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## ON-COLUMN PREPARATION OF CHEMICALLY BONDED STATIONARY PHASE WITH MAXIMUM SURFACE COVERAGE AND HIGH REPRODUCIBILITY, AND ITS APPLICATION TO PACKED MICROCAPILLARY COLUMNS

NOBUO TANAKA\*, HIROSHI KINOSHITA and MIKIO ARAKI

*Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606 (Japan)*  
and

TAKAO TSUDA

*Laboratory of Analytical Chemistry, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466 (Japan)*

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### SUMMARY

A method for the on-column preparation of chemically bonded stationary phase with monofunctional reagents was studied in conventional stainless-steel columns and in glass capillary columns for packed microcapillary chromatography. Simple reactions of the silica surface with octadecyldimethyl-N,N-diethylaminosilane in packed columns resulted in a surface coverage of more than  $3.0 \mu\text{mol}/\text{m}^2$ , comparable to the stationary phase of maximum coverage prepared in batch. The extent of surface coverage and the amount of residual silanols were carefully examined by using positively charged amines and neutral compounds. Although the results indicate a slight difference between the  $\text{C}_{18}$  phases in stainless-steel columns and in packed microcapillary columns, excellent reproducibility was found for the on-column bonding reaction, as expected for the reaction using monofunctional reagents.

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### INTRODUCTION

The preparation of chemically bonded stationary phase such as  $\text{C}_{18}$  or  $\text{C}_8$  in packed columns has been attracting considerable attention. In some areas of high-performance liquid chromatography (HPLC), on-column bonding reaction is an indispensable technique.

When columns for packed microcapillary (PMC) chromatography are prepared, glass tubing packed with silica particles is drawn at high temperatures to produce narrow-bore columns of less than  $50 \mu\text{m}$  I.D. and up to 100 m long. The preparation of chemically bonded stationary phases with these columns must be achieved after the preparation of packed columns. The preparation of chemically bonded stationary phases for open-tubular microcapillary (OTC) chromatography also requires similar procedures.

Gilpin and co-workers reported the use of alkyltrichlorosilanes for on-column bonding reactions of  $C_{18}$ <sup>1</sup> and other phases<sup>2</sup> in conventional columns. Alkylsilylation in PMC and OTC columns was carried out similarly by using octadecyltrichlorosilane<sup>3,4</sup> and alkyltriethoxysilanes<sup>5-9</sup>.

In reactions with these polyfunctional reagents, special care has to be taken concerning the purity of the reagents, the elimination of water from the silica surface and the water content of the solvents, as the reagents are very sensitive to the presence of moisture and may polymerize. Although the use of monofunctional reagents with OTC columns has been reported, the extent of surface coverage was not critically examined<sup>10</sup>. The clogging of narrow-bore columns with the use of tri- and monochlorosilanes has also been reported.

In the preparation of stationary phases for reversed-phase liquid chromatography (RPLC) in batch for conventional HPLC columns, increasing numbers of stationary phases are being prepared by using monofunctional instead of polyfunctional reagents in order to obtain high reproducibility without special precautions. The use of monofunctional reagents ensures the formation of monolayers on the silica surface, which is preferable from the chromatographic point of view<sup>11</sup>.

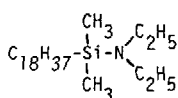
In spite of their lower reactivity than polyfunctional reagents, monofunctional reagents such as octadecyldimethylchlorosilane (ODS-Cl) show excellent reproducibility and completeness of the bonding reaction when applied with suitable amines<sup>12,13</sup>. The use of monochlorosilane, however, has been limited in the preparation of bonded phases in PMC and OTC columns, because of the lower reactivity without amines and precipitate formation with amines under the usual reaction conditions. The potential utility of PMC and OTC chromatography is well recognized<sup>14-17</sup>, and these chromatographic modes would be enhanced further by operating them under reversed-phase conditions using well characterized stationary phases that possess similar retention characteristics to those in conventional HPLC.

It is desirable to develop an on-column bonding procedure using monofunctional silanes that will allow maximum surface coverage such as is obtained in the reaction in batch without precipitate formation in the columns. In this paper we report the use of alkyldimethyl-*N,N*-dialkylaminosilanes in on-column bonding reactions and show that maximum coverages can be achieved with high reproducibility. The resulting stationary phases were equivalent to those obtained in the usual reactions in batch in terms of the surface coverage and the amount of residual silanols.

