Cholesteric bonded stationary phases for high performance liquid chromatography II: synthesis, physico-chemical characterization and chromatographic behavior of a phospho-cholesteric bonded support. A new way to mimic drug/membrane interactions?

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Cholesteric bonded stationary phases for high-performance liquid chromatography: synthesis, physicochemical characterization, and chromatographic behavior of a phospho–cholesteric bonded support. A new way to mimic drug/membrane interactions?

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Abstract Among the various methods exploitable to determine the bioavailability of drugs, reversed-phase liquid chromatography (RPLC) appears to be suited to creation of patterns of prediction. In this context a new stationary phase was designed in this work to reproduce, in terms of chemical structure, as accurately as possible, the main elements of cellular membranes; which include phospholipids and cholesterol molecules. An efficient synthetic pathway was developed to prepare ligands that contain a phosphate head, a long alkyl chain chemically bonded to silica, and a cholesteric moiety, in order to mimic both hydrophilic and hydrophobic interactions, and "membrane-like" organization, respectively. The new stationary phase was characterized by Fourier-transform infra red (FTIR) and ^{1}H–^{13}C, ^{1}H–^{31}P, and ^{1}H–^{29}Si cross-polarization magic-angle-spinning nuclear magnetic resonance (CP MAS NMR) spectroscopy. Its chromatographic behavior has been studied by classical classification tests for RPLC columns. Despite its low surface coverage, the material produced exhibits high shape selectivity, possibly due to the organization of the grafted moieties.

Keywords HPLC · CP MAS NMR · Phospho–cholestric phase · Shape selectivity · Methylene selectivity · Hydrophobicity

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Introduction

Knowledge of the oral absorption and penetration of drugs through cellular membranes is an essential aspect of determining their bioavailability. The possibility of a very early prediction of these properties has an obvious and important impact on the discovery of drugs in terms of time and cost. Among various methods exploited to achieve this, is reversed-phase liquid chromatography (RPLC), which is suitable for creation of reliable patterns of prediction. However octadecylsilyl (ODS) silica, for example, retains analytes solely on the basis of their hydrophobicity.

In this context Pidgeon et al. [1] suggested new stationary phases that mimic the phospholipids bilayer of cell membranes. These authors synthesized immobilized artificial membranes (IAMs) that are monolayers of phospholipid analogs covalently bonded to the silica surface [2, 3]. IAMs were successfully used to predict solute partitioning into the liposome membranes, to predict drug permeability through Caco-2 cells, to correlate the binding of amino acids to IAM surfaces with their brain uptake, to evaluate bile salt–membrane interactions, to predict human skin permeability of steroids and alcohols, and to obtain hydrophobicity data

for structure–activity relationship studies [4]. Indeed IAM more closely mimic the interaction of analytes with biological membranes than the classical ODS stationary phase because combinations of hydrophobic, ion pairing, and hydrogen bonding are possible.

During the same decade, cholesterol was used in RPLC for its liquid crystal behavior. Pesek et al. [5] were the first to bond a cholesteryl moiety via a terminal olefin group to a silica hydride. This modified support was obtained by a convenient process, triethoxysilane silanization [6]. Delaurent et al. [7] attempted a simpler reaction in which a cholesteric stationary phase (CP) was prepared by reacting the terminal amino-group of bonded aminopropyl silica with cholesteryl chloroformate. Buszewski et al. [8, 9] also investigated this option. The originality of their material consisted in the use of multifunctional bonded moieties such as aminopropyl, octadecyl, and cholesteryl, in order to obtain pseudo-membrane packing resembling natural systems. Chromatographic studies pointed out the great shape selectivity of CPs, a property that depends on the temperature, and that their mechanism of retention is mainly governed by the size and shape of solutes. Furthermore it has been demonstrated that the CPs can function as a chiral material for a set of enantiomeric compounds. On the other hand, several CPs were characterized by quantitative structure–retention relationships in order to test cell-membranes mimicking media in a chromatographic system [8–11]. Their chromatographic behavior urged Catabay et al. [12] to investigate the separations of four different groups of pharmaceuticals that were chosen primarily on the basis of their related structures and on their importance to pharmacologic, toxicologic, and forensic studies. CPs showed high molecular recognition capability, and thus high selectivity, which is entirely different from that of the ODS phase. Thus the implication is that the overall separation performance makes this bonded phase ideal for these types of pharmaceutical application [13].

IAMs and CPs have been a great step forward to comprehend the behavior of drugs at the cellular membrane level. However no attempt has been done to introduce, at the same time, a cholesteric and a phospholipid moiety to mimic more closely the interactions between drugs and the bilayer of cell membranes. It has been demonstrated that an ordered monolayer of immobilized lipids containing both a polar and a nonpolar region is critical for a chromatographic surface to accurately monitor the interaction between solutes and biological membranes [14]. Differences in the polar functional groups do not eliminate the ability of the surface to predict drug–membrane interaction, although a phospholipid headgroup further improved the capabilities of prediction. Furthermore, the chromatographic process is mainly governed by the organization of the bonded moieties where the molecules of cholesterol can play a key role. Indeed the presence of cholesterol has a deep impact on the state of the lipid bilayers [15].

In this work we report an efficient synthetic pathway to prepare ligands that contain a phosphate head, a long alkyl chain chemically bonded to silica, and a cholesteric moiety in order to mimic both hydrophilic and hydrophobic interactions, and "membrane-like" composition. The phospho–cholesteric phase (PCP) obtained was characterized by Fourier-transform infra red (FTIR) and cross-polarization magic-angle-spinning nuclear magnetic resonance (CP MAS NMR) spectroscopy and chromatographically.

Experimental

Chemicals

The following reagents were used for chemical modification of the support material: 9-decen-1-ol, cholesterol, triethoxysilane, and Karstedt's catalyst (platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex solution in xylene); they were from Sigma Aldrich. Phosphorus oxychloride was purchased from Fluka. All solvents were of analytical grade purity. Benzene, petroleum ether, dichloromethane, and methanol were freshly distilled under argon. Triethylamine was also freshly distilled from sodium hydroxide pellets.

LiChrospher® Si 100 (E. Merck, Darmstadt, Germany) was used as support material for preparation of the chemically bonded phase. Its physical and chemical characteristics are: nominal particle size 5 μm, pore size 10 nm, pore volume 1.25 mL g^{-1}, specific surface area of 400 m^2 g^{-1}.

