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Supercritical fluid chromatographic separation of fatty acid methyl esters on aminopropyl-bonded silica stationary phase

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ABSTRACT

Aminopropyl-bonded silica packings with various bonding densities were prepared, and the separation behaviour of fatty acid methyl esters on the prepared packings was investigated by supercritical fluid chromatography. The chromatographic behaviour of the esters could be described by two kinds of selectivity: selectivity according to the chain-length and selectivity according to the degree of unsaturation. The selectivity according to chain length increased with the aminopropyl bonding density of the packing. On the other hand, the selectivity according to degree of unsaturation decreased as the aminopropyl bonding density increased. These selectivities were useful for the characterization of commercially available amino-bonded packings.

INTRODUCTION

Amino-bonded silica has often been used for the separation of saccharides by high-performance liquid chromatography (HPLC) [1–3]. This packing material has also been used in supercritical fluid chromatography (SFC) [4,5] and found to be useful in the separation of fatty acid esters [6]. However, there are few kinds of amino-bonded packings commercially available compared with octadecyl-bonded packings, and chromatographic behaviour on an amino-bonded stationary phase has not yet been fully investigated by SFC.

In this study, aminopropyl-bonded silicas with various bonding densities were prepared, and the supercritical fluid chromatographic behaviour of fatty acid methyl esters on the prepared packings was investigated.

EXPERIMENTAL

Preparation of the packing

Aminopropyl-bonded silica was prepared as previously reported by Jones *et al.* [7]. Two types of silica, Wakosil II-5sil-100 (10 nm pore size, 5 μm, Wako, Tokyo, Japan) and Finesil 100-5 (10 nm pore size, 5 μm, Jasco, Tokyo, Japan), were used to prepare the aminopropyl-bonded packings. Silica was dried *in vacuo* at 393 K over 4 h prior to its reaction with silane. A 3-g amount of silica gel was placed in a 200-ml round-bottomed flask and refluxed in 50 ml of dry hexane (stored over molecular sieves 3A 1/16 inch) with 3-aminopropyltriethoxysilane (Aldrich, Milwaukee, WI, USA) for 30 min. After the reaction, the slurry was filtered through glass microfibre and washed with hexane, acetone and methanol. The prepared packings were then

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dried *in vacuo* at 373 K over 24 h. The carbon and nitrogen contents of the packings were determined by elemental analysis.

Preparation of the column

The packing equipment used consisted of a slurry reservoir of 20 ml and a stainless-steel column of 150 × 4.6 mm I.D. A 1.5-g amount of packing was suspended in 20 ml of water and the slurry was placed in the reservoir. The slurry was forced into the column and compressed to a compact bed by pumping methanol at 35 MPa. The solvent in the prepared column was replaced with acetone, then replaced with hexane, and finally the columns were completely purged by passing supercritical CO_2.

Supercritical fluid chromatography

The chromatographic system used has been described previously [8]. Not only the prepared packed columns but also three commercially available columns, Cosmosil NH_2 (10 μm, 150 × 4.6 mm I.D., Nacalai tesque, Kyoto, Japan), Wakosil NH_2 (5 μm, 150 × 4.6 mm I.D., Wako) and Super NH_2 (10 μm, 150 × 4.6 mm I.D., Jasco), were tested. Myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and α-linolenic acid ($C_{18:3}$) methyl esters were used as test samples. Fatty acid methyl esters derived from fish oil were also separated on each column. The eluent was monitored with a UV detector at 200 nm.

Liquid chromatography

In order to characterize the prepared packings, the retention behaviour of glucose and fructose on each packing was investigated in liquid chromatograpy. An acetonitrile–water mixture (4:1) was used as mobile phase. The saccharides were detected with a refractive index detector.