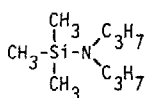
## EXPERIMENTAL

### *Materials and columns*

*Preparation of octadecyldimethyl-*N,N*-diethylaminosilane (ODS-DEA)*. Diethylamine (25 ml) was added to 300 ml of an *n*-hexane solution of 21 g of ODS-Cl. The reaction mixture was kept at 50°C for 1 h with stirring and the salt formed was



ODS-DEA



TMS-DPA

filtered off with a PTFE membrane filter. The product, ODS-DEA, was obtained directly from the reaction mixture by distillation under reduced pressure (b.p. 180°C/0.1 mmHg). Trimethyl-N,N-di-*n*-propylaminosilane (TMS-DPA) was prepared similarly from trimethylchlorosilane (TMS-Cl) and di-*n*-propylamine (b.p. 67.0°C/29 mmHg).

*Silica gel particles.* Develosil (10  $\mu\text{m}$ , 330  $\text{m}^2/\text{g}$ ) (Nomura Chemicals, Seto, Japan) was used following acid treatment<sup>18</sup>. Heat treatment of the silica gel was performed in an electric furnace at 800°C for 3 h.

*PMC columns.* Soda-lime glass tubing (0.5 mm I.D., 6 mm O.D.) was dry-packed with the silica particles, and drawn to produce PMC columns (40–50  $\mu\text{m}$  I.D., 0.5 mm O.D.) with a glass-drawing machine (GDM-1) (Shimadzu, Kyoto, Japan), as described previously<sup>8,19</sup>.

*Conventional stainless-steel columns.* Silica particles or chemically bonded C<sub>18</sub> phases were packed into stainless-steel columns (10 cm  $\times$  4.6 mm I.D.) using conventional slurry techniques.

*OTC columns.* Soda-lime glass tubing (0.4 mm I.D., 6 mm O.D.) was drawn to produce OTC columns (25  $\mu\text{m}$  I.D., 0.5 mm O.D.) in a similar manner to PMC columns. The OTC columns were treated with 0.5 *N* NaOH at 50°C for 18 h prior to the bonding reaction.

### Chemical bonding reaction

*In batch.* Octadecylsilylation of silica particles was performed as previously described<sup>12,13</sup>, using ODS-Cl and pyridine for stationary phases A–F and ODS-DEA

TABLE I  
PREPARATION METHODS OF THE VARIOUS COLUMNS

Column No.	Column type*	Stationary phase	Preparation method of bonded phase	Reagents
A-1	SS	C <sub>18</sub>	Batch	ODS-Cl
B-1	SS	C <sub>18</sub>	Batch	ODS-Cl
C-1	SS	C <sub>18</sub>	Batch	ODS-Cl
D-1	SS	C <sub>18</sub>	Batch	ODS-Cl
E-1	SS	C <sub>18</sub>	Batch	ODS-Cl
F-1	SS	C <sub>18</sub>	Batch	ODS-Cl
G-1	SS	C <sub>18</sub>	Batch	ODS-DEA
D-2	SS	C <sub>18</sub> + TMS	Batch	ODS-Cl + TMS-Cl
E-2	SS	C <sub>18</sub> + TMS	Batch	ODS-Cl + TMS-Cl
F-2	SS	C <sub>18</sub> + TMS	Batch	ODS-Cl + TMS-Cl
G-2	SS	C <sub>18</sub> + TMS	Batch	ODS-DEA + TMS-DPA
H-1	SS	C <sub>18</sub>	On-column	ODS-DEA
H-2	SS	C <sub>18</sub> + TMS	On-column	ODS-DEA + TMS-DPA
PMC-C <sub>18</sub> -1	PMC	C <sub>18</sub>	On-column	ODS-DEA
PMC-C <sub>18</sub> -2	PMC	C <sub>18</sub> + TMS	On-column	ODS-DEA + TMS-DPA
PMC-C <sub>18</sub> -3	PMC	C <sub>18</sub>	On-column	C <sub>18</sub> H <sub>37</sub> Si(OC <sub>2</sub> H <sub>5</sub> ) <sub>3</sub>

\* SS = conventional stainless-steel columns; PMC = packed microcapillary columns.

for stationary phase G (see Table I for the identity of each stationary phase). The carbon contents of stationary phases with less than the maximum coverage were controlled by the amount of ODS-Cl in the reaction mixture. Trimethylsilylation was carried out similarly using TMS-Cl and hexamethyldisilazane for stationary phases D-2-F-2 and TMS-DPA for stationary phase G-2.

*On-column.* A stainless-steel column packed with silica gel was connected to an injector equipped with a 5-ml loop. The silica gel in the column was washed with 10 ml of tetrahydrofuran (THF) at room temperature and 10–20 ml of toluene at 110°C. A 5-ml volume of 20% ODS-DEA solution in toluene was introduced into the injector loop. After pumping 2–3 ml of the reagent solution through the column, which was maintained at 105–110°C, the flow was interrupted and the silica was allowed to react with the reagent. After reaction for 1 h, the flow of toluene was resumed. The column was washed with 10 ml of THF at room temperature and 10–20 ml of toluene at 110°C. The reaction with ODS-DEA and the washing with THF and toluene were repeated again. Finally, the column was washed with methanol, then with 0.02 *M* phosphate buffer (pH 3) in 50% methanol prior to use in RPLC. On-column trimethylsilylation was performed similarly using 5 ml of 35% TMS-DPA in toluene.

