $mmol.g^{-1}$). CSP **SH2** is prepared with the optimum reaction conditions, yielding the highest obtainable density of chiral selector (0.271 $mmol.g^{-1}$).

Chromatographic Conditions

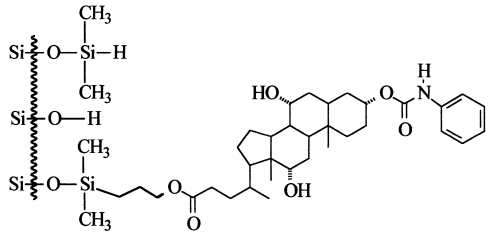
The chromatographic performance of all phases is evaluated in normal phase mode with representative solutes (the structures are presented in Figure 2). In order to elucidate the parameters responsible for enantioselective and non enantioselective retention characteristics, the behaviour of the different CSPs are compared using isoeluotropic conditions. It must be noted that identical conclusions are obtained when using the same mobile phase conditions. The chromatographic results gathered on the various phases are summarized in Tables 1 to 3. For enantiomers available in pure form, elution orders (listed in Table 1) are determined when sufficient resolution occurs, and are found to be identical on the three stationary phases.



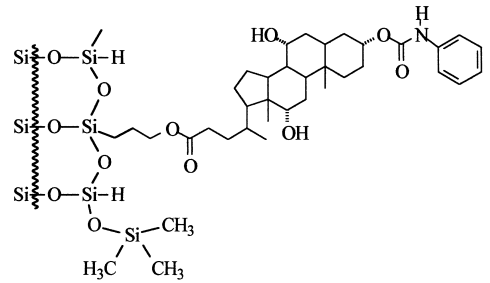
CSP SH



CSP SH after "end-capping"



CSP CDS



CSP TES

Figure 1. CSPs **SH**, **SH** after the "end-capping" treatment, **CDS** and **TES**.

Evaluation of the CSPs SH1, SH2, CDS, and TES

Although the surface coverage of chiral selectors is similar on CSPs **SH1**, **CDS,** and **TES**, the CSPs **CDS** and **TES** display enantioselectivity towards a majority of solutes (see Table 1), while no separation is achieved on CSP **SH1**

