An Investigation Concerning the Stability of a Sterically Protected Cyanopropyl Modified Silica Substrate

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AN INVESTIGATION CONCERNING THE STABILITY OF A STERICALLY PROTECTED CYANOPROPYL MODIFIED SILICA SUBSTRATE

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ABSTRACT

The hydrolytic stability of a sterically protected cyanopropyl modified silica phase, used in reversed-phase mode, has been studied. This Zorbax SB-CN stationary phase shows superior hydrolytic stability in comparison with conventional CN stationary phases against intensive flushing with aggressive eluents of high and low pH, plain and also with the addition of an organic modifier. In addition to chromatography and elemental analysis, solid-state NMR was also used to characterize the changes in the properties of these phases. Moreover, the effect of sample volume and nature of the sample solvent on overloading of the SB-CN columns was studied. Volume overloading was already observed, when 10 μl of the sample solved in pure organic modifier was injected on the column. On the other hand, if the eluent was used as the sample solvent, no significant loss of column performance could be observed, at least up to 50 μl of sample injections.

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INTRODUCTION

The most wide-spread form of reversed-phase high-performance liquid chromatography (RP-HPLC) employs stationary phases containing relatively long alkyl chains (e.g. C_8 and C_{18}). However, for some applications the use of less hydrophobic packings that do not require strong RP-eluents can be profitable. Reversed-phase separations with cyano-bonded (CN) phases potentially may have a large number of application areas, e.g., RP-HPLC analysis of proteins and peptides, have been reported [1]. One of the reasons why CN-bonded phases have not found general use up till now is the poor reproducibility of the synthesis of the stationary phases and the subsequent problems with their (in)stabilities. These latter problems are often due to a rapid loss of ligands from the silica support, especially when working with low pH mobile phases [2,3].

Recently, a new way to protect the siloxane bonds connecting the ligands to the silica surface from hydrolysis was reported [3]. The development of these sterically protected silica-based stationary phases have made it possible to obtain reproducible stationary phases with improved stability against ligand hydrolysis. For these sterically protected phases the siloxane bonds between the ligand and the silica support are sterically protected from hydrolysis by bulky side groups present on the ligand chain. Monofunctional silanes containing two isopropyl side groups instead of the conventional methyl groups were found to produce very stable packings [3].

An additional advantage of cyano-bonded silica columns is that they can be used both in the normal and the reversed-phase mode. In the normal-phase mode, the cyano column acts essentially as a deactivated silica surface resulting in the interaction of analytes with the residual silanols [4]. However, the selectivity is markedly different from that of silica. If the polarity of the solvent

in the normal phase mode is increased, then the effect of the silanols is suppressed leaving the cyano groups as the principal adsorption sites [5]. In the reversed-phase mode, the cyano column acts like a short chain alkyl silica due to the hydrophobic spacer and the bulky side groups [6]. The primary hydrophobic interaction of the analyte with the bonded phase is through the isopropyl side groups whose concentration determines strongly the hydrophobicity of the surface. The residual silanols also play a key role in the overall retention properties of these phases.

The hydrolytic stability of the sterically protected cyanopropyl modified silica, used in the reversed-phase mode, is the issue of this study. The aging effect of several eluents on the process of stationary phase deterioration was studied. Four buffering eluents of high and low pH, totally aqueous and with the addition of methanol were recirculated continuously in a closed system through the columns. Before and after these aging experiments, chromatographic tests, elemental analysis and solid-state NMR were used to characterize changes in the properties of the phases. Finally, the stabilities of these sterically protected stationary phases were compared with those of conventional CN stationary phases. It is emphasized here that by eluent recirculation further deterioration of the column may be suppressed by saturation of the eluents with ligands and/or silica material. The results from these aging experiments should, therefore, be considered as the minimal change in the properties of the stationary phase that may occur during the laboratory use of these phases in HPLC practice.

Many workers have observed that the strength of the sample solvent relative to that of the RP-HPLC mobile phase may have considerable effects on the peak shapes and peak heights of the solutes [7-12]. Peak broadening, peak distortion and multiplication of peaks has been reported when the sample solution was significantly stronger than that of the mobile phase [9-11]. Similar effects

were observed during the stability study of the sterically protected stationary phase investigated here. Therefore, the effect of sample volume and the nature of the sample solvent on the possible overloading of these columns were also studied to determine the optimal conditions for the chromatographic characterization before and after the aging experiments.