The on-column bonding reaction of PMC columns was carried out under a flow of a silylating reagent. A PMC column (3–4 m × 44 μm I.D.) was washed with THF for 30 min and with toluene for 1 h with a column inlet pressure of 200 kg/cm<sup>2</sup>. The reagent solution, 0.5 ml of 20% ODS-DEA in toluene, was passed through the column with a column inlet pressure of 150 kg/cm<sup>2</sup> at 110°C. After reaction for 1 h, the PMC column was washed with THF and toluene as indicated above. The whole process was repeated three or four times to ensure maximum surface coverage, although the reaction was almost complete after the second run. Trimethylsilylation was carried out similarly using 0.5 ml of 35% TMS-DPA in toluene. A final wash with methanol and phosphate buffer was carried out as with conventional columns.

#### *Equipment*

The conventional HPLC system consisted of an LC-3A pump (Shimadzu, Kyoto, Japan), an M-440 UV detector (Waters Assoc., Milford, MA, U.S.A.), a 98.00 RI detector (Knauer, Berlin, F.R.G.) and a 7000A data processor (System Instrument, Tokyo, Japan). PMC chromatography was performed using a Tri Rotar-V pump and a Uvidec-100-V UV detector (both from Japan Spectroscopic, Tokyo, Japan) equipped with a home-made fused-silica capillary cell (50 μm I.D., *ca.* 10 cm from the column end to the light path). Split injection was employed for PMC chromatography, using a tee-joint with an SS22RS2 micro-metering valve (Whitey, Highland Heights, OH, U.S.A.) in the waste line. The column temperature was controlled using a water-bath for stainless-steel columns and an air-bath for PMC columns.

#### *Chromatographic measurements*

Chromatographic runs were carried out in duplicate. The elution volume of <sup>2</sup>H<sub>2</sub>O was taken as *t*<sub>0</sub> for the conventional system, and that of uracil was used to determine *t*<sub>0</sub> for the PMC system using *k'* values of uracil on stainless-steel columns. Retention ratios were used instead of *k'* values for the comparison between conventional and PMC columns in order to minimize the effect of the difference between the two chromatographic systems.

## RESULTS AND DISCUSSION

In the alkylsilylation of a silica surface, polyfunctional reagents are in general more reactive than monofunctional reagents, and have been used for on-column bonding reactions. Gilpin *et al.*<sup>1</sup> used octadecyltrichlorosilane for the preparation of chemically bonded phases in conventional columns<sup>1</sup>. In batch reactions using monochlorosilanes, amines such as pyridine are added to the reaction mixture to take up the HCl formed by the reaction in order to increase the reactivity in the usual chemical bonding reactions<sup>1,2</sup>.

Körösi and Kováts<sup>20</sup> reported the alkylsilylation of a glass surface using alkyltrimethyl-N,N-dimethylaminosilanes with dimethylamine as a byproduct. We have adopted this approach in order to achieve high reactivity accompanied by a liquid byproduct in *in situ* bonding reactions. Dimethylamine has a very low boiling point of 7°C and bubbles in a column under normal reaction conditions when alkyltrimethyl-N,N-dimethylaminosilanes are applied to on-column reactions.

Diethylamine and di-*n*-propylamine have higher boiling points (55 and 110°C, respectively) and still give boiling points of octadecylsilanes low enough to allow easy distillation. Combinations of ODS-Cl and diethylamine and of TMS-Cl and di-*n*-propylamine were found to give amine hydrochloride precipitates that are easy to filter off during the preparation of the reagents. The reagents, ODS-DEA and TMS-DPA, are also reactive enough to yield readily maximum surface coverage in a short period of time, as shown below. The present method has the advantage that toluene can be used as received, without drying.

The *in situ* bonding reactions were carried out by simply introducing the aminosilane solutions in toluene from the injector loop into the silica packed column. The aminosilane solution was kept in contact with silica at 110°C for 1 h each time. The reaction was slower at lower temperatures. The reaction was repeated twice for regular stainless-steel columns and three or four times for PMC columns in order to attain maximum coverage. The total preparation time was about 4 h for stainless-steel columns including the wash time between the repeated reactions with THF and toluene. The final wash was carried out with methanol and an acidic buffer. With PMC columns the flow-rate of the reagent solution was maintained at 1–1.5  $\mu\text{l}/\text{min}$  during the reaction.

