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# Influence of the method of preparation of chiral stationary phases on enantiomer separations in high-performance liquid chromatography

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**Abstract**

Various chiral stationary phases were immobilized on supports using suitable alkyl spacers. Aminopropylsilica, which is aminopropylsilylated silica gel, is a typical spacer-carrying support for immobilization. Silanol and aminopropyl groups left unreacted on the support surface disturb the separation of enantiomers through a non-chiral interaction. The following three types of chiral stationary phases were prepared using different preparation methods and the effect of the total structure on the chiral separation was studied: (1) a chiral group was immobilized on aminopropylsilica; (2) a chiral group connected with a spacer was immobilized on silica gel; and (3) a chiral group connected with a spacer was immobilized on dimethylchlorosilane-treated silica gel. It was shown for (1) that when the amount of chiral groups immobilized was lower, free silanol groups and unreacted amino groups would preferentially interact with sample molecules. When the amount immobilized was increased, its interaction with samples became effective, and also aminopropyl groups left unreacted were thought to enhance the interaction. Too many chiral groups, however, resulted in lower separability. In case (2), a similar tendency to that with (1) but lower separabilities were obtained. A more effective performance was found with (3), with lower immobilization. For the preparation of an effective chiral stationary phase, the amount of chiral groups on the support and the structural and topographical conditions of the support material should be assessed.

## 1. Introduction

As an effective method for the separation of optical isomers, high-performance liquid chromatography with various chiral stationary phases (CSPs) has been widely used [1]. The chiral separation is often provided by the differences in the diastereomeric association energy between analytes and a chiral selector introduced to the stationary phase.

The chiral stationary phases are usually prepared by immobilizing chiral molecules on siliceous supports by way of appropriate alkyl spacers. Aminopropylsilica, prepared by aminoalkylation of silica gel [2], has frequently been used as the support material for the immobilization of chiral molecules. Unreacted silanol groups and amino groups remaining on the stationary phase after the immobilization reaction do not work for the chiral separation. The effect of residual silanol groups on the selectivity of the stationary phase has been discussed by many workers [3,4].

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Greater enantioselectivity was observed with end-capped chiral stationary phases, which were investigated by Pirkle and Readnour [5]. We have previously [6] investigated the effect of unreacted alkyl spacers on chiral separations by changing the amount of the chiral selector, and found that the total immobilized structure should be taken into consideration.

In this study, Pirkle type stationary phases [7], with which the separation mechanism has been precisely investigated, were chosen. Three types of stationary phases with the same chiral selector but differing in their total structures were prepared, and the effect of the total structure of the stationary phase including the residual silanol groups on the separation was investigated. Previously, we discussed thirteen chiral diamide-type stationary phases [8], including Pirkle-type phases. The phase that showed the highest separability was the immobilized 3,5-dimethylbenzoyl-L-valine phase for N-3,5-dinitrobenzoyl-O-isopropyl-derivatized amino acids. In this work, 3,5-dimethylbenzoyl-L-valine (DMB-L-Val) chiral selector was immobilized through different routes on the support surface, and the enantiomer separability was examined.

## 2. Experimental

### 2.1. Instrumentation

The chromatographic equipment consisted of a Shimadzu (Kyoto, Japan) Model LC-9A high-pressure pump, a Rheodyne (Cotati, CA, USA) Model 7125 sampling valve with a 25-$\mu$l loop, a Shimadzu Model SPD-6AV variable-wavelength UV–Vis spectrophotometric detector and a Shimadzu C-R4A integrator. The column (150 × 2.1 mm I.D.) was packed with modified silica gel using the slurry packing technique. The mobile phase was chosen to be 2-propanol–hexane (5:95) at a flow-rate of 250 $\mu$l/min. The detection wavelength used was 254 nm. The hold-up time was measured using the peak of 1,3,5-tri-*tert.*-butylbenzene.

### 2.2. Samples

Amino acids were used as test samples. The carboxylic group was esterified with 2 *M* HCl–2-propanol solution at 120°C for 2 h under reflux. The amino group was treated with 3,5-dinitrobenzoyl chloride to give the corresponding benzoylamide. After derivatization, the samples were purified by column chromatography using a silica gel column and hexane–ethyl acetate (10:1) as the mobile phase.

### 2.3. Synthesis of CSP-A

*Preparation of 3,5-dimethylbenzoyl-L-valine*

L-Valine was acylated with 3,5-dimethylbenzoyl chloride by the Schotten–Baumann procedure. The method of preparation in the literature [9] was partly employed.