RESULTS AND DISCUSSION

The carbon and nitrogen contents of the packings prepared at various silane concentrations are listed in Table I. Wakosil II originally contained carbon, which was probably derived from the surfactant added in the process of synthesizing silica gel. Thus, the carbon content in Table I shows the sum of carbon contents derived from the added surfactant and the bonding aminopropyl groups. In this report, the aminopropyl bonding density of each packing was calculated on the basis of its nitrogen content. A plot of aminopropyl bonding density *vs.* silane:silica ratio is shown in Fig. 1. The bonding density increased with silane:silica ratio, and above 20% (w/w) it gradually levelled off. Jones *et al.* [7] determined the support loading of the prepared packings by measuring the weight loss after combustion, and in their study the support

TABLE I

CARBON AND NITROGEN CONTENTS AND BONDING DENSITIES OF PREPARED PACKINGS

Carbon and nitrogen contents were determined by elemental analysis, and bonding density was calculated on the basis of nitrogen content.

Packing	Silane:silica ratio (%, w/w)	C (%, w/w)	N (%, w/w)	Bonding density (mmol/g)
Wakosil II	–	1.38	–	–
WN1	5.3	2.82	0.16	0.11
WN2	10.1	3.79	0.58	0.41
WN3	20.6	5.48	1.13	0.81
WN4	26.4	5.97	1.18	0.84
WN5	33.0	5.03	1.13	0.81
WN6	41.1	6.19	1.34	0.96
WN7	52.7	5.34	1.27	0.91
Finesil	–	0.08	–	–
FN1	33.0	3.78	1.08	0.77

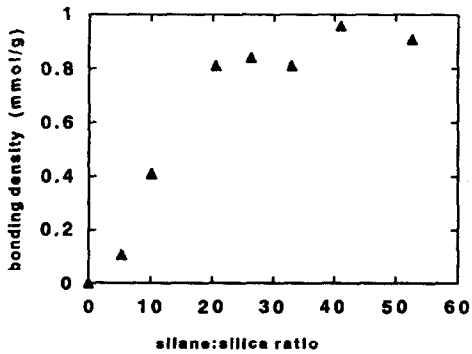

Fig. 1. Dependence of aminopropyl bonding density on silane:silica ratio (in %, w/w).

loading increased with silane:silica ratio without levelling off. They also investigated the liquid chromatographic behaviour of saccharides on the prepared packings in order to evaluate the state of bonding on the silica surface, and concluded that the polymerization of the silane monomers was predominant above a support loading of 8.5% (w/w), whereas the capacity factor was constant for a particular sugar. In this study, the retentions of glucose and fructose on the prepared packings were measured by LC, and the result is shown in Fig. 2. The capacity factors of both saccharides increased with bonding density with no plateau. Figs. 1 and 2 show that in this study silane monomers at a silane:silica ratio higher than 20% (w/w) did not take part in the reaction on the silica surface, and only very little polymerization of the silane monomers occurred on the silica surface even at high silane concentration. This is because the reaction mixtures contained little water, which causes the polymerization of silane monomers.

The highest bonding density achieved in this experiment was about 1 mmol per g of packing, and this is equivalent to 2.9 μmol/m^2 considering the surface area of Wakosil II (350 m^2/g according to the catalogue). The average value of the surface hydroxyl concentration of silica is about 8 μmol/m^2 [9,10], and one molecule of a trifunctional silane reacts with one or two hydroxyl groups on the silica surface [11]. This information shows that some hydroxyl groups remained on the surface of each prepared packing, even on the packing of maximum bonding density.

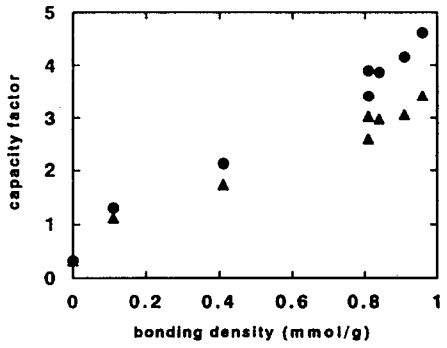

Fig. 2. Dependence of capacity factors of (●) glucose and (▲) fructose on aminopropyl bonding density in liquid chromatography. Mobile phase: acetonitrile–water (4:1). Temperature: 303 K.