In order to evaluate the surface coverage achieved by the on-column reaction, stationary phases having various carbon contents (A–F) were prepared in batch using ODS-Cl. Of these, F-1 was prepared under conditions that should give the maximum coverage with octadecylsilyl groups. It has been found that the preparation of stationary phases with maximum surface coverage using ODS-Cl in batch was highly reproducible<sup>18</sup>.

The C<sub>18</sub> phase H-1 prepared by the *in situ* reaction was compared with the C<sub>18</sub> phase G-1 prepared in batch from the same reagent, ODS-DEA, and with other stationary phases prepared by using ODS-Cl. The results are shown in Table II. The carbon content and the surface coverage of G-1 were slightly higher than those of F-1, indicating the high reactivity of ODS-DEA, as reported<sup>20</sup>. Stationary phase H-1, prepared by the *in situ* reaction, showed a very similar extent of surface coverage to G-1. This indicates that the reaction in the column using ODS-DEA can afford maximum surface coverage, as in batch reactions. The extent of surface coverage

TABLE II  
SURFACE COVERAGES AND HYDROPHOBIC PROPERTIES OF THE STATIONARY PHASES

Stationary phase	Carbon content (%)	Surface coverage ( $\mu\text{mol}/\text{m}^2$ )	$\alpha_{\text{CH}_2}^*$ (80% methanol)
A-1	8.85	1.26	0.1359
B-1	11.37	1.68	0.1479
C-1	16.97	2.74	0.1727
D-1	17.82	2.92	0.1730
E-1	18.17	3.00	0.1743
F-1	19.06	3.19	0.1755
G-1	20.06	3.42	0.1767
D-2	18.13	—	0.1771
E-2	19.06	—	0.1775
F-2	19.19	—	0.1797
G-2	20.28	—	0.1780
H-1	19.71	3.34	0.1753
H-2	19.79	—	0.1775
PMC-C <sub>18</sub> -1	—	—	0.1756
PMC-C <sub>18</sub> -2	—	—	0.1789

\* The  $\alpha_{\text{CH}_2}$  values were obtained from the slopes of the graphs of  $\log k'$  of alkylbenzenes (ethyl-, propyl-, butyl- and pentylbenzene) against the carbon number of the alkyl groups in 80% methanol at 30°C. Each regression line had a correlation coefficient of better than 0.9999.

achieved in stationary phases F, G and H agreed very well with those reported to be maximum<sup>21</sup>.

In order to confirm the maximum surface coverages indicated by the carbon content of the bonded phases, the stationary phases were further evaluated chromatographically in terms of their hydrophobic properties and the amounts of residual silanols. The retention increment due to one methylene group in a linear alkyl chain correlates very well with the hydrophobic character of the stationary phases<sup>22,23</sup>. As shown in Table II, the retention increment due to one methylene group ( $\alpha_{\text{CH}_2}$ ) increases with increase in surface coverage. Stationary phases H and PMC-C<sub>18</sub> showed very similar  $\alpha_{\text{CH}_2}$  values to stationary phases F and G, having maximum surface coverage, indicating that PMC-C<sub>18</sub> also has a high density of octadecylsilyl groups, probably higher than 3.0  $\mu\text{mol}/\text{m}^2$ , on the silica surface.

In addition to their hydrophobic character, the amounts of residual silanols on each stationary phase were compared by using so-called silanophilic solutes. This was first tried in 60% methanol using benzene derivatives as solutes. The group contribution of a polar group, or the relative retention of acetophenone (AP) with respect to benzene (B),  $\alpha_{\text{AP/B}}$ , and that of methyl benzoate (MB) to benzene,  $\alpha_{\text{MB/B}}$ , show the silanol effect<sup>13</sup>, and the group contribution of the methyl group or the relative retention of toluene (T) to benzene,  $\alpha_{\text{T/B}}$ , indicates the hydrophobic character of the stationary phase. The former decrease and the latter increases with increase in surface coverage by C<sub>18</sub> and also by trimethylsilylation, as shown in Table III.

TABLE III  
RETENTIONS ( $k'$ ) AND SEPARATION FACTORS ( $\alpha$ ) OF BENZENE DERIVATIVES

Mobile phase, 60% methanol; temperature, 30°C.