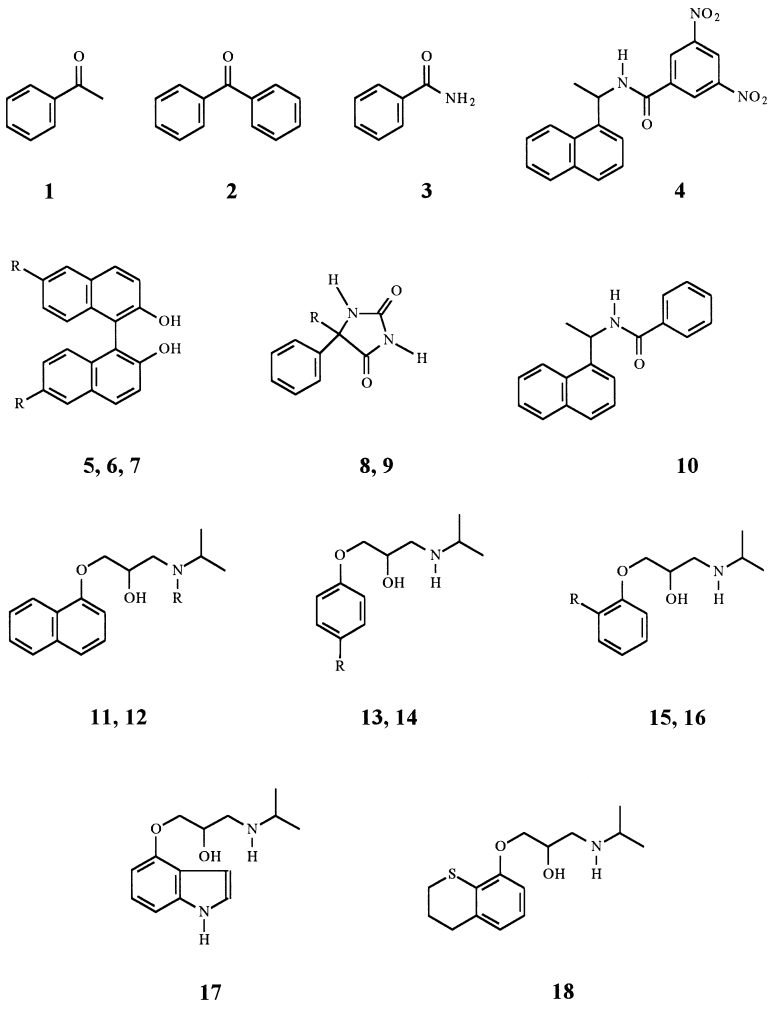


Figure 2. Structure of test solutes (**1** to **4**) and racemic analytes (**5** to **18**). **1** = acetophenone ; **2** = benzophenone ; **3** = benzamide ; **4** = R-(+)-N-3,5-dinitrobenzoyl-1-(α-naphthyl)ethylamine ; **5** = 2,2'-dihydroxy-1,1'-binaphthyl (R = H) ; **6** = 6,6'-dibromo-2,2'-dihydroxy-1,1'-binaphthyl (R = Br) ; **7** =6,6'-diyl-bis(diethylphosphonate)-2,2'-dihydroxy-1,1'-binaphthyl (R = $PO(OCH_2CH_3)_2$) ; **8** = 5-isopropyl-5-phenyl-imidazolidine-2,4-dione ; **9** = 5-ethyl-5-phenyl-imidazolidine-2,4-dione ; **10** = N-benzoyl-1-(α-naphthyl)ethylamine ; **11** = N-3,5-dinitrobenzoyl-propranolol (R = $-CO-3,5-(NO_2)-C_6H_3$) ; **12** = propranolol (R = H) ; **13** = Atenolol (R = $-CH_2-CO-NH_2$) ; **14** = Metoprolol (R = $-CH_2-CH_2-O-CH_3$) ; **15** = Alprenolol (R = $-CH_2-CH=CH_2$) ; **16** = Oxprenolol (R = $-O-CH_2-CH=CH_2$); **17** = Pindolol ; **18** = Tertatolol.

Table 1

Enantioseparation of Representative Analytes on CSP SH2, CSP CDS, and CSP TES Under Isoeluotropic Conditions

	SH2				CDS				TES			
Solutes	**Eluant**	**k_1**	**α**	**Rs**	**Eluant**	**k_1**	**α**	**Rs**	**Eluant**	**k_1**	**α**	**Rs[a]**
5	80/20	4.64	1.10	0.69	95/5	4.11	1.21	2.00	95/5	4.65	1.32	2.78(R)
6	75/25	3.98	1.11	0.66	92/8	4.08	1.31	2.39	95/5	3.74	1.33	2.92(R)
7	70/30	10.89	1.00	/	83/17	10.37	1.10	0.63	80/20	10.12	1.14	1.03(R)
8	87/13	4.87	1.00	—[b]	92/8	4.02	1.20	1.32	90/10	3.50	1.15	1.13(S)
9	87/13	3.66	1.03	/	92/8	4.93	1.12	1.39	90/10	4.97	1.12	1.10(S)
10	80/20	6.60	1.00	/	95/5	5.74	1.00	/	92/8	6.72	1.03	—[b](R)
11	80/20	10.05	1.03	—[b]	92/8	7.52	1.04	—[b]	92/8	8.66	1.08	0.74

Eluant: n-heptane/propan-2-ol (v/v). k_1: retention factor of the first eluted enantiomer. α: selectivity factor. Rs: resolution.
[a] absolute configuration of the first eluted enantiomer. [b] $0 \leq Rs \leq 0.5$.

Table 2

Column Efficiency*

Solutes	SH2	CDS	TES
1	7538	4705	5294
2	5376	4996	5650
3	3695	3978	4323
4	2030	2721	4154

* Plate number per column. Eluant: n-heptane/propan-2-ol (v/v): 70/30 for the CSP **SH2**; 85/15 for the CSPs **CDS** and **TES**.

with any tested analytes in the conditions of use. In order to enhance the performance of such a material, a stationary phase bearing a higher surface coverage of selectors, CSP **SH2**, was prepared. Indeed, a higher density of selectors is expected to increase the interactions of the chiral selector and the analytes and to strongly impede the access of solutes to deleterious silanol adsorption sites or to thioether fuctions and thiol groups. With respect to CSPs **CDS** and **TES**, CSP **SH2** exhibits higher retention, similar column efficiency (listed in Table 2), and what is more, estimable enantioselectivity. An unusual "end-capping" procedure was applied to CSPs **SH1** and **SH2**, which consists in the reaction of the accessible thiol groups with long alkenyl chains.[18] However, with both phases, this treatment does not lead to higher column performance and thus appears to have no practical interest.