EXPERIMENTAL

Chemicals and materials

Five identical sterically protected cyanopropyl modified silica columns, Zorbax SB-CN (150 mm x 4.6 mm I.D.; Rockland Technologies, Inc., Newport, DE, USA) were investigated in this study. One column was used as a reference column, while the other four columns were subjected to artificial aging procedures. The artificial aging experiments consisted of a continuous exposure of the columns separately to four different aggressive eluents. These eluents are listed in Table I. For economic and also practical reasons, these eluents were recirculated continuously in a closed system at a flow rate of 0.5 ml/min for a

Table I. Eluent compositions used for the aging experiments.

Aging exper. no.	Buffering eluent	pH	Volume fraction of methanol in eluent
1	0.05 M Phosphate	2.2	0
2	0.05 M Phosphate	2.2	0.5
3	0.05 M Phosphate	8.0	0
4	0.05 M Phosphate	8.0	0.5

period of ten days (240 hours). Hence, the columns were flushed with about 7200 column volumes of a specific eluent. As was already mentioned, possible saturation of the eluent with dissolved silica and/or ligands, may reduce the aging effect of these experiments compared to realistic laboratory practice. After finishing a typical series of aging experiments, the columns were carefully rinsed sequentially with water, water-methanol mixtures and methanol. In this way, deposition of the buffering salts in the columns was prevented. In the next step, the columns were subjected to chromatographic RP-HPLC test procedures with three test mixtures at suitable eluent compositions. Finally, the contents of the columns were used for NMR experiments and elemental analysis.

Three mixtures of test components were used for the chromatographic characterization of the stationary phases. The test components were all of reference grade. Test mixture 1 consisted of benzene, methylbenzene, ethylbenzene, n-propylbenzene and n-butylbenzene (Aldrich Chemie, Germany). Test mixture 2 consisted of methyl-p-hydroxybenzoate, ethyl-p-hydroxybenzoate, n-propyl-p-hydroxybenzoate and n-butyl-p-hydroxybenzoate (Sigma Chemical Corporation, St. Louis, MO, USA). Test mixture 3 consisted of 2-n-pentylpyridine, 2-n-hexylpyridine, 2-n-heptylpyridine and 2-n-octylpyridine (Merck, Darmstadt, Germany). All test components were dissolved in the eluent. The injection volume ranged from 2 to 50 μl and the injected amount of sample was in all cases about $6*10^{-7}$ g.

For the aging experiments and chromatographic characterizations, solvents and chemicals of analytical reagent grade (Merck) were used. For the preparation of the buffers, deionized water was used. The water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA) prior to use. All eluents were freshly prepared, filtered over 0.22 μm membrane filters (Millipore) and degassed in an ultrasonic bath prior to use.

INSTRUMENTATION AND PROCEDURES

Chromatography

The chromatographic experiments were performed with a model PU 4100 liquid chromatograph pump (Unicam, Cambridge, UK). Furthermore, a Marathon autosampler equipped with a 20 μl sample loop (Spark Holland, Emmen, The Netherlands) and a model PU 4110 UV/VIS detector (Unicam) operated at 254 nm were used. A Nelson Model 760 interface and a Nelson 3000 data system (Nelson Analytical, Cupertino, CA, USA) was applied for data acquisition and subsequent data handling.

For the aging experiments, an instrument was used with an eight-headed metering pump (Metering Pumps, London, UK). Each of these pumps can be purged separately with a specific eluent. The pumping system was provided with a home-made pulse damping system, which includes a pulse damper (Waters, Millipore), a 3 m x 0.1 mm I.D. capillary and a permanent 0.2 μm filter coupled in series. This system ensured a constant flow through the columns during the aging experiments.

Elemental Analysis

The carbon, hydrogen and nitrogen content of the original and the aged stationary phases was obtained with a Perkin Elmer Analyzer, Model 240 (Perkin Elmer Corp., Norwalk, CT, U.S.A.). Tungsten oxide was added to the packings as a catalyst for the analysis. The carbon and hydrogen contents (P_C and P_H, respectively) and the ligand surface density (α_L) of the stationary phases are given in Table II. The repeatability of these analyses was 0.3%.

