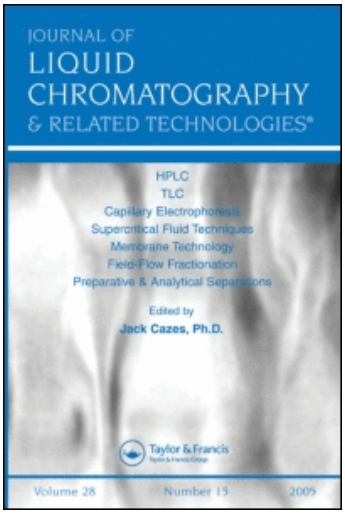

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS OF NITROSAMINES. IV. EFFECT OF TEMPERATURE ON THE SEPARATION OF NITROSAMINO CONFORMERS

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ABSTRACT

The separation of the syn and anti conformers of a selected group of nitrosamines was achieved only after the temperature of the cyclodextrin bonded silica column was lowered to ~20°C. In certain cases it was apparent that a lower temperature is required for baseline separations. The mobile phases used were 90% acetonitrile/10% triethylammonium acetate, pH 5 for the nitrosamino acids, and tetrahydrofuran for the cyclic nitrosamino isomers.

INTRODUCTION

Separations by high performance liquid chromatography (HPLC) are normally achieved at room temperature. However, in certain cases it may be necessary to carry out the experiment at above or below room temperature to

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achieve an acceptable separation. The separation of cyclic (1), acyclic (2) and conformers of nitrosamino acids (3) were carried out by high performance liquid chromatography (HPLC) at room temperature using β-cyclodextrin bonded silica and a mobile phase of acetonitrile/triethylammonium acetate (TEAA) or methanol/TEAA. The effect of pH on the separation of nitrosamino acid conformers was also investigated (3). The separation of the syn and anti conformers of the five membered ring nitrosamino acids was possible at room temperature, however; resolution of the conformers of the six member nitrosamino acids was not. These conformational isomers of heterocyclic nitrosamines, which are in equilibrium at room temperature owing to rotation about the N-N bond, may be resolved by HPLC if the rate of rotation could be slowed by reducing the temperature. This lead us to believe based on previous experience (4) that if the temperature of the column was lowered to below zero, the resolution of the isomers might be possible.

This work, therefore, deals with effect of (a) temperature and (b) ring substitution on the HPLC separation of the syn and anti conformers of a selected group of six membered ring nitrosamino acids and nitrosamines.

EXPERIMENTAL

Materials

The nitrosamines used in this study, N-nitrososarcosine (NSAR), N-nitrosothiazolidine carboxylic acid (NTCA), N-nitrosopipecolinic acid (NPPC), N-nitrosonipecotic acid (NNPC), 1,3,5-trinitroso-1,3,5-hexahydrotriazine (TNHH), 2,5-dimethyldinitrosopiperazine (2,5-DMDNP), 2,6-dimethyldinitrosopiperazine (2,6-DMDNP) and 2-methyldinitrosopiperazine (2MDNP), and dinitrosopiperazine (DNP) were synthesized in house, and their structures were confirmed by elemental analysis, mass spectrometry and nuclear magnetic resonance. Acetonitrile and tetrahydrofuran were glass-distilled UV grade (Burdick and Jackson). Water was deionized glass distilled. The 5 μm spherical

prepacked α-cyclodextrin (cyclobond III) bonded silica gel columns (150 x 4.6 mm I.D., and 250 x 4.6 mm I.D.) were purchased from Advanced Separations Technologies (Whippany, NJ).

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.

Procedure

Solutions were prepared in water to contain approximately 0.5 µg/µl. A 10 µl volume of the solution was injected, unless specified. The mobile phases were made of either pure THF or acetonitrile-triethylammonium acetate (TEAA), which was filtered and degassed before use and maintained under helium throughout the experiment. Mobile phase flow-rate was 1.5 ml/min. Absorption was monitored at 238 nm.

The TEAA solution (0.01 M) was prepared by adding 1.4 ml triethylamine to 1.0 l of water and then titrating with acetic acid to pH 5. Throughout this manuscript, TEAA will refer to a 0.01 M TEAA, pH 5 solution. pH measurements were made using a Fisher Accumet brand pH meter Model 750. The low temperatures were achieved by immersing the column and the mobile phase in an ethylene glycol/water bath. The temperature was maintained constant (±0.1°C) by using a refrigerated bath and circulator (Haake, Model 81, Saddlebrook, NJ).