Stationary phase	$k'_{AP}$ ( $\alpha_{AP/B}$ )	$k'_{MB}$ ( $\alpha_{MB/B}$ )	$k'_B$	$k'_T$ ( $\alpha_{T/B}$ )
A-1	0.94 (0.69)	1.54 (1.12)	1.37	2.28 (1.66)
B-1	1.20 (0.63)	2.04 (1.06)	1.92	3.35 (1.74)
C-1	1.59 (0.49)	3.08 (0.94)	3.26	6.21 (1.90)
D-1	1.61 (0.47)	3.17 (0.92)	3.46	6.61 (1.91)
E-1	1.60 (0.45)	3.19 (0.92)	3.57	6.88 (1.93)
F-1	1.55 (0.44)	3.09 (0.87)	3.55	6.84 (1.93)
G-1	1.49 (0.42)	3.00 (0.84)	3.57	6.91 (1.94)
D-2	1.48 (0.43)	2.97 (0.86)	3.45	6.68 (1.94)
E-2	1.46 (0.40)	2.98 (0.83)	3.61	7.00 (1.94)
F-2	1.38 (0.38)	2.83 (0.79)	3.60	7.02 (1.95)
G-2	1.35 (0.38)	2.77 (0.79)	3.51	6.82 (1.94)
H-1	1.49 (0.43)	2.99 (0.85)	3.50	6.73 (1.92)
H-2	1.32 (0.38)	2.71 (0.78)	3.46	6.70 (1.94)
PMC-C <sub>18</sub> -1	0.76 (0.47)	1.42 (0.88)	1.61	3.09 (1.92)
PMC-C <sub>18</sub> -2	0.68 (0.41)	1.34 (0.81)	1.66	3.19 (1.92)
PMC-C <sub>18</sub> -3	0.56 (0.83)	0.81 (1.21)	0.67	1.11 (1.66)

The results with stationary phases H-1 and H-2 agreed very well with those with F-1 and F-2, and with those with G-1 and G-2 having maximum surface coverages prepared in batch using ODS-Cl and ODS-DEA, respectively. With these stainless-steel columns, the  $k'$  values of benzene derivatives on stationary phase H showed excellent agreement with those on F or G, indicating similar packing densities in the columns. With PMC columns, however, the  $k'$  values of the benzene derivatives were about half of those for conventional packed columns. This is primarily due to the lower packing density of silica particles in PMC columns. PMC columns are known to show much lower flow resistance than conventional columns, by a factor of 5 or more<sup>19</sup>. The looser packing of silica in PMC columns leads to a larger inter-particle space and higher permeability. Based on  $k'$  values for non-polar solutes, benzene and toluene, the phase ratio  $V_s/V_m$  in PMC columns was estimated to be about 45% of that in conventional packed columns. This is supported by the results of studies with size exclusion chromatography using trimethylsilylated PMC columns, which showed the pore volume of the silica particles in PMC columns to be about 20% of the total column volume, compared with about 40% in conventional columns<sup>24</sup>. Therefore, the comparison of surface coverages among the stationary phases should be made on the basis of group contributions of polar and non-polar groups.

PMC-C<sub>18</sub> showed  $\alpha_{AP/B}$  and  $\alpha_{MB/B}$  values very slightly larger than those of G or H with maximum surface coverage, indicating slightly lower surface coverages of PMC columns. The  $\alpha$  values are very close to those found for stationary phases E or F, which possess 3.0–3.2  $\mu\text{mol}/\text{m}^2$  of alkylsilyl groups.

PMC-C<sub>18</sub>-3 was prepared by using octadecyltriethoxysilane according to the reported procedure<sup>9</sup>. Although the reaction was repeated three times, the  $\alpha$  values indicate a low surface coverage. This shows the much higher reactivity of aminosilanes compared with triethoxysilanes. The on-column bonding reaction using aminosilanes is also free from column clogging problems.

The retention of caffeine is much more sensitive to the amount of residual silanols. Caffeine is eluted later than phenol with peak tailing from stationary phases of low coverage. The stationary phases are compared on the basis of the relative retention of caffeine with respect to phenol. The results are shown in Table IV. Although H-2 gave similar results to F-2 and G-2, PMC-C<sub>18</sub>-2 showed slightly larger  $\alpha_{\text{caffeine/phenol}}$  values than the stationary phases with maximum surface coverage. It should be noted that the *in situ* bonding reaction employed in the preparation of stationary phase H in stainless-steel columns gave very similar surface coverages (or maximum coverage) as the conventional method in batch used to produce F or G. The same reaction conditions resulted in stationary phases with slightly more silanols for PMC-C<sub>18</sub> columns, although the surface coverages of PMC-C<sub>18</sub> columns seem to be close to the maximum, or about 3.0  $\mu\text{mol}/\text{m}^2$ . The exact reason for this result is not clear at present, and may be related to the hysteresis of silica particles in drying and the tubing-drawing process associated with PMC columns at temperatures above 750°C. The heat treatment of silica at such temperatures may reduce the number of surface hydroxyl groups and reduce the pore size<sup>2,5</sup>, leading to lower surface coverage.