From the values of the carbon content of the bulk phase and the specific surface area of the substrate, the average ligand density was calculated with an equation derived by Berendsen [13]:

Table II. A summary of carbon and hydrogen contents (P_C and P_H) and ligand surface densities (α_L) of the aged columns 1-4 and the reference column. The repeatability of the analysis is 0.3%.

packing	P_C % (w/w)	P_H % (w/w)	α_L (μmol.m^{-2})
1	4.35	0.91	2.15
2	4.06	0.83	2.0
3	3.93	0.84	1.93
4	3.88	0.82	1.91
Reference	4.29	0.82	2.12

$$\alpha_L = \frac{P_C}{S_{BET} \left(M_C - P_C \left(M_L - 1 \right) \right)} \tag{1}$$

where

α_L = ligand surface density (mol.m^{-2})

P_C = the amount of carbon (g/g)

S_{BET} = specific BET surface area of the substrate (m^2.g^{-1})

M_C = the amount of carbon per mol bonded silane (g.mol^{-1})

M_L = molecular weight of the silane molecule (g.mol^{-1})

Solid state ^{29}Si NMR measurements

The solid state ^{29}Si NMR spectra were obtained on a Bruker MSL-400 Fourier Transform NMR spectrometer at 79.49 MHz. Aluminium oxide rotors (7 mm O.D.) of the standard Bruker double bearing type were filled with representative samples of ca. 250 mg of packing and were spun at about 2.5 kHz.

^{29}Si cross polarization magic angle spinning (CP MAS) NMR spectra of all stationary phases were obtained with a cross polarization contact time of 6 ms. The pulse interval was 1 s. Typically 2000 FIDs (Free induction decay) with an acquisition time of 10 ms were accumulated in 1K datapoints and zero-filled to 8K prior to Fourier transformation. A line broadening of 20 Hz was used prior to zero-filling and Fourier transformation. The spectral width for all spectra was 20 kHz.

RESULTS AND DISCUSSION

Results of the aging process

Elemental Analysis

The carbon and hydrogen contents of the reference and of the aged packings 1 - 4 are summarized in Table II. The loss of carbon observed for all aged packings was relatively low and did not exceed 10%. Aging experiment 1 did not result in a loss of the bonded phase, while a slight loss of bonded phase was observed for the other aging experiments in the order 4 > 3 > 2 of column number.
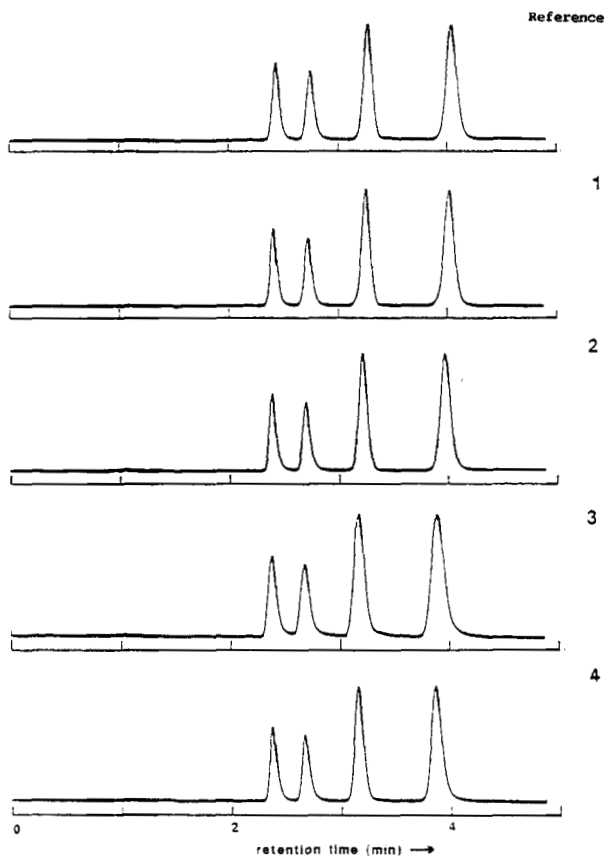
Chromatography