RESULTS AND DISCUSSION

In our previous work (3) which dealt with the separation of nitrosamino acids and their *syn* and *anti* conformers, it was found that the conformers of

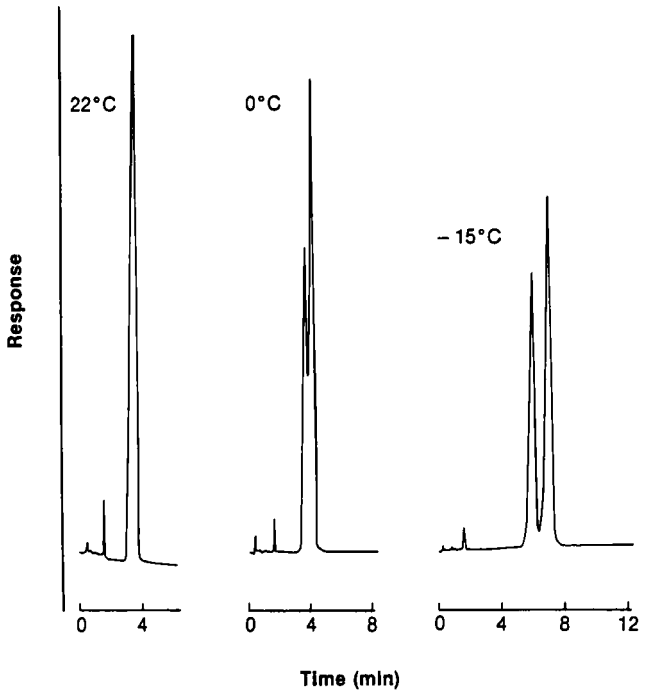


Figure 1. Chromatograms of the separation of the syn and anti conformers of N-nitrosopipecolonic acid at 22°C, 0°C and -15°C using a 150 x 4.6 mm α-cyclodextrin (cyclobond III) bonded silica gel column, 5 µm spherical, and a mobile phase of 90% acetonitrile/0.01 M TEAA, pH 5, at a flow rate of 1.5 ml/min. Detection was carried out at 238 nm.

the five membered ring nitrosamino acids were resolved at room temperature, while those of the six membered ring nitrosamino acids were at best partially resolved. It was also mentioned in that work (3) that lowering the temperature of the analysis will result in the resolution of the conformers of NPPC and NNPC (Figures 1 and 2). It is clear from the two figures that the lower the temperature the better the resolution. Using 90% acetonitrile/10%

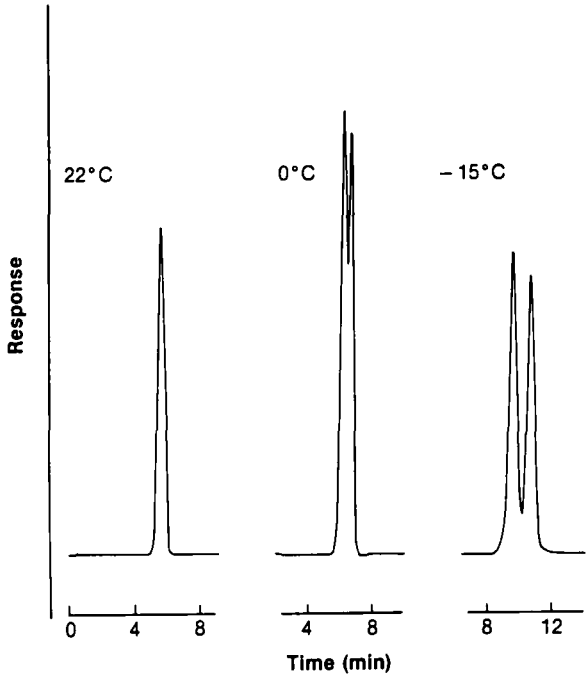


Figure 2. Chromatograms of the separation of the syn and anti conformers of N-nitrosonipecotic acid. Experimental conditions as in Figure 1.

TEAA, -15°C was the lowest temperature possible without freezing the mobile phase. In our previous work (3), a partial separation of NPPC and NNPC was achieved at room temperature using 70% acetonitrile/TEAA as the mobile phase; however, this mobile phase was not suitable for sub-zero temperatures. An improvement in the resolution of NPPC and NNPC was obtained when a 250 x 4.6 mm rather than 150 x 4.6 mm column was used (Figure 3).