FTIR spectroscopy

Spectra (4000 cm^{-1} to 650 cm^{-1}, with 4 cm^{-1} resolution and 100 scans) of each compound were recorded on a Nicolet Avatar spectrometer equipped with a DTGS detector, an Ever-Glo source, and a KBr/germanium beam splitter. Samples were deposited without preparation on an attenuated total reflectance (ATR) cell provided with a diamond crystal. Air was taken as reference for the background spectrum of each sample. Recorded spectra were normalized after correction of the baseline by the instrument software, Omnic 4.1 b (Thermo Nicolet).

Liquid and solid-state NMR

^{1}H, ^{13}C, and ^{31}P NMR spectra in solution were recorded on a Bruker Avance DPX-300. NMR data were collected at ambient temperature, and chemical shifts were given in ppm referenced to the appropriate solvent peak: $CDCl_3$ for ^{1}H and ^{13}C and 85% H_3PO_4 for ^{31}P.

^{1}H–^{31}P, ^{1}H–^{13}C, and ^{1}H–^{29}Si CP MAS NMR spectra were obtained on a Bruker Avance 400 MHz WB spectrometer. All experiments were performed with a commercial Bruker double-bearing probe with zirconium dioxide rotors of 4 mm outer diameter, and a spinning rate of 10 kHz. A ramped ^{1}H pulse was used to circumvent Hartman–Hahn mismatches [16], and to improve the resolution a dipolar decoupling TPPM-15 pulse sequence was applied during the acquisition time. To obtain a good signal-to-noise ratio typical numbers of scans were 0.5 k, 4 k, and 1 k for ^{1}H–^{31}P, ^{1}H–^{13}C, and ^{1}H–^{29}Si, respectively. The experiments were performed at ambient temperature and chemical shifts were referenced to tetramethylsilane for ^{13}C and ^{29}Si, and to a solution of H_3PO_4 (85%) for ^{31}P.

Apparatus and chromatographic conditions

HPLC was performed with a LaChrom system from VWR–Hitachi equipped with an L-7200 autosampler, an L-7100 four-channel pump, an L-7300 column oven, an L-7455 DAD detector, and a D-7000 interface. The data were collected using HSM Manager software. PCP was packed in a 250×4.6 mm column. For chromatography of analytes UV detector was set at 254 nm. All reagents and analytes employed were of highest quality commercially available. Uracil (0.01 mg mL^{-1}), benzene (0.5 mg mL^{-1}), toluene (0.5 mg mL^{-1}), ethylbenzene (0.5 mg mL^{-1}), butylbenzene (0.5 mg mL^{-1}), amylbenzene (0.5 mg mL^{-1}), triphenylene (0.5 mg mL^{-1}), and *o*-terphenyl (0.5 mg mL^{-1}) were purchased from Sigma Aldrich and the standard reference material (SRM) 869a from the standard reference materials program (NIST, Gaithersburg, MD, USA). The mobile phase consisted of methanol and water mixtures in the range 80:10 to 70:30 (*v*/*v*). The mobile phases were filtered though a 0.45 μm membrane filter. The flow rate was 1.0 mL min^{-1}. The PCP column was operated between 30 and 53°C.

Synthesis of cholesteryl 9-decenyl chlorophosphate (I)

A solution of $P(O)Cl_3$ (3.97 g, 25.86 mmol) in 50 mL benzene was added dropwise, at 0–5°C with stirring under argon, to a solution of cholesterol (10.00 g, 25.86 mmol) and Et_3N (2.61 g, 25.86 mmol) in 150 mL benzene. The reaction mixture was stirred at room temperature for 1 h. The supernatant was then transferred under argon to a dropping funnel and was added dropwise, with stirring, at 0–5°C to a solution of 9-decen-1-ol (4.04 g, 25.86 mmol) and Et_3N (2.61 g, 25.86 mmol) in 150 mL benzene. The reaction mixture was stirred at room temperature for 19 h and the supernatant was then evaporated. The product was purified by silica gel chromatography (petroleum ether–ethyl acetate, 95:5 *v*/*v*) to yield I (6.85 g, 42%) as a white solid; IR (cm^{-1}) 2927, 2851, 1637, 1463, 1380, 1279, 1015, 990, 903, 838, 802, 726; ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 5.81 (dddd, 1 H, J=16.8, 10.2, 6.6, 6.6 Hz), 5.42–5.40 (m, 1 H), 5.04–4.90 (m, 2 H), 4.39 (m, 1 H), 4.17 (m, 2 H), 2.51–2.47 (m, 2 H), 2.12–1.04 (m, 50 H), 1.02 (s, 3 H), 0.91 (d, 3 H, J=6.6 Hz), 0.86 (d, 6 H, J=6.6 Hz), 0.67 (s, 3 H); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ 138.9 (CH), 138.8 (J=31 Hz, C), 123.5 (CH), 114.1 (CH_2), 81.0 (J=31 Hz, CH), 69.5 (J=28 Hz, CH_2), 56.6 (CH), 56.1 (CH), 49.9 (CH), 42.2 (C), 39.6 (CH_2), 39.4 (CH_2), 36.8 (CH_2), 36.3 (CH_2), 36.1 (C), 35.7 (CH), 33.7 (CH_2), 31.8 (CH_2), 31.7 (CH), 29.8 (CH_2), 29.6 (CH_2), 29.2 (CH_2), 28.9 (CH_2), 28.8 (CH_2), 28.1 (CH_2), 27.9 (CH), 25.2 (CH_2), 24.2 (CH_2), 23.8 (CH_2), 22.7 (CH_3), 22.5 (CH_3), 21.0 (CH_2), 19.1 (CH_3), 18.6 (CH_3), 11.8 (CH_3); ^{31}P NMR (162.1 MHz, $CDCl_3$) δ 3.8, 3.7; ESI MS *m*/*z* $[M + NH_4]^+$ 640.6, 642.5.