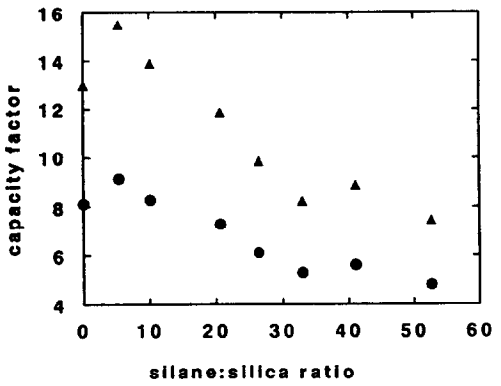

Fig. 3. Dependence of capacity factors of (●) palmitic acid and (▲) linoleic acid methyl esters on silane:silica ratio (in %, w/w). Base silica, Wakosil II; mobile phase, CO_2; temperature 313 K; outlet pressure, 15 MPa.

The capacity factors in SFC of palmitic acid and linoleic acid methyl esters are plotted as a function of silane concentration in Fig. 3. The solute retentions were measured at 313 K and 15 MPa. Except for the results on silica alone, the capacity factors of both esters decreased with an increase in the silane:silica ratio up to about 30% (w/w). Above this value, the capacity factor (k') of each ester remained constant. The same retention data are plotted as a function of the bonding density of the packings in Fig. 4. Although the capacity factor decreased with the bonding density, it varied widely at high bonding density. The variation in the k' value observed at high bonding density can be regarded as reflect-

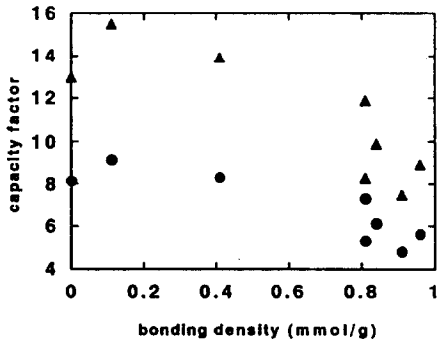

Fig. 4. Dependence of capacity factors of (●) palmitic acid and (▲) linoleic acid methyl esters on aminopropyl bonding density. See Fig. 3 for conditions.

ing a difference in the state of the bonding phase, for example a difference in the average number of hydroxyl groups reacting with one molecule of trifunctional silane (between one and two [11]), or the occurrence of uncontrollable polymerization caused by a slight amount of water in the reaction mixture. In the case of the separation of saccharides by LC, the k' value did not vary greatly at high bonding density, as shown in Fig. 2. The difference in the retention properties between SFC and LC may be because chromatographic behaviour in SFC is more sensitive to the properties of the stationary phase than in LC [5,12–16].

In our previous studies [16,17], the retention behaviour of fatty acid methyl esters on octadecylsilyl (ODS)-bonded silicas was investigated by SFC. In the case of the separation of esters by SFC, a well-end-capped ODS silica gave a smaller capacity factor than bare silica at the same pressure and temperature. For example, the k' value of methyl palmitate on ODS silica was less than 1 at 313 K and 15 MPa [16,17], and it was much smaller than the value on bare silica ($k' = 8$ on Wakosil II in Fig. 3). This suggests that the polarity of the packing contributes to the intensity of solute retention. The aminopropyl group is less polar than the hydroxyl group on a silica surface, so an increase in aminopropyl bonding density leads to a decrease in the polarity of packings and thus to a decrease in capacity factors.

On an ODS column, fatty acid esters were separated from one another depending upon the difference in chain length and the degree of unsaturation [16,17]. On an ODS packing, esters with long hydrocarbon chains and low degrees of unsaturation were retained strongly. It is reasonable to expect that the capacity factors of esters are also correlated with their chain length and degree of unsaturation on aminopropyl-bonded silicas. Fig. 5 shows the plots of capacity factor *vs.* carbon number of fatty acids on prepared packings, and Fig. 6 shows the plots of capacity factor and the number of double bonds of fatty acids. The capacity factors of myristic acid, palmitic acid and stearic acid methyl esters are shown in Fig. 5, and the capacity factors of stearic acid, oleic acid, linoleic acid and linolenic