Lowering the temperature also improves the resolution of other N-nitrosamino acid conformers. For example, syn and anti isomers of the heterocyclic NTCA are better resolved at lower temperature (Figure 4). This phenomenon is not unique only to cyclic or heterocyclic acids. The conformers

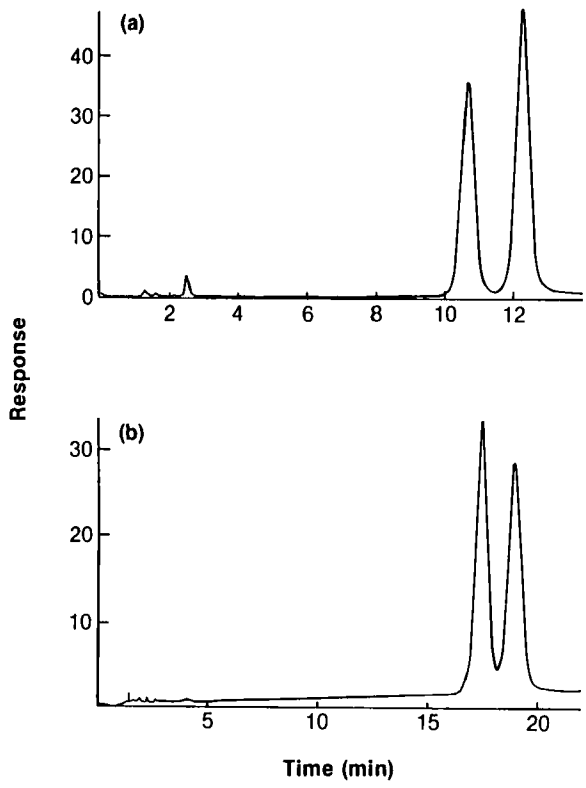


Figure 3. Chromatograms of the separation of the syn and anti conformers of (a) NNPC and (b) NPPC. Experimental conditions as in figure 1 using a 250 x 4.6 mm column.

of NSAR, an aliphatic nitrosamino acid, are better resolved at lower temperatures (Figure 5).

Effect of ring substitution on the separation of piperazines:

A probe of the effect of (a) ring substitution and (b) temperature on the resolution of the isomers of nitrosopiperazines was undertaken. The compounds studied are TNHH, DNP, 2MDNP, 2,5-DMDNP and 2,6-DMDNP. Nuclear

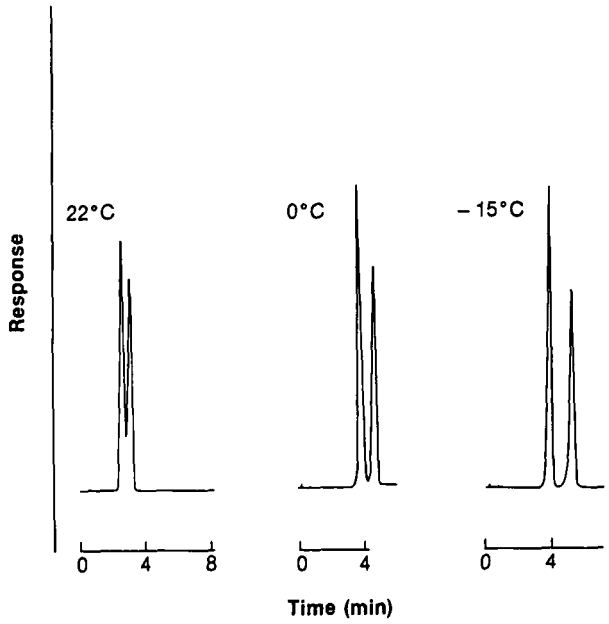
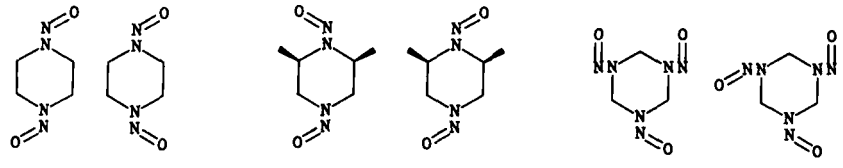


Figure 4. Chromatograms of the separation of the syn and anti conformers of N-nitrosothiazolidine-4-carboxylic acid. Experimental conditions as in Figure 1.

magnetic resonance data (6) show the presence of two isomers for both DNP and 2,6-DMDNP, corresponding to the cis or trans arrangements of the two N-nitroso groups and two isomers for TNHH as shown in chart 1.