Synthesis of cholesteryl 10-(triethoxysilyl)decyl chlorophosphate (II)

To a two-necked round-bottomed flask were added, in the following order, compound (I) (6.68 g, 10.71 mmol), triethoxysilane (20 mL, 106.42 mmol) and Karstedt's catalyst (0.17 mL, 1.7×10^{-3} Pt per Si). The reaction mixture was stirred under argon for 19 h at room temperature. Excess triethoxysilane was removed by evaporation under reduced pressure and the crude product was dissolved in dry dichloromethane and passed through a short column of dry sodium sulfate to remove the catalyst (eluent: dichloromethane) to give II (8.55 g, 99%) as a yellow liquid; IR (cm^{-1}) 2927, 2851, 1637, 1463, 1380, 1279, 1163, 1098, 1076, 1015, 990, 957, 802, 726; ^{1}H NMR (300.13 MHz, $CDCl_3$) δ 5.42–5.40 (m, 1 H), 4.39 (m, 1 H), 4.17 (m, 2 H), 3.81 (q, 6 H, J=6.9 Hz), 2.51–2.47 (m, 2 H), 2.12–1.04 (m, 51 H), 1.01 (s, 3 H), 0.91 (d, 3 H, J=6.6 Hz), 0.86 (d, 6 H, J=6.6 Hz), 0.67 (s, 3 H), 0.62 (t, 2 H, J=8.0 Hz); ^{13}C NMR (75.47 MHz, $CDCl_3$) δ 138.6 (J=26 Hz, C), 123.3 (J=13 Hz, CH), 80.8 (J=28 Hz, CH), 69.3 (J=28 Hz, CH_2), 58.0 (CH_2), 56.4 (CH), 55.9 (CH), 49.7 (CH), 42.1 (C), 39.5 (CH_2), 39.3 (CH_2), 36.6 (CH_2), 36.1 (CH_2), 36.0 (C), 35.6 (CH), 32.9 (CH_2), 31.7 (CH_2), 31.6 (CH), 29.3 (CH_2), 29.2 (CH_2), 29.0 (CH_2), 28.8 (CH_2), 28.0 (CH_2), 27.8 (CH), 25.1 (CH_2), 24.0 (CH_2), 23.6 (CH_2), 22.6 (CH_3), 22.5 (CH_2), 22.3 (CH_3), 20.8 (CH_2), 19.0 (CH_3), 18.5 (CH_3), 18.1 (CH_3), 11.6 (CH_3), 10.2 (CH_2); ^{31}P NMR (162.1 MHz, $CDCl_3$) δ 3.8, 3.7; ESI MS *m*/*z* $[M+H]^+$ 787.5, $[M+NH_4]^+$ 804.6, $[M+Na]^+$ 809.6.

Synthesis of the PCP (III)

Silica (5.30 g) was placed in a two-necked round-bottomed flask equipped with a condenser, an addition funnel with equalizing tube, a heating mantle, and a magnetic stirrer. Dioxane (114 mL) was then added, followed by a solution

of HCl (2.3 mol L^{-1}, 8 mL). The mixture was heated to about 70–80°C for 1 h, and then a solution of (II) (8.35 g, 10.60 mmol) in 43 mL dioxane was added dropwise, over a period of 30 min. The mixture was then gently heated under reflux for 2 h, after which the product was washed consecutively with 50 mL portions of methanol, methanol–water (1:1 *v*/*v*), and water (twice with each solvent). The final product was dried in an oven at 50°C for 24 h. IR (cm^{-1}) 3423, 2930, 2860, 1466, 1383; ^{1}H–^{13}C CP MAS NMR (75.47 MHz) δ 140.0, 121.8, 77.1, 67.1, 56.9, 49.8, 42.4, 39.6, 36.4, 30.2, 22.7, 12.0; ^{1}H–^{31}P CP MAS NMR (162.1 MHz) δ 2.6; ^{29}Si CP MAS NMR (79.55 MHz) δ −57.1, −67.2, −92.1, −102.0, −112.1.

Results and discussion

Synthesis of the phospho–cholesteric phase (PCP)

PCP was designed as an ordered liquid capable of reproducing ordered structures intrinsic to membrane bilayers which are important in drug–membrane interactions [17, 18]. Initial attempts to produce the expected material using the known *o*-chlorophenyl dichloro phosphate approach [3] were totally unsuccessful due to the departure of the cholesteric moiety instead of the *o*-chlorophenyl one. Therefore, we developed a new strategy involving the one-pot selective alcoholysis of phosphorus oxychloride (Fig. 1). Indeed, we were pleased to find that the reaction of phosphorus oxychloride with one equivalent of cholesterol in the presence of triethylamine followed by one equivalent of 9-decen-1-ol gave a 42% yield of compound (I). This first sequence step was monitored by ^{31}P NMR with the aim of optimizing both the time of the reaction and chronology of introduction of the two different alcohols. Thus, cholesterol has to be added first instead of 9-decen-1-ol in order to prevent competitive formation of a symmetric disubstituted phosphorus compound. Actually, we noticed that changing the chronology of addition has a dramatic effect on side-products formation and consequently on yields. As depicted in Fig. 2, total conversion of phosphorus oxychloride, characterized by the NMR peak at 4.1 ppm (Fig. 2a), was effective after only one hour. The cholesterol-monosubstituted phosphorus compound, characterized by the NMR peak at 6.0 ppm (Fig. 2b), was then reacted with 9-decen-1-ol (Fig. 2c) and, after reaction for 19 h, the two diastereomers of the desired product were formed in a 1:1 ratio (Fig. 2d). These two diastereomers are characterized by two NMR peaks in the crude material, at 4.2 and 4.3 ppm respectively. The next step was not trivial, and after optimization we found that only Karstedt's catalyst [19] enabled efficient quantitative hydrosilylation of the terminal double bond to give II. Indeed hydrosilylation of compound containing a phosphate group with classical hexachloroplatinate acid catalyst is not reported in the literature and also failed in our case, regardless of the synthetic procedure used. Finally, the PCP was obtained by the silanization between silica and ligands containing a triethoxysilane moiety according to the procedure described by Chu et al. [6]. Using a trifunctional silane in the presence of water, silanol groups and the water adsorbed can both react with the reagent, causing a cross linking reaction leading to a polymeric stationary support. It has been demonstrated that these types of support can induce problems such as low efficiency and insufficient batch-to-batch reproducibility. Despite these difficulties, polylayer bondings possess interesting chromatographic properties, such as better selectivity than monolayer bonded silicas for the separation of geometrical isomers of rigid compounds [20]. In this case, during the chromatographic process, the analyte binding occurs through two mechanisms: "penetration" of the solute between the grafted moieties and total "solubilization" of the solute in the stationary phase.