TABLE IV

$k'_X$  VALUES OF CAFFEINE AND AMINES AND THEIR RELATIVE RETENTION WITH RESPECT TO PHENOL ON STATIONARY PHASES WITH VARIOUS SURFACE COVERAGES AT 30°C

Stationary phase	$k'_X$ ( $\alpha_{X/\text{phenol}}$ )	40% methanol, pH 7.7*			
		40% methanol, pH 2.9*		40% methanol, pH 7.7*	
		PA	NAPA	PA**	NAPA**
D-1	10.32 (1.50)*	0.02 (0.01)	0.16 (0.07)	1.67 (0.73)	2.48 (1.09)
E-1	10.83 (1.56)*	0.04 (0.02)	0.18 (0.07)	1.77 (0.69)	2.63 (1.03)
F-1	10.29 (1.20)*	0.03 (0.01)	0.15 (0.06)	1.28 (0.49)	1.77 (0.67)
G-1	7.69 (0.90)	0.02 (0.01)	0.14 (0.06)	0.64 (0.24)	1.08 (0.41)
D-2	6.83 (0.85)	0.01 (0.01)	0.14 (0.06)	0.38 (0.15)	0.95 (0.37)
E-2	6.07 (0.75)	0.03 (0.01)	0.15 (0.06)	0.35 (0.14)	0.91 (0.35)
F-2	6.38 (0.73)	0.03 (0.01)	0.15 (0.06)	0.35 (0.13)	0.91 (0.34)
G-2	6.02 (0.71)	0.02 (0.01)	0.15 (0.06)	0.36 (0.14)	0.88 (0.33)
H-1	8.59 (1.01)*	0.02 (0.01)	0.15 (0.06)	1.19 (0.46)	1.46 (0.56)
H-2	5.97 (0.70)	0.02 (0.01)	0.14 (0.05)	0.39 (0.15)	0.89 (0.34)
PMC-C <sub>18</sub> -1	3.13 (0.93)*	0.09 (0.08)	0.17 (0.14)	1.15 (1.02)	1.41 (1.25)
PMC-C <sub>18</sub> -2	2.58 (0.77)	0.10 (0.08)	0.20 (0.17)	0.36 (0.30)	0.59 (0.49)

\* Tailed peak.

\*\* 0.02 M phosphate buffer was used to maintain pH.



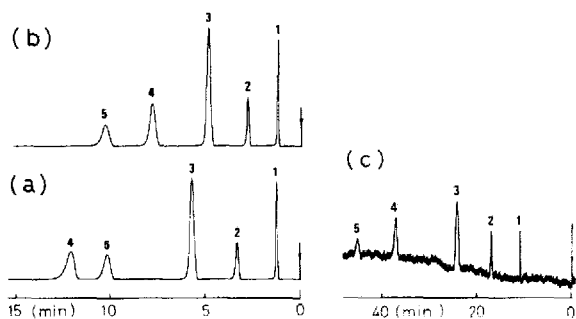


Fig. 1. Elution of uracil (1), theobromine (2), theophylline (3), caffeine (4) and phenol (5) on stationary phases F-1 (a) and F-2 (b) at a flow-rate of 1 ml/min and on PMC-C<sub>18</sub>-2 (3.6 m × 44 μm I.D.) (c) with a column inlet pressure of 250 kg/cm<sup>2</sup>. Mobile phase, 20% methanol; temperature, 30°C.

C<sub>18</sub> stationary phase prepared in batch similarly to G-1 from silica particles treated at 800°C for 3 h showed a considerably lower carbon content (16.80%), supporting the present explanation. The bonded phase in PMC-C<sub>18</sub>-2, however, apparently possesses a surface coverage of close to 3.0 μmol/m<sup>2</sup>. The similar retention ratios seen with PMC-C<sub>18</sub> phases as stationary phases prepared in batch imply little difference in the surface concentrations of silanols after the drawing process with PMC columns. As shown in Fig. 1, the chromatogram of xanthine derivatives on PMC-C<sub>18</sub>-2 was similar to that on F-2 with maximum surface coverage prepared in batch, showing the practical utility of the present on-column reaction for the preparation of reversed-phase PMC columns.

The stationary phases prepared by on-column reaction were further examined with positively charged amines as solutes. It was shown that so-called silanol effects are the combined effects of neutral silanols and some dissociated anionic sites that are present in chemically bonded phases even at acidic pH<sup>18</sup>. The former is responsible for the retention of polar neutral compounds such as caffeine, and the latter, presumably related to the highly acidic silanols or metal impurities in the silica structure, is responsible for the retention of positively charged solutes. The retention of positively charged amines such as procainamide (PA) [H<sub>2</sub>N-C<sub>6</sub>H<sub>4</sub>-CONHCH<sub>2</sub>CH<sub>2</sub>N(C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>] and N-acetylprocainamide (NAPA) [CH<sub>3</sub>CONH-C<sub>6</sub>H<sub>4</sub>-CONHCH<sub>2</sub>CH<sub>2</sub>N(C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>] at pH below 3 indicates the presence of the ion-exchange sites at such pH values, and the retention of these compounds at pH above 7 is provided by the dissociated form of silanols and the anionic sites which contribute to the retention of amines at pH below 3.