Chart 1



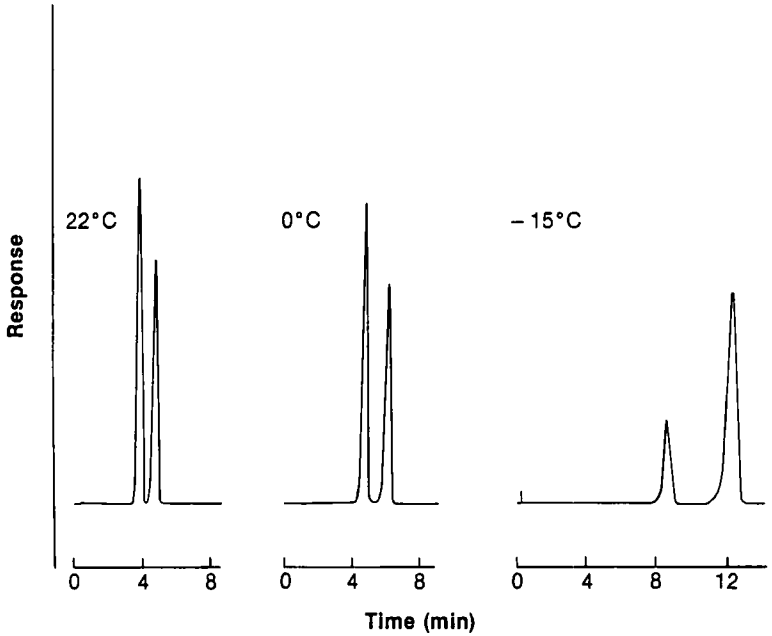


Figure 5. Chromatograms of the separation of the syn and anti conformers of N-nitrososarcosine. Experimental conditions as in Figure 1.

Three isomers were reported (6) for 2,5-DMDNP which were designated as cis, trans-syn and trans-anti. The syn and anti indicate the orientation of the N-nitroso groups with respect to the methyl substituents on the adjacent ring carbon atoms (chart 2).

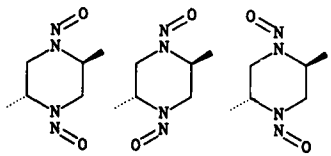


Chart 2

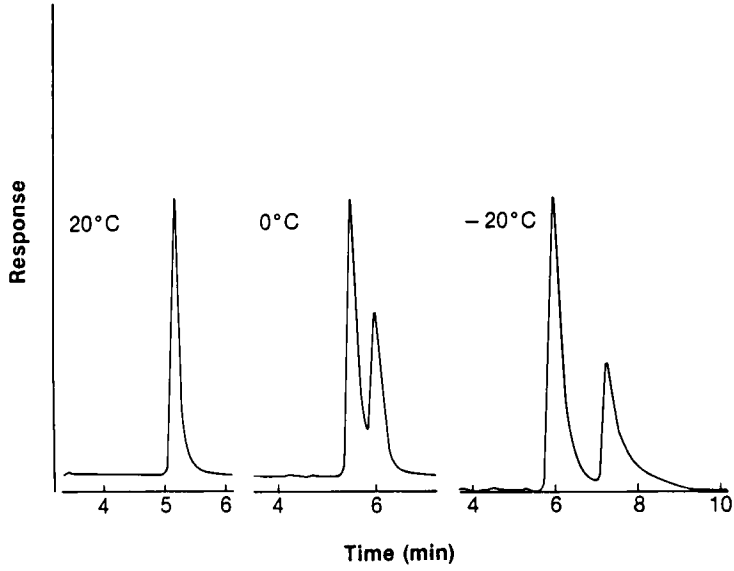


Figure 6. Chromatograms of the separation of the syn and anti isomers of 1,3,5-trinitroso-1,3,5-hexahydrotriazine at 20°C, 0°C and -20°C using a 250 x 4.6 mm α-cyclodextrin (cyclobond III) bonded silica gel column, 5 µm spherical, and a mobile phase of neat tetrahydrofuran, at a flow rate of 1 ml/min. Detection was carried out at 238 nm.

DMDNP was reported (7) to have four isomers (chart 3).

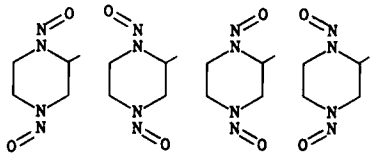


Chart 3

Figure 6 shows that the two isomers of TNHH are almost baseline resolved at -20°C. The ratio of the concentrations of the isomers, measured by the areas under the two peaks is 2:1.

