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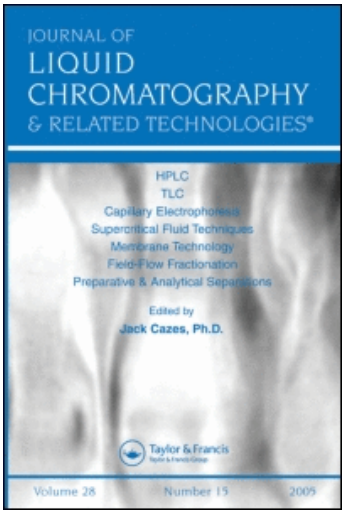

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A NOVEL PACKING MATERIAL FOR RP-HPLC

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A NOVEL PACKING MATERIAL FOR RP-HPLC

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ABSTRACT

A simple, practical, and economical procedure had been developed to prepare novel C18 ether-bond reversed phase packing. A soft and long C_{18} alkyl chain was coupled onto silica (Sinopak-S, particle size d = 5μm, pore diameter Dp=11nm, and surface area s=170m^2/g) with γ-glycidoxypropyltrimethoxysilane as a coupling agent. Characterization of prepared packing was carried out with elemental analysis and solid-state ^{13}C NMR. Chromatographic evaluation indicates that its reversed phase behavior was similar to ordinary ODS.

INTRODUCTION

Reversed-phase packings have found widespread application in the fields of biology, chemistry, drug industries, and so on. According to references,[1,2] RPLC is still the workhorse of the chromatographic methods. Today more than 600 RP columns are commercially available worldwide; a number of new and better RP columns are pushed onto market.[3] In addition to the stationary phase selection between those of alkyl chain lengths from C_1 to C_{18}, there are many kinds of C_{18} phases available that can provide different selectivities. It is common to carry mobile phase optimization to optimize separation in RP-HPLC with one of these columns. In practice, however, the application of different types of columns is sometimes much more effective in the development of a separation for closely related compounds with simple mobile phase.[4,5]

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A variety of stationary phases were bonded to siliceous supports through intermediate silane coupling agents containing active functional groups (i.e., amino-, epoxy-) for further modification. γ-Glycidoxypro-pylrimethoxysilane is a widely used important intermediate silane coupling agent. Chang et. al.[6] described the use of oxiranes in the preparation of bonded phase supports. So far a number of packings prepared through intermediate silane containing epoxy groups are used as HIC packings,[7-10] SEC packings,[8,11] HPLAC packings,[12-15] ion exchanger packings,[6] and so on . The epoxy group on the KH560 is highly active,[16] which might result in some by-products when media for reaction (i.e. water, toluene, xylene) and some conditions (pH, concentration of solvent) could not be considered properly. Porsch investigated the optimization of surface bonding reaction on epoxy- and diol-modified silica in detail.[16] He confirmed that the bonding reaction in toluene might result in an "ether" bonded phase which could not be modified further due to the disappearance of the epoxy group. According to Engelhardt et al., however, toluene is the best medium for silanization of trialkoxy-n-alkylsilane on silica.[17]

This paper describes a novel procedure for the preparation of C_{18} ether-bonded RPLC packing.[18] Preliminary chromatographic evolution was also carried out.

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EXPERIMENTAL

Materials

Spherical porous silica (Sinopak-S, mean particle diameter d=5μm, pore diameter Dp=11nm, surface area S=170m^2/g) was home-made. The γ-glycidoxypropyltrimethoxysilane was purchased from Yingkou Chemical Engineering Corp. (China). $BF_3.Et_2O$ (analytical-reagent grade, Beijing Chemicals Factory), n-$C_{18}H_{37}OH$ (n-Octadecyl Alcohol, imported reagents by Shanghai Chemicals Corp.(Shanghai, China)), Acetonitrile (HPLC grade, Fisher Chemicals), TFA (Protein sequencing grade, SIGMA). Methanol (Excellent Reagents, Beijing Chemicals Factory). Other reagents utilized are all analytical-reagent grade. Peptides used were synthesized by FMOC-strategy with sequences of YSSKQA, YRSKQA, and FLAG.

Apparatus

The HPLC analyses were carried out on a system comprising a Beckmann Liquid-delivery system, a KARATOS Spectroflow 783 Programmable Absorbance Detector, and a chart recorder (Hitach Co., Ltd). A pneumatic amplification pump (Chemco Packer, Japan) was used for packing of the column.