*Preparation of aminopropyl-functionalized silica*

A porous silica (Develosil silica 100-5, mean particle size 5 $\mu$m, mean pore size 100 Å and specific surface area 350 $m^2/g$) (Nomura Chemical) was dried at 160°C and 20 Pa for 5 h. An excess of aminopropyldimethylethoxysilane (Sinetsu Chemical) in 13.2 ml (70 mmol) of toluene was added to 10 g of dried silica gel. The mixture was heated at reflux until ethanol was no longer removed azeotropically. After cooling, the modified gel was filtered and washed successively with toluene, tetrahydrofuran, methanol, acetone and diethyl ether and finally dried under vacuum. Elemental analysis gave the amount of the aminopropyl groups as 0.987 mmol/g.

*Preparation of CSP-A*

Different amounts of 3,5-dimethylbenzoyl-L-valine(DMB-L-Val) in 100 ml of dry tetrahydrofuran were mixed with 1.2 g of aminopropyl-functionalized silica gel and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in 1.2 times the molar amount of DMB-L-Val was added [10,11]. The suspension was stirred for 1 day under an argon atmosphere at room temperature. The bonded phase was filtered and washed with tetrahydrofuran, methanol, acetone

and diethyl ether. After drying, the coverage densities of the chiral selector were calculated from the carbon contents obtained by elemental analysis. All of these compounds were confirmed by $^1H$ NMR and fast atom bombardment MS.

### 2.4. Synthesis of CSP-B

*Preparation of 3,5-dimethylbenzoyl-L-valine allylamide*

Six grams (24 mmol) of DMB-L-Val were dissolved in 200 ml of dry ethyl acetate. To the solution, stirred and cooled to −18°C by a CTP-100 cooling thermo pump (Tokyo Scientific Chemical), was added 3.3 g (29 mmol) of N-hydroxysuccinimide and 5.9 g (29 mmol) of dicyclohexylcarbodiimide under an argon atmosphere. After stirring the reaction mixture for 24 h at −18°C, the suspension was warmed slowly (12 h) to room temperature under continuous stirring. Subsequently, the white precipitate of dicyclohexylurea thus formed was removed by filtration, and the resulting clear solution was evaporated. To the residual oil were added 200 ml of dry tetrahydrofuran and the mixture was cooled to −18°C under an argon atmosphere. Then, a mixture of 3.7 ml (34 mmol) of N-methylmorpholine and 2.6 ml (34 mmol) of allylamine in 30 ml of dry tetrahydrofuran were dropwise added to the stirred solution over 1 h. After the solution had been kept stirred for 48 h, the solvent was evaporated. The residue was dissolved in chloroform and the solution was washed with 0.1 *M* hydrochloric acid and then with 5% sodium hydrogen carbonate, and finally dried under vacuum.

*Preparation of 3,5-dimethylbenzoyl-L-valine dimethylchlorosilylpropylamide*

To a solution of DMB-L-Val allylamide, 1.8 g (6.4 mmol) and 0.044 g (0.064 mmol) of $H_2PtCl_6$ in 70 ml of chloroform were added 11.3 ml (102 mmol) of dimethylchlorosilane. The mixture was then heated at reflux for 5 h, the solvent and excess silane were removed in vacuo and the residue was used in the next step without purification.

*Preparation of CSP-B*

Different amounts of silylated DMB-L-Val in 50 ml of dry toluene were mixed with 1.6 g of dried silica gel (160°C, 5 h), and a small amount of pyridine was added. The suspension was stirred for 1 day under an argon atmosphere at room temperature. The bonded phase was filtered and washed with chloroform, toluene, tetrahydrofuran, methanol, acetone and diethyl ether.

### 2.5. Synthesis of end-capped CSP-B

CSP-B (0.8 g) was suspended in 50 ml of dry toluene and 1.2 ml (5.6 mmol) of 1,1,1,3,3,3-hexamethyldisilazane were added. After refluxing the mixture for 3 h under an argon atmosphere, the modified gel was collected by filtration and washed successively with toluene, tetrahydrofuran, methanol, acetone and diethyl ether.