Stationary phase H showed almost no retention of PA and NAPA at pH below 3 in 40% methanol, indicating the presence of essentially no anionic sites on the silica surface, as is the case with stationary phases prepared in batch. This is to be expected from the acid treatment of the silica gel used in the preparation of these stationary phases<sup>18</sup>. Stationary phase H-2 also showed a very low retention at pH 7.7, indicating that very few silanols are present as in stationary phases D-2-G-2. The results confirm the completeness of surface coverage by the *in situ* reaction under the present reaction conditions.

PMC-C<sub>18</sub>-2, however, showed a slightly higher retention of PA and NAPA at pH 2.9, indicating the presence of small amount of ion-exchange sites. PMC-C<sub>18</sub>-2 also showed a slight retention of PA and NAPA at pH 7.7, indicating the presence of dissociated silanols and ion-exchange sites. It has been shown that silanols in the form of SiOH are not responsible for the retention of positively charged amines, but silanols do contribute to the retention of such compounds at pH above 7 where silanols undergo dissociation<sup>18</sup>. If one compares the results obtained with PMC-C<sub>18</sub>-2 with those for D-1, E-1 and F-1, which were not trimethylsilylated, the retention of PA on PMC-C<sub>18</sub>-2 was higher than on D-1, E-1 and F-1 at pH 2.9, indicating the presence of more ion-exchange sites on PMC-C<sub>18</sub>-2. At pH 7.7,  $k'_{PA}$  on PMC-C<sub>18</sub>-2 was lower than the value on D-1, E-1 and F-1. These stationary phases possess more silanols than PMC-C<sub>18</sub>-2, as shown in Tables III and IV. These results indicate that the retention of PA and NAPA on PMC-C<sub>18</sub>-2 is mainly caused by the presence of ion-exchange sites. The possible source of such dissociated anionic sites in PMC-C<sub>18</sub> phases is the glass wall of PMC columns. In addition to these ion-exchange sites, small amounts of silanols may be left unreacted owing to the modified surface structure or the reduced pore size of silica particles, as mentioned earlier. Further study is needed to explain in detail the small difference between conventional and PMC columns.

In spite of the presence of small numbers of ion-exchange sites and silanols, the performance of PMC-C<sub>18</sub>-2 obtained with the present *in situ* bonding reaction for silanophilic solutes will be comparable to that of the best stationary phases commercially available, judging from the retention of PA and NAPA at pH 2.9 and 7.7<sup>26</sup>.

The completeness of surface coverage of the on-column bonding reaction was shown above. Another important factor in the chemical bonding reaction is its re-

TABLE V  
REPRODUCIBILITY OF ON-COLUMN REACTION

Mobile phase, 60% methanol.

Column type	Run No.	Stationary phase	$k'_{AP}$ ( $\alpha_{AP/B}$ )	$k'_{MB}$ ( $\alpha_{MB/B}$ )	$k'_B$	$k'_T$ ( $\alpha_{T/B}$ )
Stainless-steel	1	C <sub>18</sub>	1.42 (0.42)	2.83 (0.84)	3.38	6.51 (1.93)
	2	C <sub>18</sub>	1.45 (0.42)	2.92 (0.84)	3.48	6.71 (1.93)
	3	C <sub>18</sub>	1.50 (0.42)	3.01 (0.85)	3.55	6.85 (1.93)
	4	C <sub>18</sub>	1.51 (0.43)	3.01 (0.85)	3.55	6.83 (1.92)
	5	C <sub>18</sub>	1.49 (0.43)	2.99 (0.85)	3.50	6.73 (1.92)
	6	C <sub>18</sub> + TMS	1.32 (0.39)	2.70 (0.79)	3.41	6.60 (1.94)
	7	C <sub>18</sub> + TMS	1.32 (0.38)	2.71 (0.78)	3.46	6.70 (1.94)
PMC	1	C <sub>18</sub>	0.73 (0.48)	1.35 (0.89)	1.51	2.86 (1.89)
	2	C <sub>18</sub>	0.69 (0.47)	1.29 (0.88)	1.46	2.79 (1.91)
	3	C <sub>18</sub>	0.76 (0.47)	1.42 (0.88)	1.61	3.09 (1.92)
	4	C <sub>18</sub> + TMS	0.60 (0.39)	1.23 (0.80)	1.54	2.95 (1.92)
	5	C <sub>18</sub> + TMS	0.68 (0.41)	1.34 (0.81)	1.66	3.19 (1.92)

