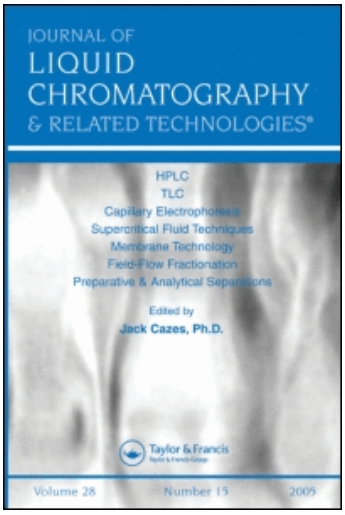

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS OF NITROSAMINES. II. ACYCLIC NITROSAMINES

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ABSTRACT

The separation of acyclic nitrosamines by HPLC using a β-cyclodextrin bonded silica gel column and a C_{18} reversed phase column was evaluated. Four groups of nitrosamines were used to evaluate the utility of both columns: (1.) methylmethyl-, methylethyl-, methylpropyl and methylbutyl-; (2.) ethylmethyl-, ethylethyl-, and ethylpropyl-; (3.) propylmethyl-, propylpropyl-, and propylbutyl-; and (4.) butylmethyl-, butylethyl-, butylpropyl-, and butylbutyl nitrosamines.

The results show that both columns performed well in separating the nitrosamines in each group, with the C_{18} column requiring a higher

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percentage of organic modifier (methanol) than the β-cyclodextrin column. The β-cyclodextrin column was superior in separating all the E to Z isomers of short chain alkyl nitrosamines, while the C_{18} column was superior in separating long alkyl chain nitrosamines and their E and Z isomers.

It was also found that the longer the alkyl chain the longer the retention time, while nitrosamines having the same number of carbons, for example ethylbutyl-and propylpropyl- eluted at virtually the same time, using both columns.

INTRODUCTION

In a previous study (1) we showed that the separation of cyclic nitrosamines by HPLC using a β-cyclodextrin bonded silica gel column and a mobile phase of methanol water was possible. In this study the separation of acyclic nitrosamines using basically the same column and mobile phase mixture was undertaken. In addition, the results obtained were compared to those achieved by using a reversed phase C_{18} column and methanol/water. Four groups of nitrosamines were used for this study: (1) methylmethyl-, methylethyl-, methylpropyl- and methylbutyl nitrosamines; (2) Ethylmethyl-, ethylethyl-, and ethylbutyl nitrosamines; (3) propylmethyl-, propylpropyl-, and propylbutyl nitrosamines; and (4) butylmethyl-, butylethyl-, butylpropyl-, and butylbutyl nitrosamines. In addition, longer alkyl chain nitrosamines were studied to evaluate the utility of the β-cyclodextrin, γ-cyclodextrin bonded silica gel and a C_{18} reversed phase columns.

Effect of volume of organic modifier in the mobile phase on the separation of the E and Z isomers of nitrosamines, where applicable, was evaluated.

EXPERIMENTAL

Materials

The nitrosamines used in this study were synthesized in house and their structures were confirmed by mass spectrometry and nuclear magnetic

resonance. Methanol was glass distilled uv grade (Burdick and Jackson, Muskegon, MI). Water was deionized glass distilled. The β-cyclodextrin (Cyclobond I) and γ-cyclodextrin (Cyclobond II) bonded silica gel columns were purchased from Advanced Separations Technologies, Inc. (Whippany, NJ). The reversed phase C_{18} column Partisil 5μ ODS-3 was purchased from Whatman. All columns had the same dimensions, 25cm x 4.6mm.

Apparatus

A Hewlett-Packard Model 1090 Liquid Chromatograph equipped with a photodiode array detector, an automatic injector, a strip chart recorder, a Hewlett-Packard Model 3392A integrator and a Hewlett-Packard Model 85 computer/controller was used. Cyclobond I, Cyclobond II and C_{18} reversed phase columns were used. Five μl of solution were injected, unless specified, and the absorption was monitored at 254 nm. The mobile phase was methanol/water, which was filtered and degassed before use and maintained under helium throughout the experiment. Mobile phase flow rate was 1 ml/min.