The effect of long-term exposure of the SB-CN columns to aggressive eluents on their chromatographic properties is illustrated in the Figures 1, 2 and 3. These figures depict the chromatograms of the test mixtures 1, 2 and 3, respectively, eluted on the columns before and after the aging experiments. The chromatogram indicated as "reference" represents the reference column, while the other chromatograms represent the aged columns; chromatograms 1 - 4. The numbers on the right side of the chromatograms correspond to the number of the aging experiment as listed in Table I. In addition to that a number of
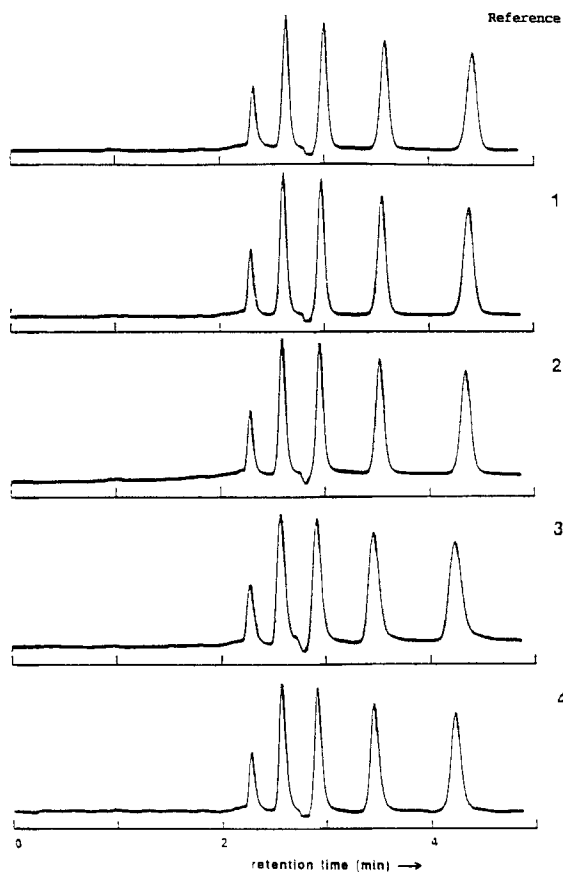
Figure 1.
Chromatograms of the n-alkylbenzenes test mixture eluted on the Zorbax SB-CN stationary phases before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the number of the aging experiment as outlined in Table I.
Chromatographic test conditions: mobile phase: methanol-water (60/40, v/v); flow rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 20 μl; sample solvent: methanol-water (60/40, v/v); injected amount: $6*10^{-7}$ g.

Figure 2.

Chromatograms of the n-alkyl esters of p-hydroxybenzoic acid test mixture eluted on the Zorbax SB-CN stationary phase before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I.
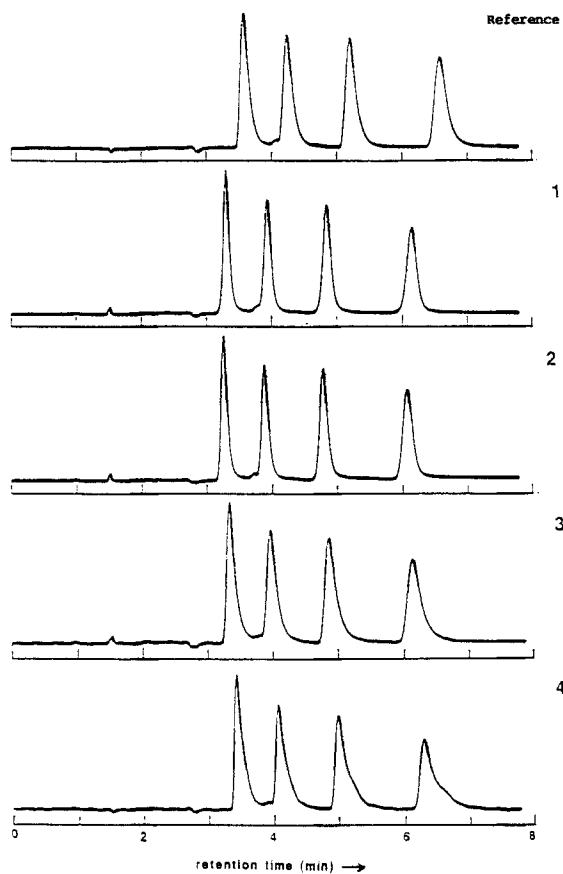
Chromatographic test conditions: mobile phase: methanol-water (55/45, v/v); flow rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 20 μl; sample solvent: methanol-water (55/45, v/v); injected amount: $6*10^{-7}$ g.