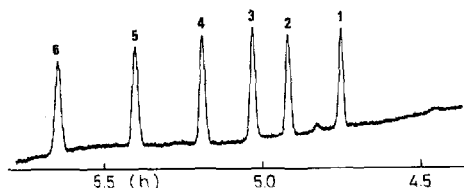


Fig. 2. Separation of benzene (1), toluene (2), ethylbenzene (3), propylbenzene (4), butylbenzene (5) and pentylbenzene (6) on a PMC-C<sub>18</sub> column (16.2 m × 44 μm I.D.) with a column inlet pressure of 100 kg/cm<sup>2</sup>. Mobile phase, methanol; temperature, 30°C.

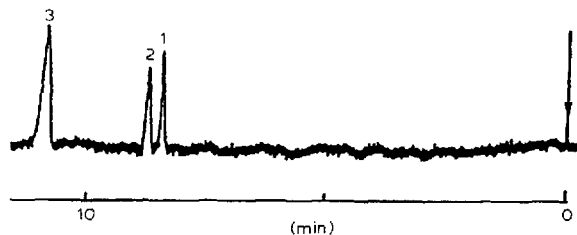


Fig. 3. Elution of uracil (1), phenol (2) and acetophenone (3) on an OTC-C<sub>18</sub> column (4.5 m × 25 μm I.D.) prepared from soda-lime glass with a column inlet pressure of 20 kg/cm<sup>2</sup>. Mobile phase, water; temperature, 30°C.

producibility. This can be seen in Table V. For conventional columns, excellent reproducibility was achieved with the selectivity between benzene derivatives and also with the  $k'$  values, which can be affected by the packing density of silica particles in the columns. The variation of  $k'$  with PMC columns was slightly greater than with conventional columns. This probably reflects the slight variation in the packing density of silica particles in glass capillary tubing prepared separately. The reproducibility in terms of  $\alpha$ , however, was excellent, as is the case with stainless-steel columns.

Long reversed-phase PMC columns can now be prepared easily and reproducibly at close to maximum surface coverage. Fig. 2 shows the performance of a 16.2-m PMC-C<sub>18</sub> column with alkylbenzenes. This column afforded more than  $5 \cdot 10^5$  plates per column in absolute methanol at 30°C for ethylbenzene with a  $k'$  value of 0.11 at a mobile phase linear velocity of 6 cm/min. This approach to attaining a very high number of theoretical plates is very promising in comparison with conventional or micro-bore columns<sup>27</sup>, as the PMC columns show a much lower flow resistance than the usual packed columns. A theoretical study and practical applications of these PMC columns with maximum surface coverage are currently in progress.

Apart from ODS-Cl, any type of chlorosilanes can be used for the *in situ* bonding reaction following the preparation of aminosilanes. The preparation of the reagent is facile, provided that the functional group is compatible with aminosilane functions. The present method also allows the regeneration of stationary phases in conventional reversed-phase columns when they lost some alkylsilyl groups during use.

The on-column bonding reaction is naturally applicable to OTC columns. Fig. 3 shows the chromatogram obtained with an OTC-C<sub>18</sub> column (4.5 m × 25 μm I.D.) prepared under the present reaction conditions after base treatment of the soda-lime glass tubing. The extent of surface coverage is yet to be determined in this instance, but the results show the potential of the procedures reported here in the preparation of this type of reversed-phase column.

## CONCLUSION

The on-column preparation of chemically bonded stationary phases was studied using the monofunctional reagents ODS-DEA and TMS-DPA. By the simple reaction of the silica surface with the aminosilanes, a monomeric C<sub>18</sub> stationary phase was prepared on silica packed in columns with maximum surface coverage and high reproducibility, comparable to those for stationary phases prepared in batch using ODS-Cl or ODS-DEA. The method was also applied to the preparation of reversed-phase PMC columns. The PMC-C<sub>18</sub> columns also showed near maximum surface coverage, estimated to be *ca.* 3 μmol/m<sup>2</sup>. Although the PMC-C<sub>18</sub> stationary phases were shown to possess slightly more residual silanols and anionic sites, their retention characteristics were very similar to those of stationary phases with maximum surface coverage.

The proposed method allows the reproducible preparation of very long reversed-phase PMC columns to generate large numbers of theoretical plates inexpensively. The on-column reaction also allows the preparation of reversed-phase OTC columns and the regeneration of chemically bonded stationary phases in conventional stainless-steel columns.

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