FTIR investigations

Examination of the absorption spectrum (Fig. 3) of both native and modified silicas shows the successful completion of the bonding reaction, by monitoring the reduction of the intensity of the band at about 3400 cm^{-1}, corresponding to residual silanols on the modified silica, relative to bare silica. The two regions of the spectrum that are relatively unobscured and correspond to areas of interest for normal alkanes are in the 3100–2700 cm^{-1} and 1600–1320 cm^{-1} ranges. The two peaks at 2930 cm^{-1} and 2860 cm^{-1} in the bonded silica spectrum are due to the carbon–hydrogen stretching frequency for methyl and methylene groups, while the two peaks at 1465 cm^{-1} and 1383 cm^{-1} are attributed to carbon–hydrogen bending, scissoring, and wagging.

^{1}H-^{29}Si CP MAS NMR investigations

^{1}H–^{29}Si CP MAS NMR spectroscopy was used to investigate the different types of surface species formed on the silica surface after bonding of the moieties. Indeed, by use of the cross-polarization technique, in which magnetization is transferred from protons to close silicon atoms, and it is possible to examine bonded phase features within few Angstroms from the surface of the chromatographic sorbents [21]. The various surface species formed on silica after modification with a trifunctional silane reagent are shown in Fig. 4 labeled in the conventional manner [21]. The structures of the native silica gel groups are also included. The differences among structures are the number and types of siloxane bonds and the number of residual

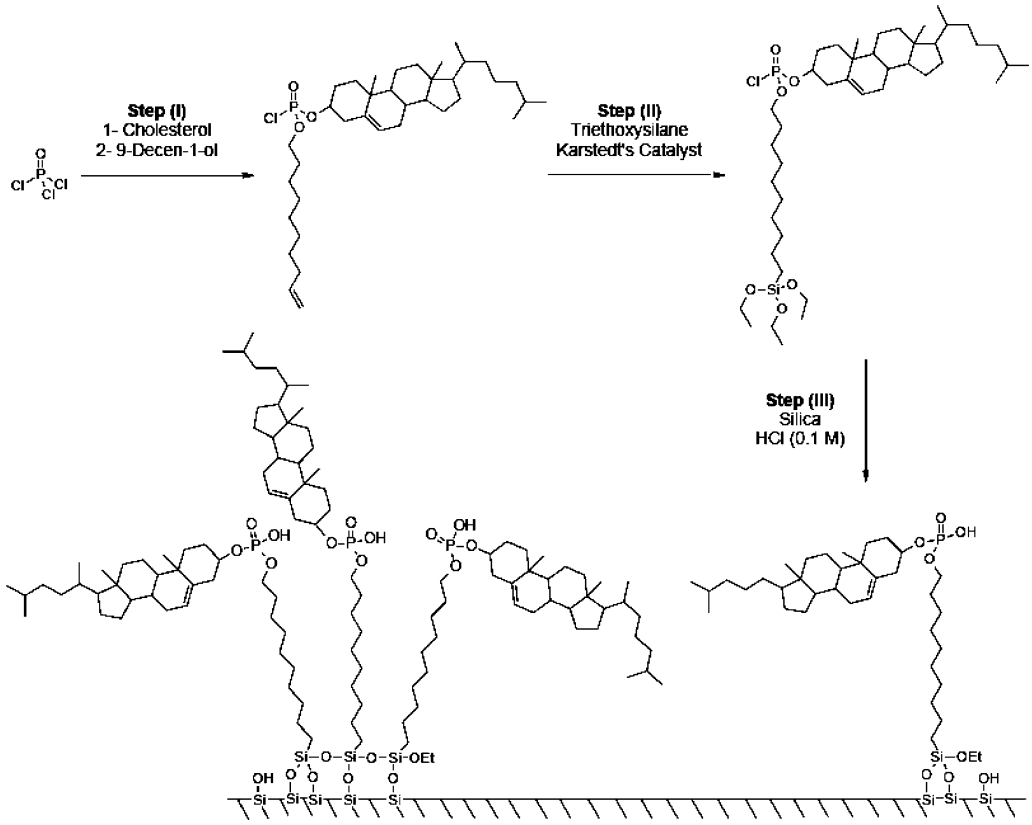

Fig. 1 Synthetic pathway of the PCP

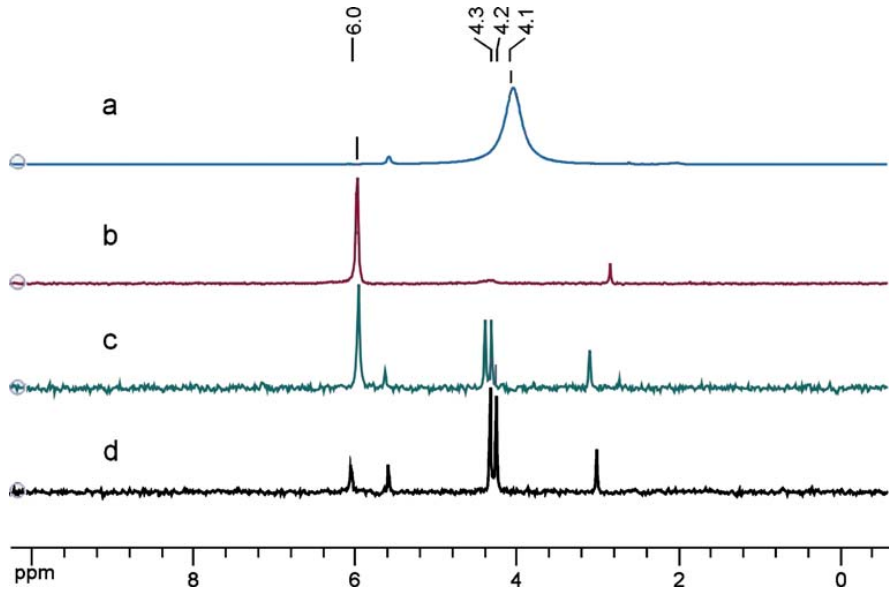

Fig. 2 Reaction progress monitored by ^{31}P NMR: (**a**) mono-substitution of $POCl_3$ by cholesterol (after addition); (**b**) mono-substitution of $POCl_3$ by cholesterol (after 1 h); (**c**) formation of compound I (after addition of 9-decen-1-ol); (**d**) compound I (after 19 h)