^{1}H and ^{13}C NMR spectra were obtained at 200MHz and 50.3MHz, respectively, on a Varian XL200 NMR spectrometer equipped with an ADVANCE data system. The same solutions (ca. 0.05 ml of solute in 0.5 ml of "100%" MSD $CDCl_3$) were used for both ^{1}H and ^{13}C. To rule out the possibility of solvent effects on the ratio of isomers, quantitative ^{1}H and ^{13}C spectra of methylethyl nitrosamine and ethylbutyl nitrosamine were obtained using the same concentrations in methanol-d_4/deuterium oxide that were used for HPLC evaluation. The following internal references were used: ^{1}H: $CDCl_3$ - TMS; MeOD-D_2O-TSP and ^{13}C: $CDCl_3$, solvent assigned δ = 77.00 and MeOD-D_2O, methanol assigned δ = 49.00. All spectra were obtained at ambient

temperature, ca. 19°C. Quadrature detection was used for all spectra. Quantitative ^{1}H spectra were obtained with a flip angle of 20° (PW90=22.5 μsec.), a SW = 3000 Hz, an acquisition time of 4.0 sec., with zero filling of 32K, and a preparation period of 30 sec. Quantitative ^{13}C spectra were obtained with a flip angle of 47° (PW=9.6μsec), a SW = 12500 Hz, an acquisition time of 1 sec., with a zero filling of 64K, a preparation period of 20 sec. and with hardware Waltz decoupling of ca. 0.5 watt (YH_2 = 2KHz).

RESULTS AND DISCUSSION

The separation of all the nitrosamines in the four groups using the β-cyclodextrin column was achieved by employing a mobile phase of 30% methanol/water (Table 1). The results show that although all the nitrosamines within a group were separated, some gave two peaks while others gave a shoulder and the rest gave one peak. Those which appeared as a shoulder at 30% methanol/water (methylpropyl- and methylbutyl nitrosamines) gave 2 peaks when 10% methanol/water was used. It was suspected that the two peaks obtained for some of the nitrosamines were the E and Z isomers. Nuclear magnetic resonance data (for example, Fig. 1 and 2) show that the 2 peaks obtained are the E and Z isomers, the ratios of which are given in Table 2. (This is discussed in detail later). The ratios obtained are consistent with steric effects; for example, the ratio of E to Z isomers of methylethyl nitrosamine is 3:1, while that of ethylbutyl nitrosamine is 1:1. Also, the NMR and chromatographic quanitificaiton data were comparable.

Unlike some of the E and Z isomers of cyclic nitrosamines, which were not resolved at room temperature (2), the E and Z isomers of these alkyl nitrosamines were found to be very stable not only at room temperature but at higher temperatures, ranging up to 70°C. For example, the E and Z isomers of ethylbutyl nitrosamine were partially resolved at elevated temperatures, Table 3.

Table 1

Separation of nitrosamines on a β-cyclodextrin column using 30% methanol/water and 10% methanol/water.

30% Methanol/Water (Rt/min.)	α	Compound	10% Methanol/Water Rt(min.)	α
3.57	-	Me-N-Me NO	4.3	-
4.2, 4.4	1.05	Me-N-Et NO	6.6, 7.3	1.11
5.1 (sh*)	1.0	Me-N-Pr NO	9.9, 11.2	1.13
6.0 (sh*)	1.0	Me-N-Bu NO	16.5, 18.2	1.10
4.2, 4.4	1.05	Et-N-Me NO	6.6, 7.3	1.11
5.6	-	Et-N-Et NO	-	-
7.8, 9.0	1.15	Et-N-Bu NO	-	-
5.0 (sh)*	1.0	Pr-N-Me NO	9.9, 11.2	1.13
7.3	-	Pr-N-Pr NO	-	-
9.2, 9.9	1.08	Pr-N-Bu NO	-	-
6.0 (sh)*	1.0	Bu-N-Me NO	16.5, 18.2	1.10
7.6, 8.9	1.17	Bu-N-Et NO	-	-
9.8, 10.6	1.08	Bu-N-Pr NO	-	-
13.5	-	Bu-N-Bu NO	-	-