Regarding CSPs **CDS** and **TES**, the samples elute in similar mobile phases with close retention factors. As seen in Table 2, both these stationary phases can be estimated to behave similarly in term of efficiency for solutes **1** to **3**. The mass transfer kinetics are thus comparable, regardless of the monomeric or polymeric support. By affording high column efficiency, especially with compound **4**, the cross-linked hydride monolayer of CSP **TES** proved to cover, effectively, the silanols on the silica surface.

An increase in enantioselectivity can be observed for most solutes when considering respectively the CSPs **SH2** after "end-capping", **SH2**, **CDS**, and **TES**. Non-specific adsorption can not totally account for the lower enantioselectivity of CSPs **SH2**, which may be caused by additional achiral interactions. Moreover, the long tether connecting the chiral selector to the silica surface may induce mobility of the chiral selector, which, in turn, could be detrimental for the enantioselectivity. CSP **TES** affords generally greater enantioselectivity and superior resolution relative to CSP **CDS**. Especially high resolution values are obtained on CSP **TES** for solutes 5, 6, or for the newly synthesized ana-

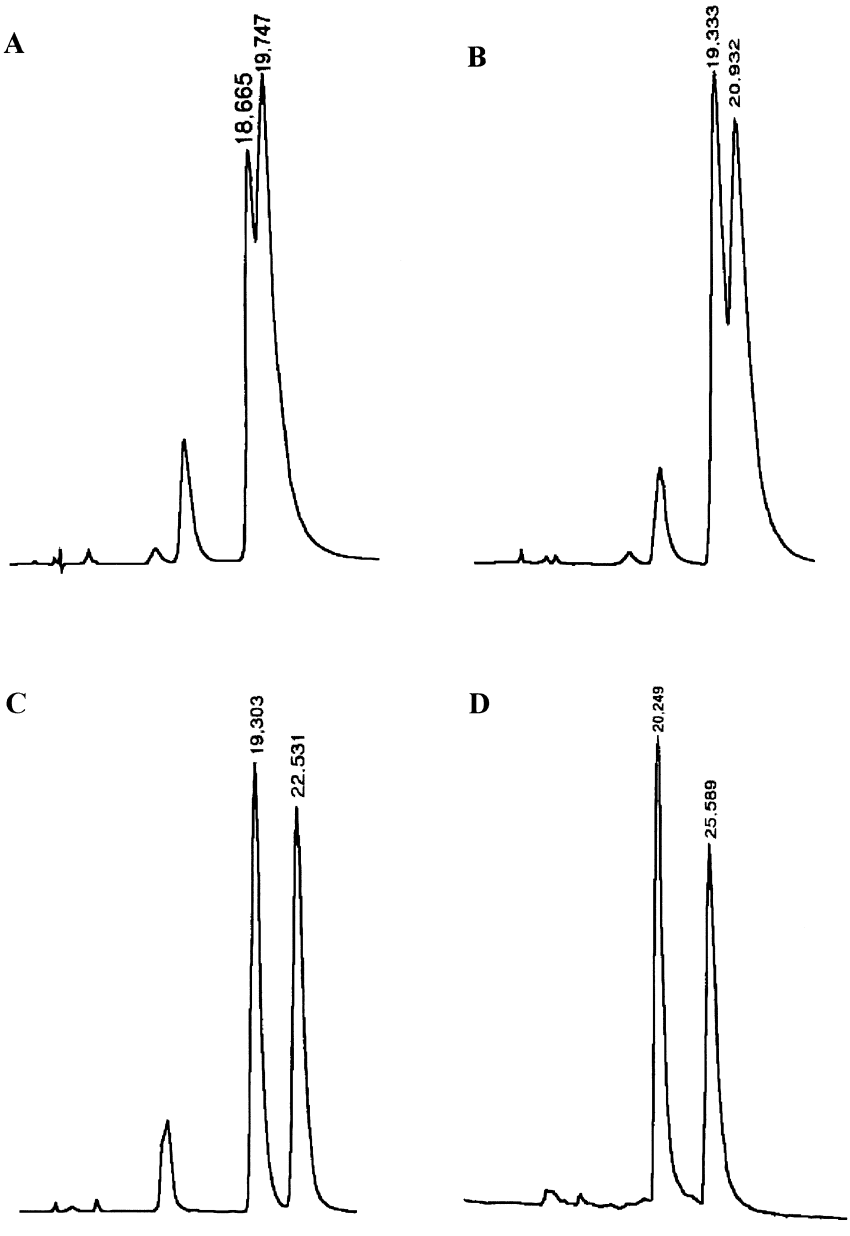


Figure 3. Chromatograms of the enantioseparation of solute **5** on : (A) CSP **SH2** after the "end-capping" treatment, (B) CSP **SH2**, (C) CSP **CDS** and (D) CSP **TES**.

Table 3

Enantioseparation of β-Blocker Solutes on CSP CDS and CSP TES

		CDS			TES	
Solutes	**Eluant**	k_1	α	**Eluant**	k_1	α
12	90/10	4.35	1.00	90/10	3.16	1.09
13	70/30	6.68	1.00	70/30	5.04	1.00
14	90/10	4.15	1.00	85/15	3.09	1.07
15	95/5	4.29	1.04	90/10	1.94	1.08
16	90/10	4.02	1.00	90/10	4.06	1.08
17	85/15	8.48	1.00	85/15	7.68	1.00
18	90/10	3.75	1.00	90/10	3.24	1.10

Eluant (v/v): n-heptane/propan-2-ol, 0.1% (v/v) triethylamine, 0.1% (v/v) trifluoroacetic acid. k_1: retention factor of the first eluted enantiomer.
α: selectivity factor.

lyte 7,[19] highlighting the real ability of the cholic acid moiety to the chiral recognition of 1,1'-binaphthyl derivatives. Figure 3, showing chromatograms of 5, illustrates the different chromatographic performance of the four stationary phases.