Synthetic Procedures

Preparation of C_{18} Ether-Bonded Silanization Reagents

n-$C_{18}H_{37}OH$ (6.5g) was dissolved in a 250 mL three-neck round-bottom flask with 50 mL of toluene, then 0.5 mL of fresh distilled $BF_3.Et_2O$ was added while stirring. After 5 minutes, the flask was placed in an oil bath at 90°C and then 5 mL of γ-glycidoxypropyltrimethoxysilane was added while stirring. The temperature of the oil bath was maintained at 90°C for 8h to ensure complete reaction of the ring-opening of the epoxide.[16] The target product was purified and stored for use at any time.

Preparation of C_{18}-Ether-Bond Reversed-Phase Packing

Dried silica gel (5.0g) was placed in a 250 mL three-neck round-bottom flask, and heated up to 150°C under vacuum for 4h. Then, ether-bonded silanization reagent was drawn into the flask by the vacuum within. The slurry was agitated at a refluxing temperature in an oil bath for 6-8h. The packing, prepared with the procedure, was filtered and washed with toluene and acetone, respectively, then dried in vacuo at 60°C for 2h..

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After-Treatment of the Ether-Bonded Packing

The bonded phase was placed into a 250 mL three-neck round-bottom flask, then 100 mL of 70% ethanol aqueous solution (pH=3.0) was added. The slurry formed was heated at a refluxing temperature for 8h, then was filtered and washed with acetone and treated at 150°C under vacuum for 4h.

End-Capping with Trimethylchlorosilane

The bonded packing treated with the above procedure was end-capped with an excess of trimethylchlorosilane according to published procedures.[18]

Product Evaluation and Characterization

Elemental and Spectroscopic Analysis

Elemental analysis was performed on an ST-02 Elemental Analyser. Solid-state ^{13}C CP-MAS-NMR spectra were obtained with a Bruker Spectrometer.

Chromatographic Studies

The ether-bonded phase packings were packed into a 250×5.0 i.d. stainless steel column using a pneumatic amplification pump at 400kg/cm^2. Test solutes included benzene, naphthalene, phenanthrene, aniline, pyridine, and three peptides.

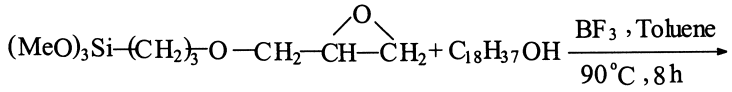
Stability Studies

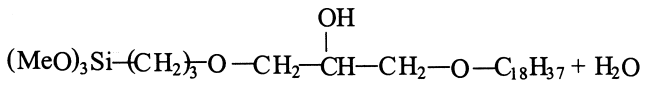
A methanolic aqueous solution (70%) was used as the mobile phase. A sample mixture (benzene, naphthelene, and phenanthrene) was injected after 1000 column volumes of mobile phase.

RESULTS AND DISCUSSION

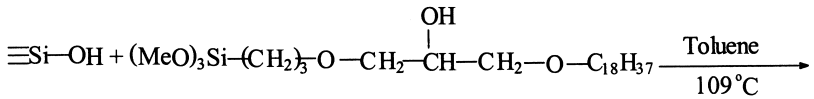
Synthetic Reaction

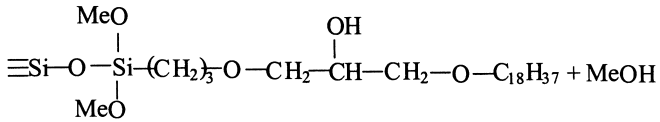
The packing was synthesized with the following reactions:





Bonding of the terminal C_{18} chain onto γ-glycidoxypropyltrimethoxysilane was through ring opening reaction then followed by silanization with silica gel as follows:





It is clear that packing prepared with the above procedure can be hydrolyzed to produce more silanol groups. Existence of silanol groups induces non-specific adsorption, as well as a general dec9+-+line in performance of the column. For this reason, the bonded phase was hydrolyzed with 70% ethanol aqueous solution (pH=3.0) to release the silanol groups, then gelated at 150°C under vacuum to eliminate silanol groups. Finally the bonded phase was capped with trimethylchlorosilane as reported in the literature.[19] Strictly speaking, the packing prepared by the procedure given above is not actually a conventional C_{18} RPLC packing in the usual sense, since the C_{18} hydrophobic chain is much similar to commercial C_{18} RPLC packings. On the other hand, this packing might have special selectivities due to its specific polar groups.