* sh = shoulder

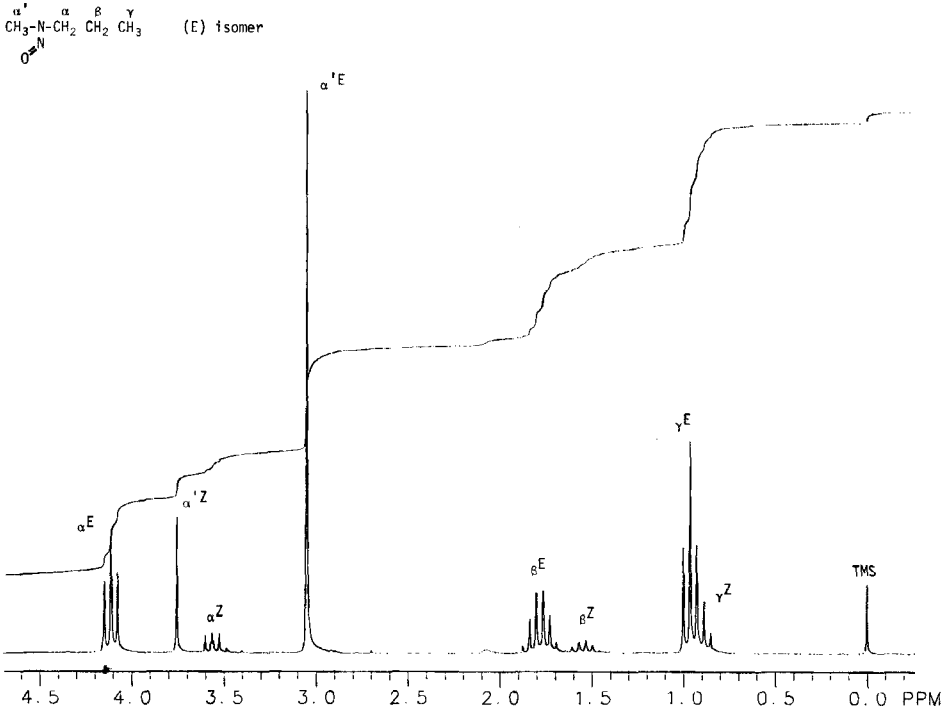


Figure 1. Typical ^{1}H NMR spectrum of a mixture of E and Z isomers of methylpropyl nitrosamine in $CDCl_3$ (see Experimental section for details).

Table 1 also shows, that within a group, methyl-, ethyl-, propyl-, butyl-, the longer the alkyl chain, the longer the retention time. Nitrosamines having the same number of carbons, for example, propylpropyl- and ethylbutyl; and methylpropyl- and ethylethyl-, eluted at almost the same retention times (Table 4).

Table 5 shows the separation of the four groups of nitrosamines using a reversed phase C_{18} column and a mobile phase of methanol/water. The results obtained for the separation of the nitrosamines in the four groups are comparable to those obtained with the β-cyclodextrin column except for the E