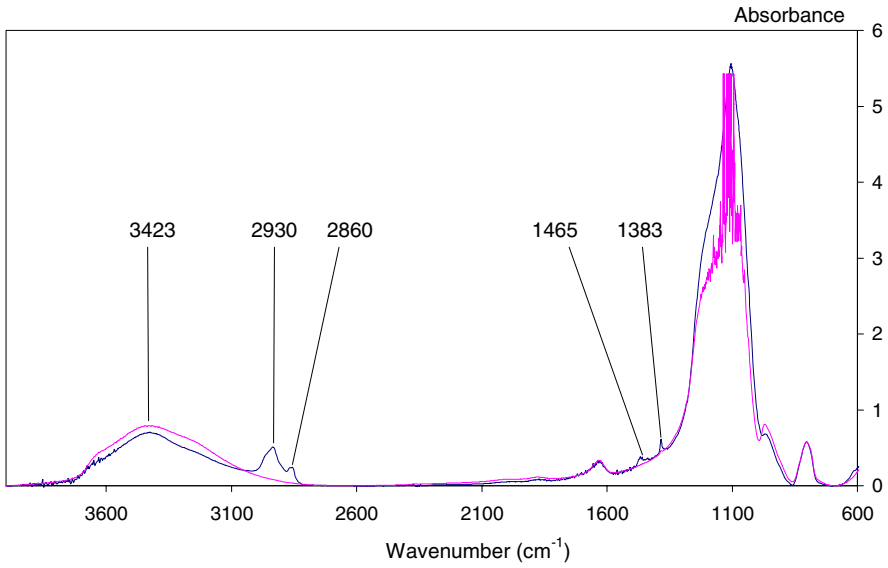

Fig. 3 FTIR of PCP and of LiChrospher® Si 100. The band close to 3400 cm^{-1} is due to residual silanols, the bands close to 2900 cm^{-1} are due to C–H stretching of methyl and methylene groups, and bands close to 1400 cm^{-1} are due C–H bending, scissoring, and wagging

ethoxy or uncondensed hydroxyl groups per attached silane moiety.

Figure 5 illustrates the ^{1}H–^{29}Si CP MAS NMR spectra of the native silica and of the PCP produced after the reaction of silanol groups on the native silica with the ethoxy groups of the phospho–cholesteric alkoxysilane moiety. As expected, peaks representing siloxane groups Q^4, single silanol groups Q^3, and geminal silanol groups Q^2 from the native silica can be seen in both spectra at −112.1, −102.0, and −92.1 ppm, respectively. The peaks at −67.2 and −57.1 ppm that appeared in the spectrum of the bonded phase can be assigned to T^3 and T^2 bonding types. This indicates polymeric bonding of the trifunctional silane to the surface of native silica. The degree of cross-linking is calculated from the integrals of the T^n signals with the equation: T (%) = $1/3T^1$ (%) + $2/3T^2$ (%) + T^3 (%) [21]. This is believed to be an important factor in stationary phase stability, since ligand hydrolysis is hindered by the formation of multiple bonds. Here the T value is about 90%.

The presence of two peaks representing surface modified species T^3 and T^2, and the decrease of those representing species Q^3 and Q^2 confirmed that the chemical immobilization [22] of phospho–cholesteric moieties on native silica has occurred by means of the silanization reaction.

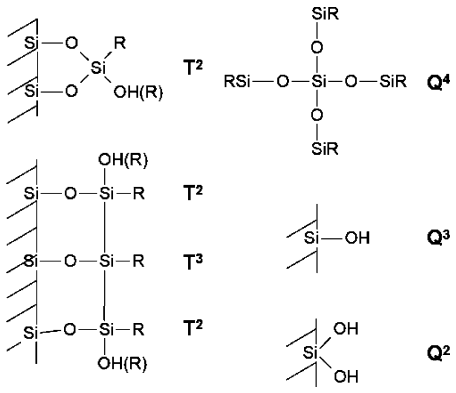

Fig. 4 Structures of silica surface species labeled by the conventional scheme [21]

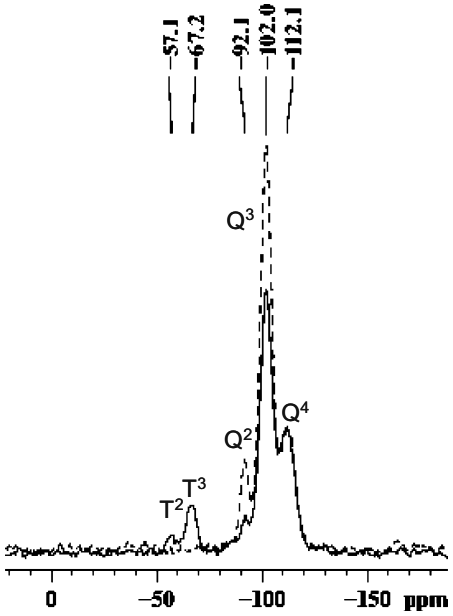

Fig. 5 ^{1}H–^{29}Si CP MAS NMR of PCP (*continuous line*) and native silica (*dashed line*). The appearance of peaks T^2 and T^3 and the decrease of peaks Q^2 and Q^3 confirmed the polymeric bonding reaction of (II) (Fig. 1)

^{1}H–^{13}C CP MAS NMR investigations

Additional information about the phospho–cholesteric moiety chemically bonded to the surface of native silica is obtained from the ^{1}H–^{13}C CP MAS NMR spectrum that is shown in Fig. 6. The spectrum exhibits peaks at 140.0 and 121.8 ppm that can be attributed to the two allylic carbons of the cholesterol, and peaks at 77.1 and 67.1 ppm corresponding to the carbons of the cholesterol and of the alkyl chain directly linked to the phosphate head, respectively. The peaks in the 12–57 ppm range are due to the carbons of cholesterol except for the peak at 30.2 ppm that is globally assigned to methylene units of the alkyl chain and the peak at 12.0 ppm that characterizes the carbon of the C–Si bond. The peaks at 22.7 and also 12.0 ppm are due to the terminal methyl groups of the cholesterol. As expected, these results indicate that the moieties chemically bonded to the surface of the chromatographic sorbent correspond to phospho–cholesteric molecules. However a ^{1}H–^{31}P CP MAS NMR study is needed in order to establish the nature of the phosphate head.