### 2.6. Synthesis of CSP-C

*Preparation of silanized silica gel (pre-end-capped silica)*

A 15-ml volume (14 mmol) of dimethylchlorosilane (Sinetsu Chemical) in 150 ml of toluene was added to 10 g of dried silica gel. The mixture was stirred for 24 h under an argon atmosphere at room temperature. Then, the pre-end-capped silica gel was filtered and washed with toluene, tetrahydrofuran, methanol, acetone and diethyl ether and dried under vacuum. The amount of the dimethylchlorosilyl function was determined to be 0.598 mmol/g from elemental analysis.

*Preparation of CSP-C*

Different amounts of DMB-L-Val allylamide in 100 ml of dry tetrahydrofuran were mixed with 0.8 g of pre-end-capped silica gel. After adding a small amount of dicyclopentadienylplatinum dichloride as the platinum catalyst, the mixture was stirred for 5 h under an argon atmosphere at 60°C. The bonded phase was filtered and washed with tetrahydrofuran, methanol, acetone and diethyl ether.

## 3. Results and discussion

In the case of CSP-A type immobilization, the chiral moiety was immobilized after attaching the alkyl spacer to the silica gel. A certain amount of unreacted amino groups was present on the silica gel after reaction as shown in Fig. 1. In order to examine the effects of the extent of bonding of the chiral moiety, and the remaining amount of amino groups, CSPs with different coverage densities of the chiral moiety were prepared by changing the mixing ratio of DMB-L-Val to aminoalkylated silica gel (coverage density 1.70 groups/nm$^2$). The amount of the chiral selector was estimated by elemental analysis of carbon. Then the number of groups per unit surface area of silica gel was calculated. These phases were evaluated mainly by separation factors and capacity factors of the derivatized enantiomer samples. Separation factors on six different CSP-A type phases are shown in Fig. 2. The phases with coverage densities of not more than 0.1 groups/nm$^2$ showed a very low separability of enantiomers. Considering that the remaining aminopropyl groups do not work for the chiral separation, when the amount of the immobilized chiral selector is small, analytes are retained mainly by the residual amino and silanol groups. When the coverage densities were between 0.3 and 0.5 groups/nm$^2$, the residual aminopropyl groups were thought to enhance the enantio-

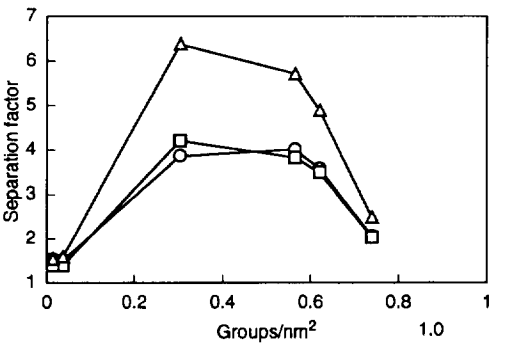


Fig. 2. Relationship between separation factor and the coverage density of CSP-A phase. ○ = N-(3,5-Dinitrobenzoyl)alanine isopropyl ester; □ = N-(3,5-dinitrobenzoyl)-valine isopropyl ester; △ = N-(3,5-dinitrobenzoyl)leucine isopropyl ester. Column, 150 × 2.1 mm I.D.; mobile phase, 2-propanol–*n*-hexane (5:95, v/v); flow-rate, 250 μl/min; column temperature, room temperature; detection, UV at 254 nm.

selectivity. As in the usual cases, the separation factor of D,L-leucine derivatives is far higher than those of alanine and valine. Coverage densities higher than 0.5 groups/nm$^2$ did not result in a higher separation factor. This result demonstrates that optimum amounts of the chiral selector exist for the separation of enantiomers. When the density of the chiral selector is too high, the interaction between the selector and the analyte might be blocked. We speculate that the self-association, described in a previous paper [12], of the selectors occurs in that region.

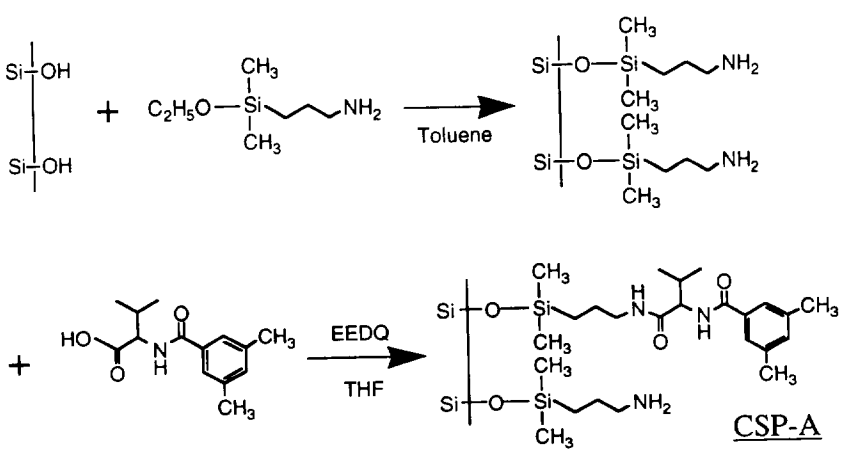


Fig. 1. Preparation scheme for CSP-A phase.