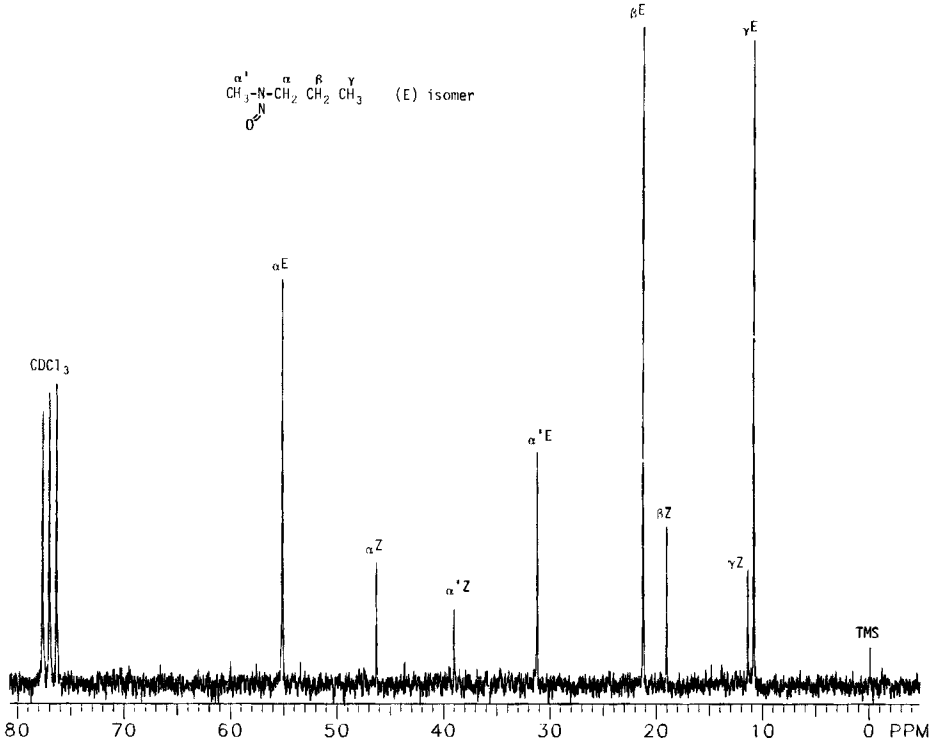


Figure 2. Typical ^{13}C NMR spectrum of a mixture of E and Z isomers of methylpropyl nitrosamine in $CDCl_3$ (see Experimental section for details).

and Z isomers. When the ß-cyclodextrin column was used not all the E and Z isomers in the tested mixtures were resolved in 30% methanol/water, but all were separated in 10% methanol/water (Table 1). With the reversed phase column the E and Z isomers were resolved in 30% methanol/water except for the propylbutyl nitrosamine which eluted after 99.5 minutes as one peak (Table 5).

Another interesting observation of comparing the separation using both columns (Tables 1 and 5) is the different mechanism of separation. This is apparent when the retention times are compared. Note that all the

TABLE 2

Quantitative data of E and Z isomers of nitrosamines separated on ß-cyclodextrin column and a mobile phase of 10% methanol/water.

Compound	% E	% Z
Me-N-Et N O	71.9	28.1
Me-N-Pr N O	72.8	27.2
Me-N-Bu N O	71.5	28.5
Et-N-Bu N O	55.4	44.6
Pr-N-Bu N O	55.7	44.3

Table 3

Effect of temperature on the separation of E and Z isomers of ethylbutyl nitrosamine on ß-cyclodextrin column and a mobile phase of 30% methanol/water (in text)

Temperature	Rt_E, Rt_Z	Δ^*	α^{**}
28°	7.55, 8.60	1.05	1.14
40°	5.75, 6.46	0.61	1.11
50°	4.98, 5.40	0.42	1.08
60°	4.40, 4.68	0.28	1.06
70°	4.03, 4.19	0.16	1.04

* $\Delta = Rt_Z - Rt_E$

** $\alpha = Rt_Z/Rt_E$

Table 4

Separation of nitrosamines having the same number of carbon atoms on β-cyclodextrin and C_{18} columns.

Compound/ Mobile phase	β-cyclodextrin		C_{18}	
	30% MeOH (min.)	20% MeOH (min.)	30% MeOH (min.)	50% MeOH (min.)
Me-N-Pr N O	5.1	6.7, 7.3	10.4, 11.6	5.7, 5.9
Et-N-Et N O	5.6	7.4	10.5	5.6
Et-N-Bu N O	7.7, 8.9	9.4, 10.8	45.1, 47.4	12.2, 12.6
Pr-N-Pr N O	7.3	9.1	41.0	11.8

experimental conditions such as flow rate, mobile phase composition, injection volume, temperature of mobile phase and column, and column dimensions are the same except for the column packings. For example, at 30% methanol/water the ethylethyl- eluted in 5.6 min. (β-cyclodextrin) and in 10.5 min. (C_{18}); propylpropyl- eluted in 7.3 min. (β-cyclodextrin) and in 41.0 min. (C_{18}); and butylbutyl- eluted in 13.5 min. (β-cyclodextrin) but did not elute after 100 min. from the C_{18} column. This may be attributed to the fact that the separation mechanism using a β-cyclodextrin bonded silica gel column is an inclusion mechanism where the larger molecule takes longer to elute. This has been found to be consistent with the experimental data,

Table 5

Separation of nitrosamines on reversed phase C_{18} in 30% methanol/water and 50% methanol/water