Figure 3.
Chromatograms of the n-alkylpyridines test mixture eluted on the Zorbax SB-CN stationary phase before (chromatogram indicated as "reference") and after the aging experiments, chromatograms 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I. Chromatographic test conditions as in Figure 1.

Table III. Summary of the capacity factors (k') of n-heptylbenzene, selectivity (α) between ethylbenzene and n-heptylbenzene, plate numbers (N) and asymmetry factors (A_S) both determined for n-heptylbenzene. The chromatographic measurements were performed on aged columns 1-4 and on the reference column. The plate numbers were calculated taking into account the asymmetry factor measured at 10% of the total peak height [14].

column	k'	α	N	A_S
1	5.8	5.34	6800	1.0
2	5.8	5.36	6420	0.97
3	5.7	5.09	3930	1.14
4	5.8	5.0	5520	0.99
Reference	5.6	5.54	5910	1.0

chromatographic data calculated from the chromatograms for ethyl- and heptylbenzene are presented in Table III as a example.

The chromatographic characteristics of the columns did not change much after flushing the columns with the aggressive eluents applied in this study. The retention times of the n-alkylbenzenes and the n-alkyl esters of p-hydroxybenzoic acid homologous series, as well as the selectivity between ethylbenzene and n-heptylbenzene dropped somewhat on the columns 3 and 4 after the aging with alkaline eluents of pH=8. Also a decrease in efficiency was observed for column number 3. These observations of a slight loss of ligands made by chromatography and elemental analysis correspond to the somewhat increased peak asymmetries measured for the n-alkylpyridines for the aging experiments 3 and 4. These latter substances being very sensitive to silanophilic interactions of the stationary phase will respond to the slightly increased amount of silanols due to the loss of ligands. The observed increased asymmetry factors may also be explained by a slight

dissolution of the silica substrate and consequently of a partial collapse of the column bed structure. However, generally spoken, it can be concluded that the aging effect of aggressive eluents on the column performance is low, even if plain aqueous buffers are used as the eluents.

Solid state ^{29}Si NMR measurements

To confirm the observations made by chromatography and by elemental analysis also the reference phase as well as the aged phases were analyzed by NMR spectroscopy. ^{29}Si CP MAS NMR spectra of the reference packing indicated as "reference" and of the aged stationary phases 1-4 are shown in Figure 4 The numbers on the right side of the spectra correspond to the number of the aging experiment, as listed in Table I. Here, too, no significant loss of ligands (signal at 12 ppm) was observed. These observations are in good agreement with the results of the chromatography and with elemental analysis.

The results described above, correspond to those already reported, on the stability of this type of stationary phase [3]. In [3] the decrease in retention on the sterically protected diisopropyl-3-cyanopropyl stationary phase (Zorbax SB-CN) was compared with the decrease for the corresponding conventional dimethyl-3-cyanopropyl phase (Zorbax CN). The stationary phases were subjected to degradation experiments with alternating isocratic runs, followed by gradient elution under water/acetonitrile/TFA conditions. These data demonstrated that the isopropyl protecting groups are very effective in stabilizing the bonded phases and protecting those from hydrolytic deterioration. No measurable loss of the diisopropyl-3-cyanopropyl phase was observed by elemental analysis measurements of carbon, hydrogen and nitrogen content after a 4-day test period. These observations by Kirkland et al. are in good agreement with our results. The test conditions applied in the previous work of Kirkland differ strongly from those used in our study and were performed over a 4-day period instead of a 10-day period in our work. However, as in [3] the SB-CN phases kept their original
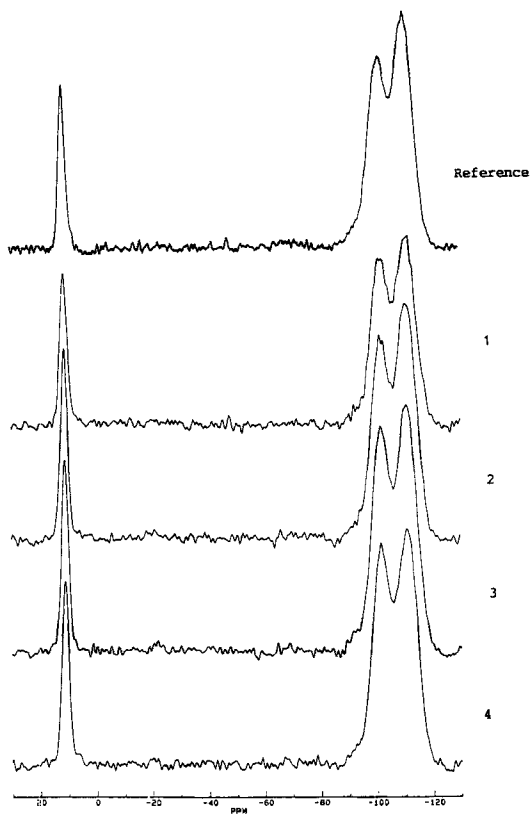
Figure 4.