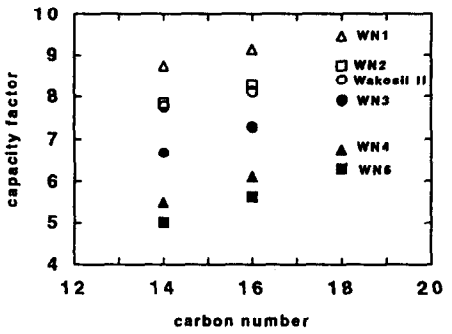

Fig. 5. Plots of capacity factors of saturated fatty acid methyl esters on each prepared packing *vs.* carbon number of fatty acids. Myristic acid, palmitic acid and stearic acid methyl esters were used as samples. See Fig. 3 for conditions.

acid methyl esters are shown in Fig. 6. Esters with more double bonds were strongly retained, the opposite to what was observed on ODS silicas.

As Figs. 5 and 6 show, the retention of fatty acid methyl esters depends on their chain length and degree of unsaturation. Thus the selectivity of esters can be divided into two types: selectivity according to the difference in carbon number and selectivity according to the difference in the number of double bonds. In this study, the former and the latter are indicated by α_{CN} and α_{DB}, respectively, and defined as follows:

$$\alpha_{CN} = k'(C_{16:0})/k'(C_{14:0})$$

$$\alpha_{DB} = k'(C_{18:1})/k'(C_{18:0})$$

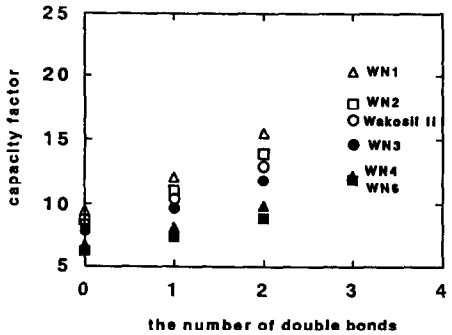

Fig. 6. Plots of capacity factors of fatty acid methyl esters on each prepared packing *vs.* the number of double bonds of fatty acids. Stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and α-linolenic acid ($C_{18:3}$) methyl esters were used as samples. See Fig. 3 for conditions.

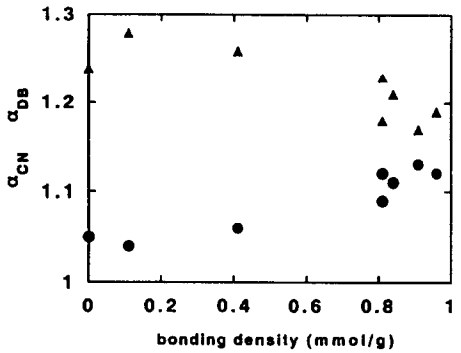

Fig. 7. Dependence of (●) α_{CN} and (▲) α_{DB} on aminopropyl bonding density. See Fig. 3 for conditions.

Fig. 7 shows the relation between the two selectivities and bonding density. With the exception of the value on bare silica α_{DB} decreased as bonding density increased. On the other hand, α_{CN} increased with bonding density. As described above, the ester with more double bonds was retained strongly on aminopropyl-bonded silicas ($\alpha_{DB} > 1$); on the other hand, it was retained weakly on ODS silicas ($\alpha_{DB} < 1$). This suggests that α_{DB} should be closely related to the polarity of packings. The decrease in α_{DB} can be attributed to the decrease in packing polarity with the increase in bonding density. The increase in α_{CN} with bonding density can be attributed to the increase in the number of hydrocarbon chains on the silica surface. This view is supported by the high α_{CN} value observed on an ODS packing that has longer hydrocarbon chains on its surface. For example, the value of α_{CN} on a commercial ODS column was about 1.4 at 313 K and 15 MPa [16,17], although the value of α_{CN} on each aminopropyl-bonded silica was below 1.13. It is also well known that in reversed-phase LC chromatographic selectivity depends strongly on the bonding density or carbon content of the packings.