The optimum values of coverage density were different from analyte to analyte, and the reason was considered to be due in part to the difference in the spaces needed for the formation of complexes between the stationary phase and analytes.

With CSP-B type phases, DMB-L-Val was first reacted with the spacer, allylamine, and then the product was reacted with dimethylchlorosilane. This chiral selector was directly reacted with the silica gel, so that the prepared CSP was completely free from residual amino groups. As shown in Fig. 3A, however, this CSP has remaining silanol groups on the silica gel surface. These residual silanol groups show a non-chiral interaction with analytes, and therefore are considered to suppress the enantioselectivity. Thus, end-capped CSP-B type phases were further modified by end-capping, which minimized the number of silanol groups on the surface as shown in Fig. 3B.

The separation factors on five different CSP-Bs and end-capped CSP-Bs with different coverage densities of chiral selector are shown in Figs. 4 and 5. Similarly to CSP-A, the CSP-Bs in Fig. 4 with a coverage density of chiral selector of not

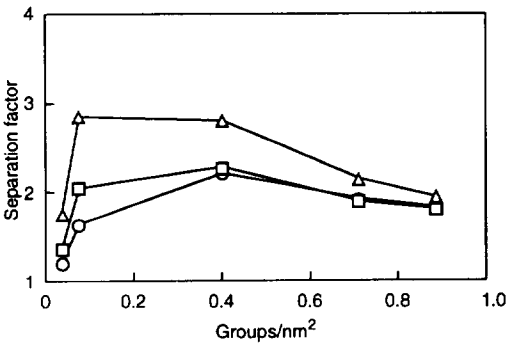


Fig. 4. Relationship between separation factor and the coverage density of CSP-B phase. Conditions as in Fig. 2.

less than 0.4 group/nm$^2$ showed lower separation factors. A low coverage density resulted in an extremely low performance, which was considered to be due to the presence of silanol groups on the silica gel surface. On the end-capped CSP-B with a coverage density not more than 0.2 groups/nm$^2$, a higher selectivity in chiral separation was observed. CSP-Bs with coverage densities higher than 0.3–0.4 groups/nm$^2$ have almost no end-capping effect. Therefore, the silanol groups still remaining were not considered to have a significant effect on the

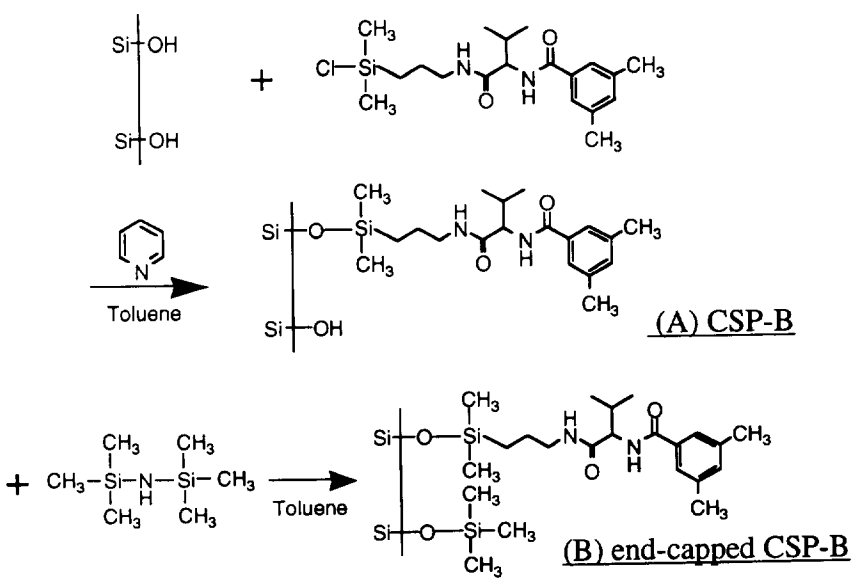


Fig. 3. Preparation schemes for (A) CSP-B and (B) end-capped CSP-B phases.