	30% Methanol	α	50% Methanol	α
Me-N-Me NO	4.6	-	3.9	-
Me-N-Et NO	6.4, 6.7	1.05	4.5	1.0
Me-N-Pr NO	10.4, 11.6	1.12	5.7, 5.9	1.04
Me-N-Bu NO	21.6, 24.6	1.14	8.2, 8.8	1.07
Et-N-Me NO	6.4, 6.7	1.05	4.5	1.00
Et-N-Et NO	10.5	-	5.6	-
Et-N-Bu NO	45.1, 47.4	1.05	12.2, 12.6	1.03
Pr-N-Me NO	10.4, 11.6	1.12	5.7, 5.9	1.04
Pr-N-Pr NO	41.0	-	11.8	-
Pr-N-Bu NO	99.5	1.00	20.2	1.00
Bu-N-Me NO	21.6, 24.6	1.14	8.2, 8.8	1.07
Bu-N-Et NO	45.1, 47.4	1.05	12.2, 12.6	1.03
Bu-N-Pr NO	99.5	1.00	20.2	1.00
Bu-N-Bu NO	-	-	37.1	-

which also suggests that the length -to-breadth ratio of each nitrosamine may be important. The larger the ratio the longer the elution time. With the C_{18} column the retention of the molecules in the columns is much stronger than with the β-cyclodextrin column. Since the nitrosamine mixtures are aliphatic and the C_{18} is aliphatic in nature, the old theory of likes dissolves likes is especially true. The interaction of the solute (nitrosamine) with the stationary phase (C_{18}) is much stronger than with the eluent (methanol/water) which is less hydrocarbon in nature. As mentioned earlier, we can not talk of solute solubility effects because in both cases, C_{18} and β-cyclodextrin columns, the same mobile phase (30% methanol/water) was used. This is a case where the longer the alkyl chain, the more hydrocarbon in nature the molecule and the more " soluble" it is in the C_{18} group of the stationary phase.

Figure 3 shows the separation of another group of nitrosamines using a β-cyclodextrin column, and a mobile phase of 30% methanol/water. The elution order is consistent with the inclusion mechanism, where branching of the molecule promotes solute stationary phase interaction and causes it to elute at a later time than a straight akyl chain molecule. This is true if we examine peaks 4 and 6 in Figure 3 which belong to branched molecules.

Figure 4 compares the separation of long chain alkyl nitrosamines, $CH_3(CH_2)_7\text{-}\underset{\underset{O}{\|}}{N}\text{-}CH_3$, $CH_3(CH_2)_8\text{-}\underset{\underset{O}{\|}}{N}\text{-}CH_3$ and $CH_3(CH_2)_9\text{-}\underset{\underset{O}{\|}}{N}\text{-}CH_3$, by the β-cyclodextrin column (45% methanol/water) and reversed phase C_{18} column (75% methanol/water). The results show that the C_{18} column gives better resolution of the E and Z isomers of these nitrosamines. The results also show the poor interaction (solubility) of these long alkyl chain molecules with the mobile phase (45% methanol/water).

Increasing the volume of the organic modifier resulted in poorer resolution of the E and Z isomers using the β-cyclodextrin column. Another

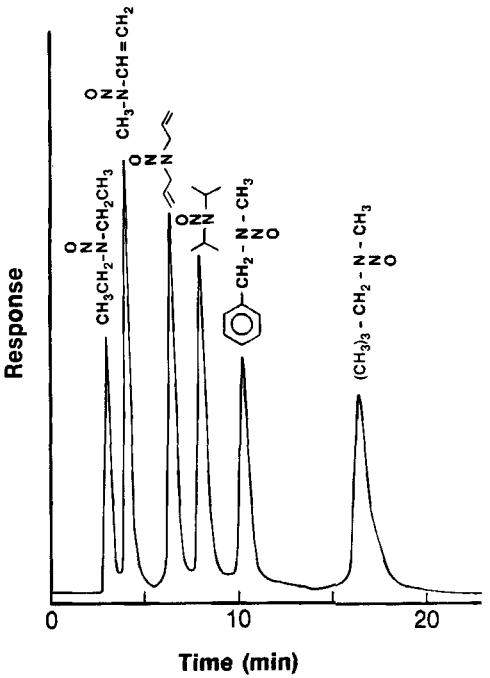


Figure 3. Separation of short alkyl chain nitrosamines using a β-cyclodextrin column and a mobile phase of 30% methanol water.

explaination for the broadness of the peaks is that these molecules are larger than the β-cyclodextrin cavity (7.5 A°), which results in a strong inclusion complex. This was found to be true using the γ-cyclodextrin which has a larger cavity (9 A°) bonded silica gel column, Figure 5, which shows that at 45% methanol/water the peaks eluted in much faster times than with the β-cyclodextrin column, compare Figures 4(b) and 5(a). Figure 5(b) shows that decreasing the volume of the organic modifier from 45% to 40% increased the retention time from 7.5 min. to 12.5 min. for the last eluting peak. Since no column is suitable for the separation of all types of molecules, it is clear that the cyclodextrin columns fall into that category.