^{1}H–^{31}P CP MAS NMR investigations

The ^{1}H–^{31}P CP MAS NMR spectrum of PCP shown in Fig. 7, presents two peaks. The peak at −2.6 ppm is due to the expected bonded phosphonic acid moiety. It confirms

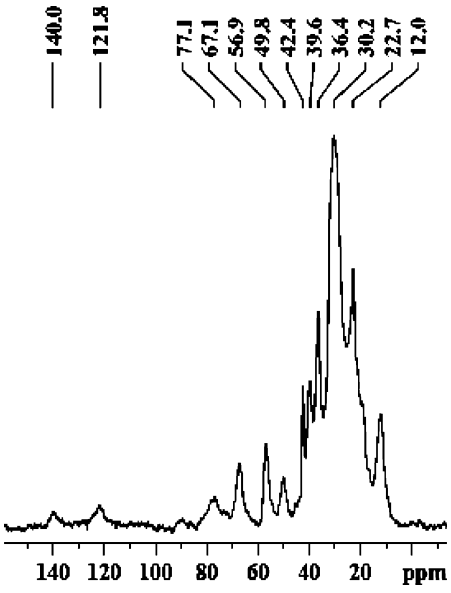

Fig. 6 ^{1}H–^{13}C CP MAS NMR of PCP. Peaks at 140.0 and 121.8 ppm correspond to the olefin, peaks at 77.1 and 67.1 ppm correspond to the carbons directly bonded to the phosphonic acid group, peaks in the 56.9–12 ppm range correspond to the cholesteryl and alkyl moieties, and the peak at 12 ppm corresponds to the carbon directly bonded to the silicone (Fig. 1)

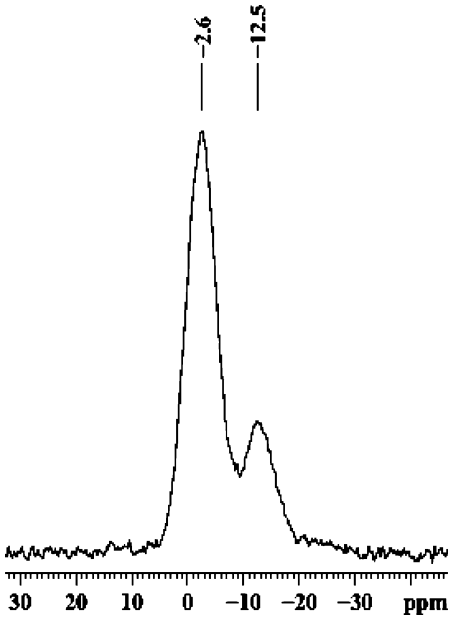

Fig. 7 ^{1}H–^{31}P CP MAS NMR of PCP. The peak at −2.6 ppm corresponds to the phosphonic acid group of (III) and the peak at −12.5 ppm corresponds to the dimer of the grafted moiety (Fig. 1)

the hydrolysis of the chlorophosphonate head of the phospho–cholesteric alkoxysilane moiety during the silanization reaction. Indeed the ^{31}P NMR spectrum of the moiety, with the chloro phosphate head, before bonding to the silica surface, shows two peaks at 3.8 and 3.7 ppm that can be assigned to the two diastereoisomers.

The peaks at −12.5 ppm can be attributed to the dimer of the phosphate head $(RO)_2P(O)OP(O)(RO)_2$ that was also produced during the hydrolysis of the moiety at a rate less than 20%.

Hydrolysis was also confirmed by synthesis of the free phosphonic acid, cholesteryl 9-decenyl phosphonic acid, that was characterized by ^{1}H, ^{13}C, DEPT 135, and ^{31}P NMR spectroscopy and mass spectrometry. The ^{31}P NMR spectrum exhibits a peak at −2.6 ppm and the molecular weight corresponds to the expected compound. Furthermore another compound has been synthesized during the reaction that was identified as the dimer after the same characterization.

HPLC investigations

Chromatographic test procedures remain the most effective way to analyze RPLC columns. Subtle yet decisive differences in the chromatographic properties of RPLC columns can only be detected using these methods. What may seem to be a minor difference in the properties of two different columns can mean success or failure in a separation

method. Many procedures have been developed in order to classify columns according to their separation mechanism [23]. Most chromatographic test procedures focus on one particular property of the column. Properties such as hydrophobicity, silanol activity, shape selectivity, and metal impurity level can all be characterized using this type of method. In accordance with a previous work conducted to evaluate CPs properties [24], we will focus solely on hydrophobicity and shape selectivity.

Among solutes commonly used to characterize the hydrophobic property of columns, alkylbenzenes are the most employed compounds. Tests of hydrophobicity of Tanaka and Engelhardt (Th and Eh) [25, 26] measure methylene selectivity, that is the ratio between the retention factors of two consecutive homologous alkylbenzenes. Methylene selectivity could be also defined by the slope of a line fit to a plot of log k vs. homolog number (methylene selectivity = e^{slope}) [27]. Alternatively, the test of Galushko [28] measures the average of retention factors of toluene and benzene.

A homologous series of alkylbenzenes from benzene to amylbenzene was injected at controlled temperatures. All methods provided similar results (Table 1). Methylene selectivity and retention factors (Fig. 8) were observed to increase with decreasing temperature and with decreasing mobile phase strength as described in the literature [27] for alkyl bonded stationary phases. Methylene selectivity was found to vary only over a narrow range for the various conditions and was lower than for conventional alkyl bonded stationary phases independently their chain length and their bonding density [27, 29]. These results led us to assume that PCP may have a low bonding density due to the high ratio between the size of (II) and the pore diameter of silica, which implies a large number of unreacted silanols during the bonding procedure. This factor is all the more relevant since, during the polymeric bonding procedure, moieties polymerize in a first fast step and then slowly react with surface silanols. This hypothesis was confirmed by a bonding density of 1.1 μmol m^{-2} calculated from a measured carbon content of 15.4%. At any rate, as Sentell and Dorsey [30] have concluded for alkyl bonded phases, methylene selectivity is not influenced by bonding density, and the degree of ordering incurred with high bonding density phases does not affect the "non specific hydrophobic interactions" that cause methylene selectivity.