The prepared aminopropyl-bonded silica columns and three commercial amino-bonded silica columns were used for the separation of methyl esters derived from fish oil. The supercritical fluid chromatograms of the esters derived from fish oil are shown in Fig. 8. The esters were separated at 313 K, and the separation pressures were 20 MPa for Wakosil II and WN2, 15 MPa for WN4, WN6, FN1 and Super NH_2, 12 MPa for Wakosil NH_2, and 10 MPa for Cosmosil NH_2. On bare silica packing (Wakosil II), the esters were separated according to their degree of unsaturation. On the aminopropyl-bonded silica packings of high bonding density, the esters were better separated from one another because of the increase in the selectivity according to chain length.

Some differences in the separation behaviour of the esters were observed with the commercial amino-bonded silica columns. The observed differences could arise from differences in the properties of the base silica used as well as the aminopropyl bonding density. For the characterization of the aminopropyl-bonded silica packings, the effects of chain length and degree of unsaturation of esters on the chromatographic retention and selectivity were investigated. Figs. 9 and 10 show the plots of k' ($C_{18:1}$) *vs.* k' ($C_{16:0}$) and α_{DB} *vs.* α_{CN}, respectively. A plot of k' ($C_{18:1}$) *vs.* k' ($C_{16:0}$) gives a straight line regardless of the type of base silica used. Fig. 10 indicates that aminopropyl-bonded packings with higher α_{CN} values generally exhibit lower α_{DB} values. The plots for the packings with the same base silica, WN1 to WN7, made one curve, and the points of the commercial packings deviated from the curve. The difference in chromatographic behaviour observed among commercial amino-bonded packings in Fig. 8 is based on the difference in α_{CN} and α_{DB}. Fig. 10 is useful for the evaluation of amino-bonded packings.

CONCLUSIONS

Aminopropyl-bonded silicas with varying bonding densities were prepared, and the supercritical fluid chromatographic behaviour of the fatty acid methyl esters on the prepared packings was investigated. As the aminopropyl bonding density increased, the selectivity according to the chain length of esters increased. On the other hand, the selectivity according to the degree of unsaturation of esters decreased with the increase in the aminopropyl bonding density. As to the separation of fatty acid esters, the aminopropyl-bonded silicas, including commercially