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Effect of Silanization Time on the Content of Bonded Ligands

It is reported that, if γ-glycidoxypropyltrimethoxysilane is bonded onto silica first, then followed with a ring-opening reaction under the catalysis of $BF_3.Et_2O$, it will result in the formation of diol.[16] To avoid opening of the oxirane ring during the silanizaton, a ring-opening reaction was performed first. Doing so can not only guarantee the completeness of ring-opening reaction, but also easily obtain the pure silanization reagent through purification. Ring-opening reaction under the catalysis of $BF_3.Et_2O$ is very fast;[16] Eight hours is enough for the completeness of the reaction between γ-glycidoxypropyltrimethoxysilane and n-octadecyl alcohol. As a result, the silanization was chosen to be investigated in detail. During the period 20.5h of silanization, five samples at different reaction times were taken to perform elemental analysis. According to literature,[17] toluene was chosen to be the solvent through the synthetic process. The correlation between the carbon content and silanization time is shown in Figure 1.

There is a plateau after 5h, which indicates that 5-8h is long enough for silanization.

Characterization of the Packing

The final product was synthesized within 6h with 1.8 μmol/m^2 of ligand concentration. Solid-state ^{13}C CP-MAS-NMR was used to characterize the final product. The success of the bonding reaction can be confirmed by the solid-state ^{13}C spectrum of the ether-bonded phase. The peak positions in the spectrum are similar to those obtained for bonding similar species to a silica surface.[20, 21] Owing to the absence of an absorption at 50 ppm, we can conclude that the silica derivative does not contain a methoxy group or it contains very few methoxy groups. In other words, the methoxy group was hydrolyzed com-

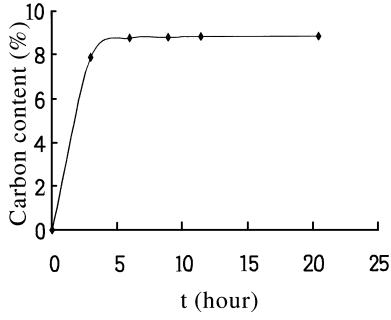

Figure 1. The curve of correlation between C% and silanization time.

pletely. Peaks in the range of 10 to 35 ppm indicate the successful bonding of target long-chain-alkyl-bonded phases . The spectrum is shown in Figure 2.

Chromatographic Evaluation

The packing, which had different silanization times, was packed into a stainless-steel column (ϕ5.0 × 250). Observation of the chromatographic behaviors of these columns indicated that the packings which had more than 5h of synthesis time exhibited similar good performance. The packing, which had 5h of silanization time was selected to be investigated in detail. Benzene, naphthalene, phananthrene, aniline, and pyridine were used as test solutes. The chromatogram for this column is shown in Figure 3.

The theoretical plate number calculated was 45,500 plates/m, the k' (aniline) and k' (pyridine) were 0.90 and 1.64, respectively; the γ (aniline) and

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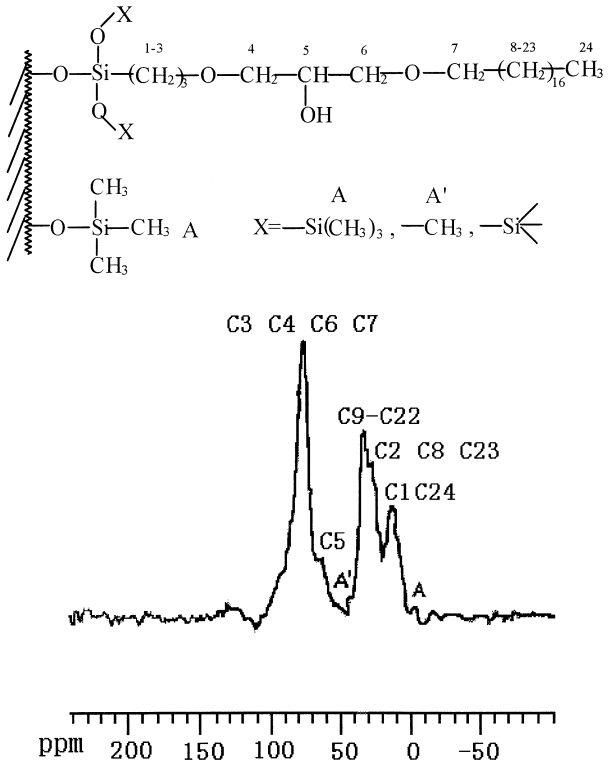

Figure 2. Solid state ^{13}C-CP-MAS-NMR spectrum of the bonded phase.