The chromatographic behavior of the PCP was also evaluated by the shape selectivity according to the test of Tanaka/Engelhardt (E/Ts) [25, 31] and Sander and Wise (S&W) [32]. The test of E/Ts measures the selectivity between the couple triphenylene and *o*-terphenyl whereas the test of S&W evaluates the retention order of the three compounds benzo[*a*]pyrene (BaP), dibenzo[*g*,*p*]chrysene (TBN), and phenanthro[3,4-*c*]phenanthrene (PhPh). Preliminary results obtained from the test of E/Ts have emphasized the great shape selectivity of the PCP (Fig. 9). Indeed the shape selectivity estimated according to test of E/Ts [29, 31] has been related to the classification of Sander and Wise [32]: monomeric-like alkyl phases are characterized by values below 2, intermediate-like ones by values between 2 and 3.4, and polymeric-like ones by values greater than 3.4 up to about 4. Results described in our previous paper with CPs are between 3.5 and 4.5 [24]. Here, the shape selectivity of the PCP is always higher than 5 independently of the temperature and of the mobile phase in the studied ranges. This means that the shape selectivity of the PCP is 1.5 times higher than obtained with CPs, which are known for their high shape selectivity, and about twice as high as the values obtained with selective conventional ODS phases. This observation is all the more impressive since, contrary to our case, it has been suggested that "the combination of high group density and wide pore diameter seems to be essential for stationary phase prepared for shape recognition" [29, 33]. Furthermore, the shape selectivity of the PCP decreases when the temperature increases, consistent with the reversed-phase liquid chromatographic process (Fig. 9). On the other hand, the test of S&W shows the following elution order of solutes: BaP < PhPh < TBN, which indicates that the column has monomeric-like properties [32]. The shape selectivity decreases with increasing size of the solutes, as was previously observed on CPs, but in a way less significant [24], leading to a phase truly intermediate between monomeric ODS phases and the unique liquid crystal phase. Catabay et al. [34] attribute this unique property to the basic structure of the cholesteric bonded phase, one side of which is relatively planar whereas the other is not. Coupled with the

Table 1 Summary of methylene selectivity and hydrophobicity of PCP

Temperature (°C)			30	40	40	50
Mobile phase: MeOH–H_2O (*v*/*v*)			80:20	80 /20	70:30	70:30
Tests	Tanaka	$k_{amylbenzene}/k_{butylbenzene}$	1.33	1.31	1.44	1.40
	Engelhardt	$k_{ethylbenzene}/k_{toluene}$	1.21	1.21	1.35	1.28
	–	$k_{toluene}/k_{benzene}$	1.35	1.33	1.47	1.38
	e^{slope}	–	1.12	1.11	1.16	1.14
	Galusko	$(k_{toluene} + k_{benzene})/2$	0.31	0.29	0.48	0.46

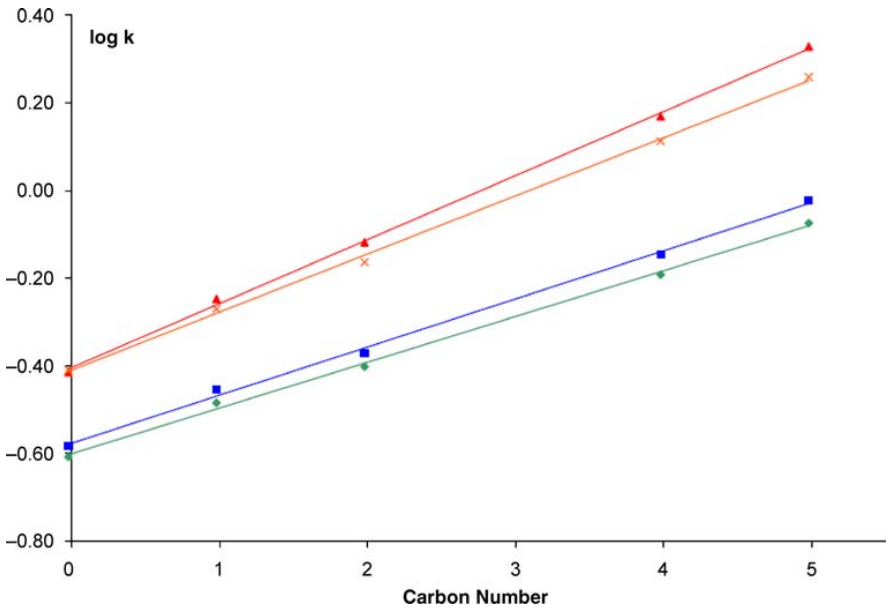

Fig. 8 Effect of temperature and mobile phase composition on the retention factor of alkylbenzenes in the PCP column: *diamonds*, T=40°C, MeOH–H_2O 80:20 (*v*/*v*); *squares*, T=30°C, MeOH–H_2O 80:20 (*v*/*v*); *crosses*, T=50°C, MeOH–H_2O 70:30 (*v*/*v*); *triangles*, T=40°C, MeOH–H_2O 70:30 (*v*/*v*)

flexible movement of long alkyl chains between the cholesteryl moiety and silica surface, the ability to retain planar molecules which are larger than four rings is seriously reduced. The mechanism of separation of PCP seems to be mainly governed by the "penetration" and the total "solubilization" of solutes but also by their size and shape as was previously demonstrated with CPs [24]. However, the phenomena observed are more impressive on PCP than on CPs.

The preliminary results obtained in this work with PCP show high shape selectivity for small analytes and a low surface coverage. This is at variance with a study of shape selectivity of conventional ODS phases where the authors assumed that the higher the density of bonded functional groups, the better the differentiation between planar and twisted polyaromatic hydrocarbons [31]. Further research is thus required to comprehend the molecular processes involved in chromatographic separations in PCP.