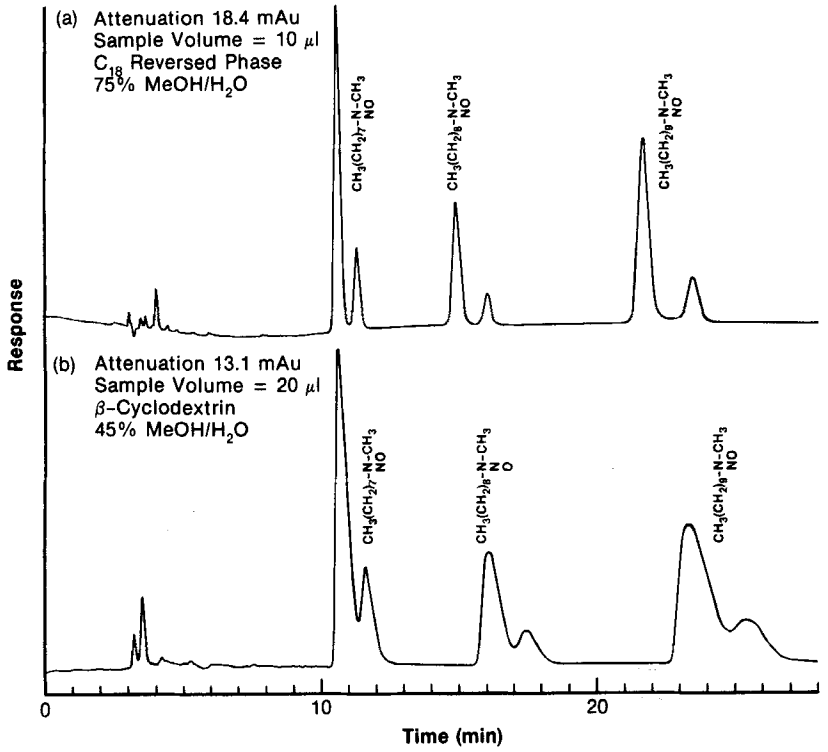
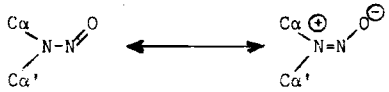


Figure 4. Separation of long alkyl chain nitrosamines by (a) β-cyclodextrin column and (b) C_{18} reversed phase column.

NMR spectroscopy assignments:

NMR spectroscopy was used to identify the Z (cis, syn) and E (trans, anti) isomers and to confirm quantitatively the ratios of these isomers. The following resonance structures of the N-nitrosamino group and α-carbons produce a planar structure with double bond character between the two nitrogen atoms resulting in hindered rotation about the N-N bond.