^{29}Si CP-MAS NMR spectra of the Zorbax SB-CN stationary phases before (spectrum indicated as "reference") and after the aging experiments, spectra 1-4. The numbers on the right side of the chromatograms correspond to the numbers of the aging experiments as outlined in Table I.

Measurement conditions: N_S: 2000; contact-time: 6 ms; pulse interval time: 1 s; acquisition time: 10 ms; line broadening: 20 Hz.

chromatographic behaviour to a large extent, even when the phases were used under aggressive eluent conditions.

Sample injection studies

In order to determine the optimal chromatographic test conditions some SB-CN columns were used for a number of different injection experiments prior to the aging experiments. Test mixture 1, consisting of benzene, methylbenzene, ethylbenzene, n-propylbenzene and n-butylbenzene, was used for these tests. This mixture was dissolved in two different sample solvents consisting of pure methanol or the eluent. By varying the concentration of the different test solutions the absolute injected amount of n-alkylbenzenes on the columns was kept constant at 6.10^{-7} g. In this way, volume overloading could be studied independently of mass overloading. The applied injection volumes ranged from 2 to 50 μl. Plate numbers and asymmetry factors were calculated from the chromatograms.
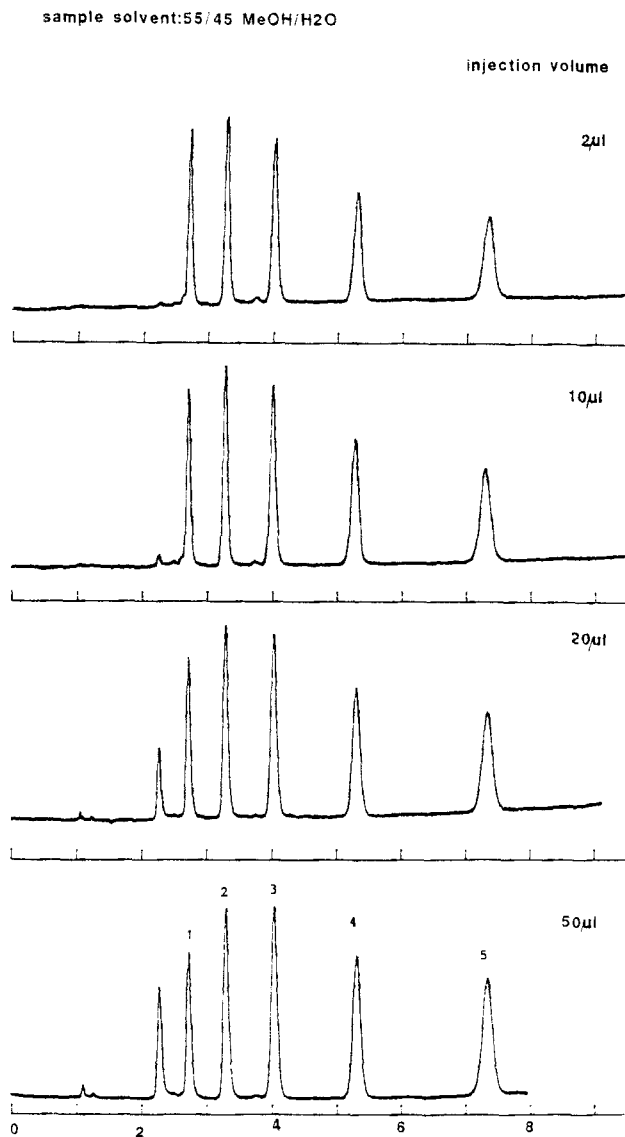
In Figures 5 and 6 the chromatograms of the n-alkylbenzenes dissolved in pure methanol and in the eluent, respectively, are presented. The numbers on the right side of the chromatograms correspond to the applied injection volume, in μl, of a specific sample solution. From these figures it can be seen, that volume overloading can be already observed, when 10 μl of the sample dissolved in methanol is injected on the column. On the other hand, if eluent was used as the sample solvent, no significant loss of column performance could be observed, even when 50 μl of the sample was injected. A summary of column plate numbers and asymmetry factors, calculated for n-butylbenzene, is presented in Table IV. These data clearly show that a strong decrease in plate number, as well as an increase in asymmetry factor, is already caused by the injection a few μl of test mixture 1 dissolved in methanol. Opposite to that, the column performance is hardly influenced by injection of relatively high volumes of this test mixture dissolved in the eluent. To avoid any interference by this overloading effect during our further study of the Zorbax SB-CN columns, all chromatographic tests