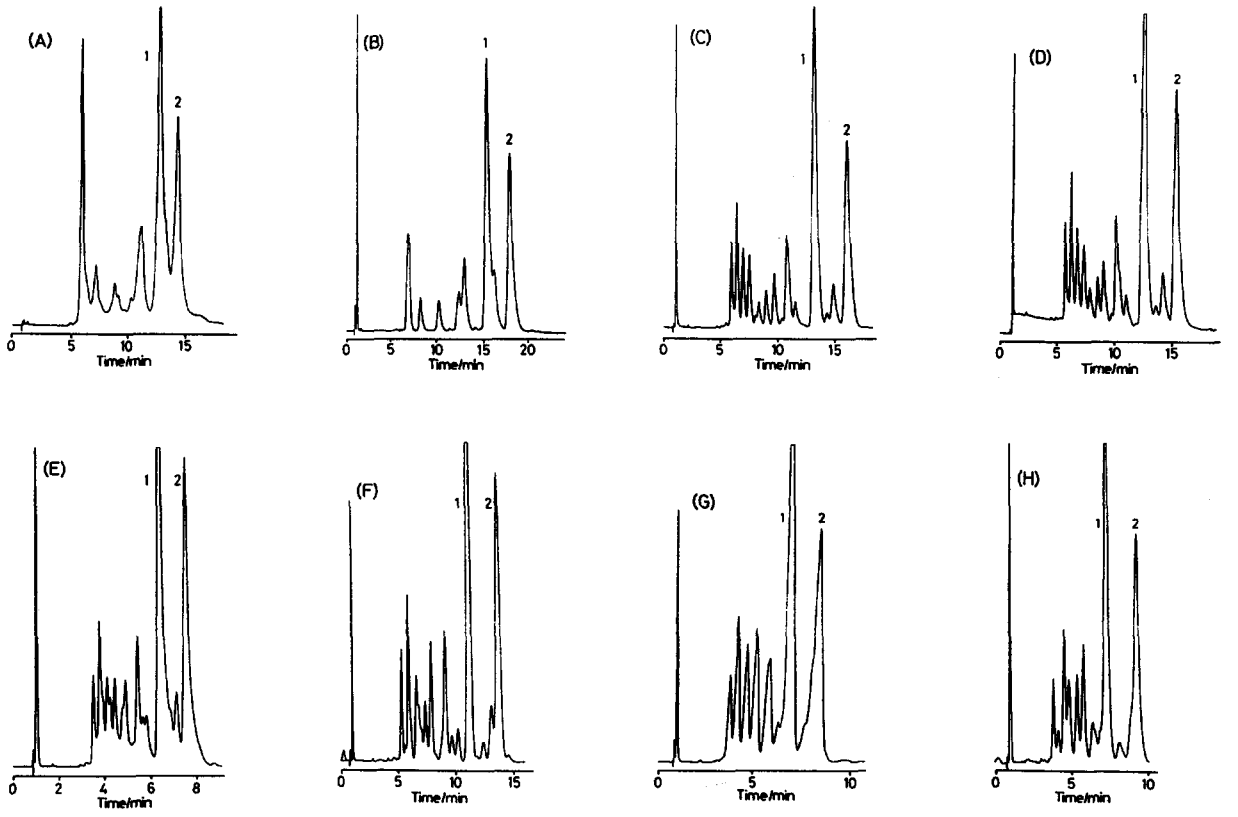

Fig. 8. Separation of methyl esters derived from fish oil. Temperature 313 K, flow-rate 1.4 l/min. (A) Column Wakosil II, outlet pressure 20 MPa; (B) column WN2, outlet pressure 20 MPa; (C) column WN4, outlet pressure 15 MPa; (D) column WN6, outlet pressure 15 MPa; (E) column FN1, outlet pressure 15 MPa; (F) column Super NH_2, outlet pressure 15 MPa; (G) column Wakosil NH_2, outlet pressure 12 MPa; (H) column Cosmosil NH_2, outlet pressure 10 MPa. 1 = eicosapentaenoic acid ($C_{20:5}$) methyl ester; 2 = docosahexaenoic acid ($C_{22:6}$) methyl ester.

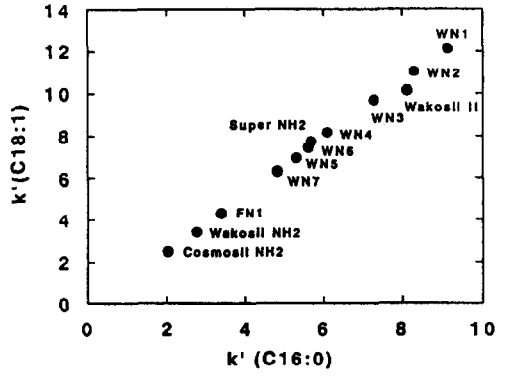

Fig. 9. Correlation between k' ($C_{18:1}$) and k' ($C_{16:0}$) on various packings. See Fig. 3 for conditions.

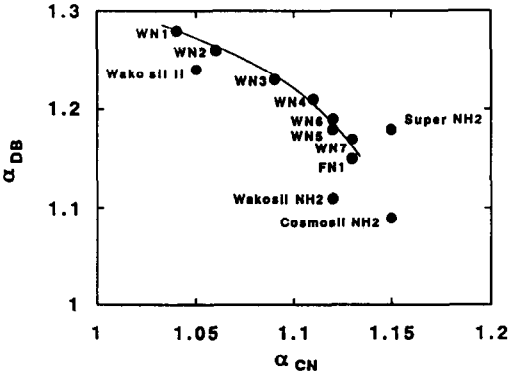

Fig. 10. Correlation between α_{DB} and α_{CN} on various packings. See Fig. 3 for conditions.

available amino-bonded silicas, were well characterized by two kinds of selectivities, α_{CN} and α_{DB}.

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