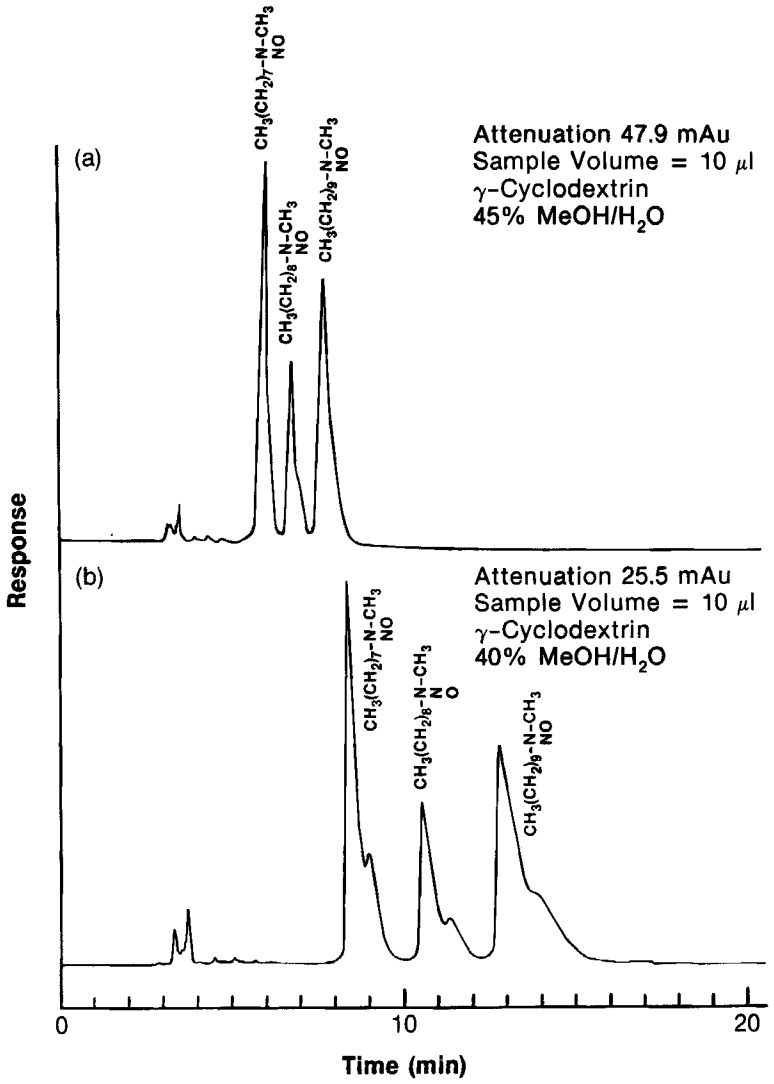


Figure 5. Separation of long chain nitrosamines using a Y-cyclodextrin column and a mobile phase of (a) 45% methanol/water and (b) 40% methanol/water.

This fact is discussed in detail by Cooney, et al. (3). The proton NMR shielding effects can be seen if the shielding cones are described as a semicircular shielding zone bent towards the Z side (4). That is, the methyl (methylene) group in a Z configuration will be in the shielding cone and appear upfield to that of a group in an E configuration. In Figure 1, the proton NMR spectrum of methylpropyl nitrosamine in $CDCl_3$ is shown. The α CH_2(t) centered at δ=3.56 is therefore assigned to the α CH_2 cis to the NO group because it is shielded with respect to the α CH_2(t) centered at δ=4.12 for the α-CH_2 trans to the NO group. The CH_3(s)'s are then assigned as follows: δ=3.76 trans to the NO group and δ=3.05 cis to the NO group. Using these assignments, the major component is the E isomer, i.e. α CH_2 trans to NO group, α CH_3 cis to the NO group. The proton spectra for the other compounds were assigned in a similar manner.

Carbon-13 NMR spectra (Figure 2) show similar results, i.e. the major isomer has CH_2 at lower field and CH_3 at higher field than those for the minor isomer. However, the magnitudes of the chemical shift differences can not be explained on the basis of shielding cones alone, although the effect is certainly present. (Assignments of the CH_2 vs. CH_3 carbons were made on the basis of APT (5) spectra and the large chemical shift differences between carbon attached to nitrogen and carbon attached to carbon. The assignment of the major component to the E isomer could also be made from the proton NMR results on the same sample.) The E - Z α-carbon difference in chemical shift has been attributed to electric field effects (6) or steric compression effects at the Z α carbon (7). Fraser and Grindley (7) make a comparison to the steric compression arguments accepted for amides and oximes and support the fact that it should be true for nitrosamines as follows. For α carbons, Δδ values are about 11 ppm for each rigid nitrosamine in their study while the magnitude is reduced in more flexible

dialkyl nitrosamines studied by Pregosin and Randall (8). (Hawkes et al. (9) support the steric compression argument for ketoximes in the case of cyclobutanone oxime which exhibits a very small difference in chemical shifts for the E and Z α carbons. These carbons are in a less hindered environment because of the cyclobutyl ring constraints). Until calculations show otherwise (6), we tend to favor the steric compression arguments of Fraser et al. (7) to explain the magnitude of the ^{13}C shift differences observed.

To insure that solvent effects were unimportant to the E and Z assignments and subsequently their quantitative ratios, several samples with concentrations and solvent mixtures identical to those in the HPLC studies were investigated by both ^{1}H amd ^{13}C NMR. No significant differences were found.

CONCLUSION

The results show that the separation of aliphatic nitrosamines by HPLC using reversed phase C_{18} or cyclodextrin bonded columns is possible. It also appears that both reversed phase and β-cyclodextrin columns complement each other, in the separation of the E and Z isomers, with the C_{18} being more efficient when the alkyl groups on both sides of the nitroso group are widely different, while β-cyclodextrin is more efficient when the size of the aliphatic groups differ by a methyl- or ethyl- group.

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