sample solvent:methanol

injection volume

2μl

10μl

20μl

0   2   4   6   8   10
retention time(min) —>
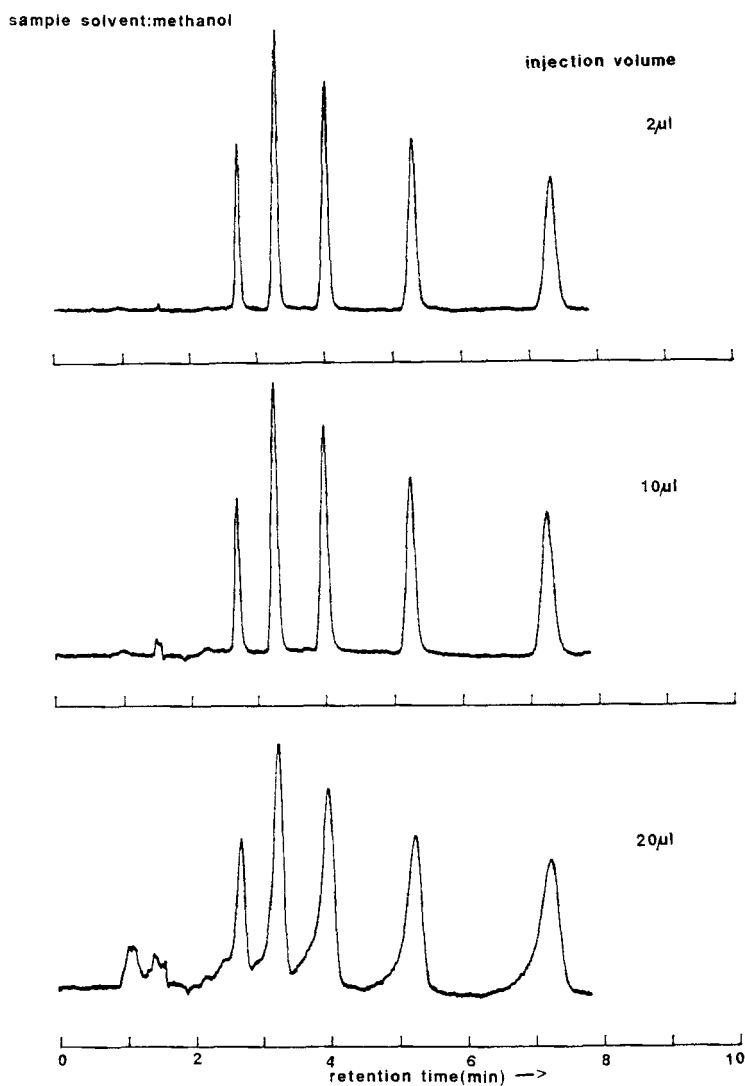
Figure 5.
Chromatograms of the n-alkylbenzenes, dissolved in pure methanol, eluted on the
Zorbax SB-CN stationary phase. The numbers on the right side of the
chromatograms correspond to the applied injection volume of the solution in μl.
Chromatographic test conditions: mobile phase: methanol-water (55/45, v/v); flow
rate: 1.0 ml/min; detection: UV at 254 nm, 0.01 AUFS; sample volume: 2, 10
or 20 μl; sample solvent: methanol; injected amount: $6*10^{-7}$ g.