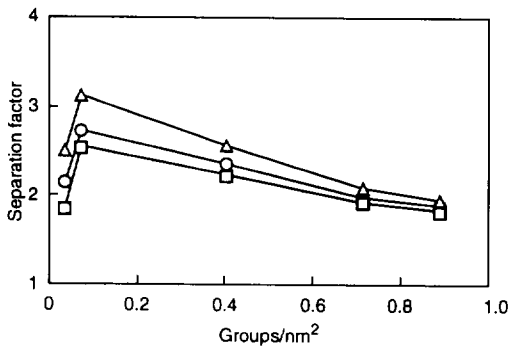


Fig. 5. Relationship between separation factor and the coverage density of end-capped CSP-B phase. Conditions as in Fig. 2.

performance. When the coverage density was extremely low, CSP-B type phases showed a higher performance than CSP-A type stationary phases after end-capping. Previously, Brügger and Arm [13] synthesized urea-linked CSPs, silica gel bonded with different amount of silylurea, with or without end-capped silanol groups. According to Brügger and Arm, silanol groups may interfere with the interactions between the analyte and the chiral selector. For the non-end-capped CSPs, the separation factors decrease with decrease in coverage density. However, on the end-capped CSPs a lower coverage density leads to reduced non-chiral interactions. In this study, with diamide-type CSPs, similar results were obtained.

In order to cover the silanol groups on the surface of silica gel more effectively, we tried another immobilization method, a pre-end-capping method, expecting that the silanol groups on the surface would be more efficiently covered. With the CSP-C type immobilization method shown in Fig. 6, dimethylchlorosilane was first reacted with silica gel (coverage density 1.03 groups/nm$^2$). We chose monochlorosilane as a silylating reagent. Such a type of silane has high reactivity and no possibility of regenerating the silanol groups [14]. Then, DMB-L-Val allylamide was immobilized using a hydrosilylation reaction. By changing the amount of DMB-L-Val allylamide, CSP-Cs with different coverage densities were prepared. Platinum catalyst, $H_2PtCl_6$, is often used in hydrosilylation reactions. It does not work, however, in a highly polar field such as on the silica gel surface, and leads to an extremely low yield of immobilization. Ohtsu et al. [15] synthesized ODS-silica gel by hydrosilylation of Si–H after suppressing the polarity of silica gel by a polysiloxane coating. In this study, however, we found that dicyclopentadienylplatinum dichloride [16] could efficiently catalyse the hydrosilylation of Si–H on the silanized silica gel surface. An immobilization yield as high as about 70% was achieved.

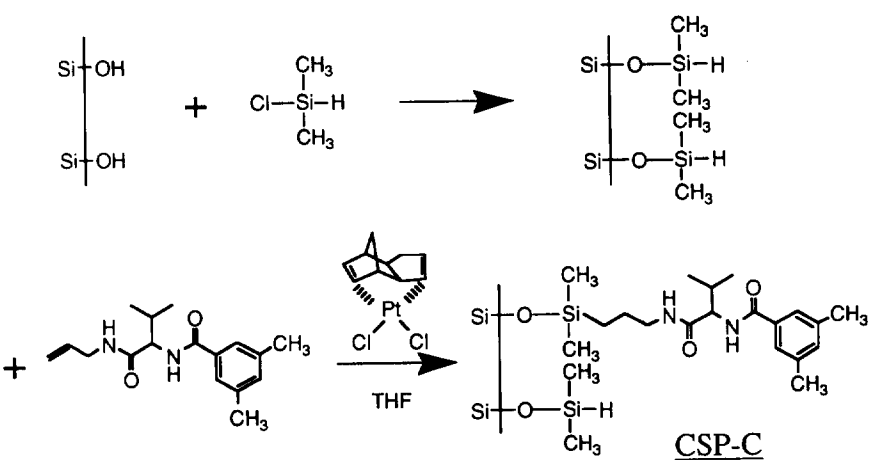


Fig. 6. Preparation scheme for CSP-C phase.

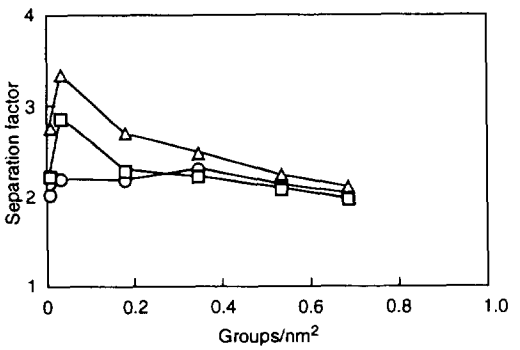


Fig. 7. Relationship between separation factor and the coverage density of CSP-C phase. Conditions as in Fig. 2.