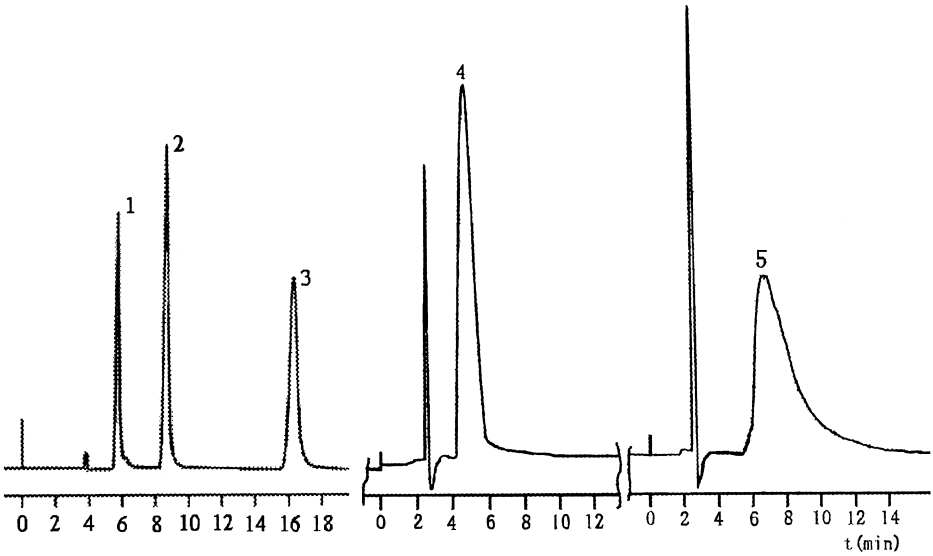

Figure 3. Chromatogram of (1) benzene, (2) naphthalene, (3) phenanthrene, (4) aniline, and (5) pyridine. Column: 5.0 x 250 mm; Detection: 254 nm; 0.1 AUFS; Eluent: 70% aqueous methanol solution; Flow rate: 1.0 mL/min.

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γ′(pyridine) were 1.05 and 4.0, respectively. The solutes above were injected after more than 1000 column volumes of mobile phase; the chromatogram was not changed too much. The performance remained unchanged. The N = 45,600 plate/m, k' (aniline) = 1.02 and k' (pyridine) = 1.89, γ'(aniline) = 1.08 γ′ (pyridine) = 4.0. The pH stability studies demonstrated that the column could be used in the range of pH 2-8 for thousands of column volumes of mobile phase without any loss in performance. Both the composition of the eluent (70%MeOH-30%H_2O) and the chromatographic behavior indicated the unique RP properties of the packing, which might be of importance for special uses.

Separation of Some Peptides

Three peptides were separated on the column. The sequence of peptides and their retention times are shown in Table 1. The chromatogram of the three peptides is shown in Figure 4**.**

Peptides 1 and 2 have sharp peaks on chromatogram, which indicates that the column can be applied to the separation of low MW biomolecules. The ion-pair formed between peptide 2 and TFA is a little bit more hydrophobic than the ion-pair formed between peptide 1 and TFA, which can also be demonstrated

Table 1

The Sequence of the Peptides

Number	Sequence	Retention Time (min)
1#	Tyr-Ser-Ser-Lys-Gln-Ala	5.0
2#	Tyr-Arg-Ser-Lys-Gln-Ala	6.72
3#	Val-Phe-Leu-Ala-Gly	23

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with the structural difference between peptides 1 and 2 (on peptide 2, the second amino acid residue is Arg, which is more hydrophobic than Ser in acidic solution). Peptide 3 is highly hydrophobic, which results in its long retention time. However, the peptides above will be well separated under optimized conditions. This indicates that the column is, indeed, an RPLC column as was predicted.

CONCLUSIONS

Bonding of $C_{18}H_{37}$-ether to the surface of silica in toluene can yield a high level of hydrophobic bonded ligand. The formation of the ether bond and the simultaneous opening of the oxirane ring are performed first, which can prevent the oxirane ring from opening at silanization. The corresponding bonded phase exhibits excellent RPLC behavior.

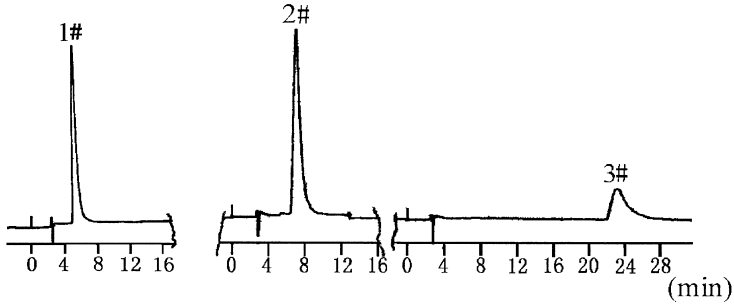

Figure 4. Chromatogram of three peptides. Column: 5.0 x 250 mm; Eluent: 8% aqueous ACN solution (0.1% TFA); Detection: 220 nm, 0.1 AUFS; Flow rate: 1.0 mL/min.

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