sample solvent:55/45 MeOH/H2O

injection volume

Figure 6.
Chromatograms of the n-alkylbenzenes, dissolved in eluent, eluted on the Zorbax SB-CN stationary phase. The numbers on the right side of the chromatograms correspond to the applied injection volume (in μl) of the solution.
Chromatographic test conditions: eluent: methanol-water 55/45 v/v; flow rate: 1.0 ml/min; detection UV at 254 nm, 0.01 AUFS; sample volumes 2, 10, 20 or 50 μl; sample solvent: methanol-water 55/45 v/v; injected amount of sample $6*10^{-7}$ gr.

Table IV. A summary of plate numbers (N) and peak asymmetry factors (A$_S$) both calculated for n-butyl benzene. The plate number was calculated taking into account the asymmetry factor measured at 10% of the total peak height [14].

injection volume (μl)	sample solvent: methanol		sample solvent: eluent	
	N	A$_S$	N	A$_S$
2	8700	1.05	8300	0.95
10	6800	1.14	8800	0.97
20	2400	0.44	9200	0.95
50	-	-	8600	0.99

in this study were continued with 20 μl sample injections, using the eluent as the sample solvent.

CONCLUSIONS

The Zorbax SB-CN stationary phases investigated in the present study show high stability against intensive flushing with aggressive eluents used in the aging experiments. Only the packings aged with alkaline eluents exhibit a slight loss in retention and selectivity, accompanied by a decrease in column efficiency for the packing aged with plain buffer of pH 8. As a consequence for these packings aged with alkaline eluents, a slight loss of bonded phase is observed by elemental analysis.

In this study it is also shown that the sterically protected cyanopropyl modified silica can be applied very well as a reversed-phase stationary phase.

As also observed for other types of reversed phase packings the volume loadability of the Zorbax SB-CN columns is limited, when pure organic modifier

is used as the sample solvent. In these cases a strong decrease in plate number, as well as an increase in asymmetry factor, was observed after the injection of only a few μl of sample. However, this problem can easily be overcome by dissolving the sample in the eluent. Little change in the performance of these columns was observed in the injection range from 2 to 50 μl, when the sample was dissolved in the eluent.

ACKNOWLEDGEMENT

REFERENCES

1. N.E. Zhou, C.T. Mant, J.J. Kirkland, R.S. Hodges. J. Chromatogr. 548 (1991) 179.

2. D.R. Knighton, D.R.K. Harding, J.R. Napier, W.S. Hancock. J. Chromatogr. 347 (1985) 237.

3. J.J. Kirkland, J.L. Glajch, R.D. Farlee. Anal. Chem. 61 (1989) 2.

4. E.L. Weiser, A.W. Salotto, S.M. Flach, L.R. Snyder. J. Chromatogr. 303 (1984) 1.

5. J.F. Schapbron, R.J. Hurtubise, H.F. Silver. Anal. Chem. 50 (1978) 1911.

6. R.E. Majors. J. Chromatogr. Sci. 18 (1980) 488.

7. G.K.C. Low, P.R. Haddad, A.M. Duffield. J. Chromatogr. 336 (1984) 15.

8. S. Perlman, J.J. Kirschbaum. J. Chromatogr. 357 (1986) 39.

9. K.J. Williams, A.Li Wan Po, W.J. Irwin. J. Chromatogr. 194 (1980) 217.

10.   M. Tsimidou, R. Macrae. J. Chromatogr. Sci. 23 (1985) 155.

11.   N.E. Hoffman, S.Pan, A.M. Rustum. J. Chromatogr. 465 (1989) 189.

12.   D. Vukmanic, M. Chiba. J. Chromatogr. 483 (1989) 189.

13.   G.E. Berendsen. Ph.D. Thesis, Delft, The Netherlands, (1980).

14.   J.P. Foley, J.G. Dorsey, Anal. Chem., 55 (1983) 730.