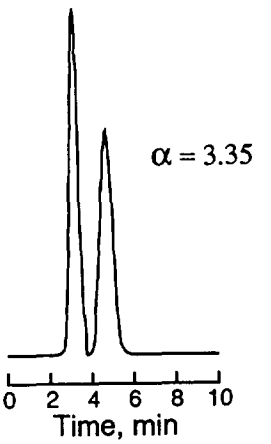


Fig. 8. Chromatogram of N-(3,5-dinitrobenzoyl)leucine isopropyl ester on CSP-C column (coverage density 0.033 groups/nm$^2$). Conditions as in Fig. 2.

The separation factors on six different CSP-Cs with different coverage densities are shown in Fig. 7. The CSP-Cs with a coverage density of not less than 0.3 groups/nm$^2$ showed almost the same properties as CSP-B and end-capped CSP-B, and the pre-end-capping effect was not observed. Therefore, it was concluded that in this instance the chiral separability depends on the coverage density of the chiral selector, and that the effect of the silica gel surface can be neglected. When the coverage density is not higher than 0.2 groups/nm$^2$, however, no decrease in separation factors even at very low coverage density is observed. Even with a coverage density not greater than 0.05 groups/nm$^2$, CSP-C showed a higher enantioselectivity than CSP-A and CSP-B. It could be concluded that the adsorption on silanol groups could be eliminated effectively through the post-end-capping method. The chromatogram shown in Fig. 8 demonstrates a typical separation of D,L-leucine derivatives on CSP-C. It shows a good resolution and small capacity factor.

In Fig. 9, the correlations between the coverage densities and $k'$ values on various CSPs prepared in the present study are shown. On either type of CSPs, the coverage density of the chiral selector showed a positive correlation with capacity factors ($k'$). The increase in $k'$ value with increase in the coverage density of CSPs was similar to those on the non-chiral phases. The decrease in the performance of chiral separation along with the increase in the coverage density of the chiral selector, such as shown in Figs. 2, 4 and 5, means that retention unrelated to the chiral separation occurs predominantly in the interaction between the stationary phase and the analytes. In this study, we used Pirkle-type chiral stationary phases, which utilize hydrogen bonding and the $\pi$-donor–$\pi$-acceptor group interaction. It was considered that the mutual interaction of the selector groups in the stationary phase was increased at higher coverage densities, which leads to a possible inefficiency of the interaction between the analytes and the chiral selector. Therefore, it is necessary to

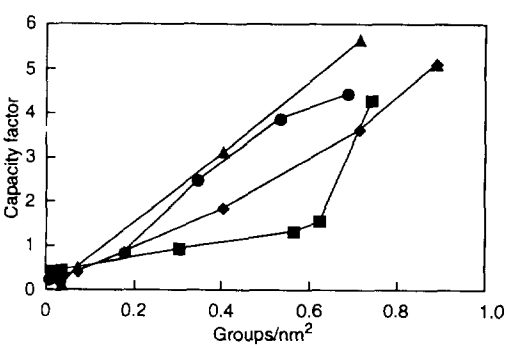


Fig. 9. Capacity factors of N-(3,5-dinitrobenzoyl)-D-leucine isopropyl ester on (■) CSP-A, (◆) CSP-B, (▲) end-capped CSP-B and (●) CSP-C as a function of the coverage density. Conditions as in Fig. 2.

examine the effect of the surface coverage density on the chiral separability of other chiral phases that have different interaction mechanisms.

## 4. Conclusions

The behaviours of the chiral stationary phases were demonstrated to be affected by the method of immobilization of the chiral selector. The chiral separability also varied depending on the coverage density of the chiral selector and the environmental conditions near the chiral selector. Therefore, in order to evaluate a chiral stationary phase, these factors should be taken into consideration. Of the immobilization methods studied, the pre-end-capping method was found to be useful, as it can reduce the amount of chiral selectors required for immobilization. As the time required for analysis is very short owing to the small $k'$ value, this pre-end-capping method could be very suitable for the preparation of other chiral stationary phases. These findings will be applied in our future development of new chiral stationary phases.

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