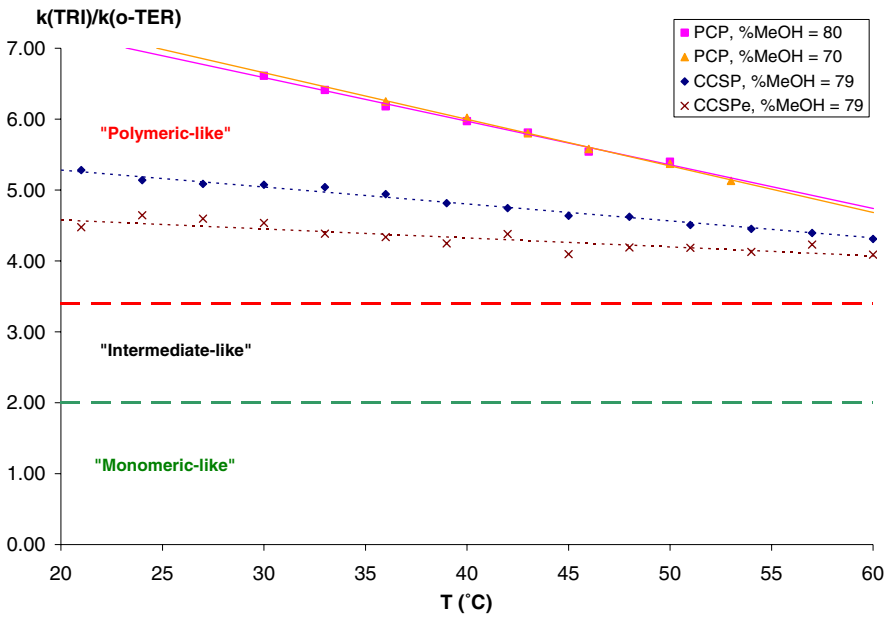

Fig. 9 Effect of temperature on shape selectivity in the PCP column (*continuous line*) and cholesteric carbamate stationary phase (CCSP) and CCSP end-capped columns (*dashed lines*) according to the Tanaka/Engelhardt's test with a methanol and water mobile phase

Conclusion

The novel phospho–cholesteric phase (PCP) synthesized in this work is a unique separation material whose analytical capabilities are just beginning to be explored. It presents properties and a range of separation features that are not possessed by any currently available stationary phases. PCP exhibits high resolving power for probe solutes that might be ascribed to liquid crystal properties of cholesterol in the native state. Even though the bonding density estimated by hydrophobicity tests appears to be low, this first stage of characterization of the material shows an excellent potential of PCP to separate geometrical isomers. In addition, the durability of PCP is excellent, as evidenced by nearly identical chromatograms obtained on two columns after more than one year of use.

Notwithstanding, much remains to be investigated about the properties of PCP in order to properly assess its role as a tool to reproduce as accurately as possible the organization between the molecules of phospholipid and cholesterol in cellular membranes and to better define the complete separation capabilities.

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References

1. Pidgeon C, Venkataram UV (1989) Anal Biochem 176:36–47
2. Ong S, Cai SJ, Bernal C, Rhee D, Qiu X, Pidgeon C (1994) Anal Chem 66:782–792
3. Qiu X, Ong S, Bernal C, Rhee D, Pidgeon C (1994) J Org Chem 59:537–543
4. Yang CY, Cai SJ, Liu H, Pidgeon C (1996) Adv Drug Del Rev 23:229–256
5. Pesek JJ, Matyska MT, Williamsen EJ, Tam R (1995) Chromatographia 41:301–310
6. Chu CH, Jonsson E, Auvinen M, Pesek JJ, Sandoval JE (1993) Anal Chem 65:808–816
7. Delaurent C, Tomao V, Siouffi AM (1997) Chromatographia 45: 355–363
8. Buszewski B, Jezierska M, Ostrowska-Gumkowska B (2001) Mater Chem Phys 72:30–41
9. Buszewski B, Jezierska-Switala M, Kaliszan R, Wojtczak A, Albert K, Bachmann S, Matyska MT, Pesek JJ (2001) Chromatographia 53:S204–S212
10. Buszewski B, Jezierska M, Welniak M, Kaliszan RJ (1999) J Chromatogr A 845:433–445
11. Al-Haj MA, Haber P, Kaliszan R, Buszewski B, Jezierska M, Chilmonzyk ZJ (1998) Pharm Biomed Anal 18:721–728
12. Catabay AP, Pesek JJ, Matyska MT, Jinno KJ (1999) J Liq Chromatogr Related Technol 22:953–967
13. Pesek JJ, Matyska MT, Brent Dawson G, Wilsdorf A, Marc P, Padki MJ (2003) J Chromatogr A 986:253–262
14. Liu H, Ong S, Glunz L, Pidgeon C (1995) Anal Chem 67:3550–3557
15. McMullen TPW, Lewis RNAH, McElhaney RN (2004) Curr Opin Colloid Interface Sci 8:459–468
16. Peersen OB, Wu X, Kustanovich I, Smith SOJ (1993) Magn Reson 104:334–339
17. Betageri GV, Rogers JA (1989) Pharm Res 6:399–403
18. Choi YW, Rogers JA (1990) Pharm Res 7:508–512
19. Beyou E, Humbert J, Chaumont P (2003) e-Polymers 20:1–9
20. Stella C, Rudaz S, Veuthey J-L, Tchapla A (2001) Chromatographia 53:S113–S131
21. Pursch M, Brindle R, Ellwanger A, Sander LC, Bell CM, Händel H, Albert K (1997) Solid State NMR 9:191–201
22. Sindorf DW, Maciel GE (1982) J Phys Chem 86:5208–5219
23. Stella C, Rudaz S, Veuthey J-L, Tchapla A (2001) Chromatographia 53:S132–S140
24. Courtois C, Pagès G, Caldarelli S, Delaurent C (2008) Anal Bioanal Chem; on line doi:10.1007/s00216–008–2276–5
25. Kimata K, Iwaguchi K, Onishi S, Jinno K, Eksteen R, Hosoya K, Araki M, Tanaka N (1989) J Chromatogr Sci 27:721–728
26. Engelhardt H, Jungheim M (1990) Chromatographia 29:59–68
27. Rimmer CA, Sander LC, Wise SA, Dorsey JG (2003) J Chromatogr A 1007:11–20
28. Galushko SV (1993) Chromatographia 36:39–42
29. Claessens HA, van Straten MA, Cramers CA, Jezierska M, Buszewski B (1999) J Chromatogr A 826:135–156
30. Sentell KB, Dorsey JG (1989) J Chromatogr 461:193–207
31. Engelhardt H, Nikolov M, Arangio M, Scherer M (1998) Chromatographia 48:183–189
32. Sander LC, Wise SA (1984) Anal Chem 56:504–510
33. Sander LC, Wise SA (1984) J Chromatogr 316:163–181
34. Catabay AP, Saito Y, Okumura C, Pesek JJ, Williamsen E (1997) J Microcolumn Sep 9:81–85