The different behaviour of CSPs **SH2**, **CDS**, and **TES** is confirmed by the chromatographic data obtained for some underivatized amino alcohols of the β-blocker series (shown in Table 3). The chromatographic analyses are performed by using the joined addition of triethylamine and trifluoroacetic acid in the mobile phase, which is needed to reduce analysis time and peak tailing. Nevertheless, even when using these optimum conditions, no separation is observed on CSP **SH2** and exclusively alprenolol enantiomers 15 are separated to a small extent on CSP **CDS**. In fact, only CSP **TES** shows appreciable enantioselectivity towards the β-blocker analytes.

These results emphasize the different behaviour of monomeric versus polymeric packing of CSPs **CDS** and **TES** respectively, and the enhanced chromatographic performance of CSP **TES**. Moreover, CSP **TES** shows a great stability over months of use, despite the use of aggressive mobile phases. CSPs **CDS** and **TES** bear identical chemical functions. Both these phases differ only from the cross-linked hydride monolayer on CSP **TES** performed by the triethoxysilane treatment and from the methyl groups brought by the dimethylsilane on CSP **CDS**. Hence, subtil changes in the chemical structure of the sta-

tionary phase greatly influence the chiral chromatographic properties of the column. The importance of this observation is not generally appreciated even though it has profound implications regarding CSP design. The results reported in this paper are in agreement with the secondary effect of the hydride monolayer mentioned by Pesek et al.[9] However, this secondary effect can not be merely explained by the influence of adsorption sites as silanols. As mentioned above, there is no evidence that non enantioselective interaction sites occur differently on CSPs **CDS** and **TES**. Accordingly, further work is needed to understand and convincingly explain the different comportment of such similar packings.

CONCLUSION

The chromatographic performance of chiral stationary phases was demonstrated to be greatly affected by the method of immobilization of the chiral selector onto the silica surface. From the methods studied, the CSP prepared from the triethoxysilane-treated silica gel exhibits the greatest separations of enantiomers. This is especially emphasized with the enantiomeric recognition of some β-blocker solutes. The silanization of the silica surface with triethoxysilane followed by hydrosilylation appears to be a very suitable procedure for the preparation of new chiral stationary phases. Further studies are currently underway with new cholic acid derivatives as chiral selectors.

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