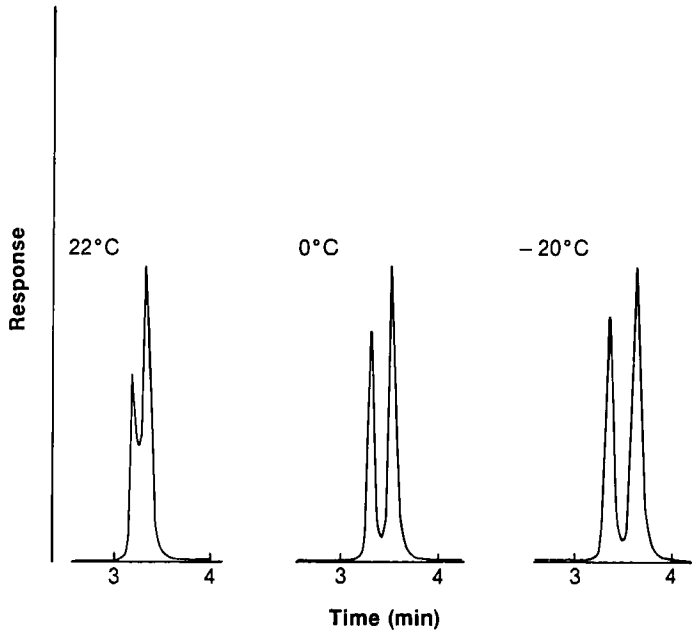


Figure 7. Chromatograms of the separation of the syn and anti conformers of dinitrosopiperazine. Experimental conditons as in Figure 6.

Figure 7 is a chromatogram of the resolution of the cis and trans isomers of DNP. At 22°C partial resolution of the isomers is observed while at -20°C baseline resolution was achieved.

A comparison of the chromatograms in Figures 6 and 7 reveals that although DNP was resolved at room temperature, the three nitroso substituted ring compound gave only one sharp peak which was not resolved into two peaks until the temperature was lowered at 0°C, Figure 6. However, at -20°C the conformers of these two compounds were equally resolved. This lead to a study of the effect of the substitution on the ring.

Figure 8 shows the separation of the isomers of 2MDNP. The figure shows that although improvement in the resolution was observed by lowering the

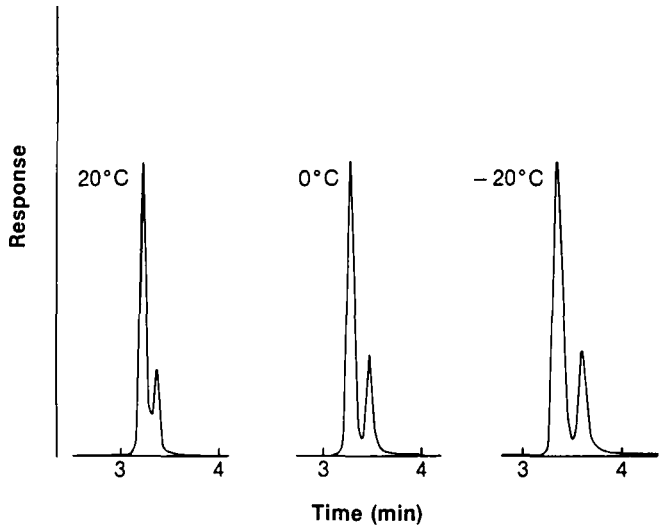


Figure 8. Chromatograms of the separation of syn and anti isomers of 2-methyl-dinitrosopiperazine. Experimental conditions as in Figure 6.

temperature from 22°C to 0°C, there was no appreciable improvement in the resolution when the temperature was lowered from 0°C to -20°C. As mentioned earlier (7), this compound should have 4 isomers. It is possible that a much lower temperature is needed for this separation as was observed earlier (4) when silica gel thin layer chromatography was used. The temperature had to be lowered to -77°C to achieve complete resolution of syn and anti conformers of nitrosopiperazines. This is especially true for 2,6-DMDNP (Figure 9) and 2,5-DMDNP (Figure 10) where complete resolution of the conformers at -20°C was not possible.

A comparison of the chromatograms of 2MDNP, 2,6-DMDNP and 2,5-DMDNP, Figures 8-10, reveals that when one methyl group is on the ring (2-MDNP) the resolution of only two of the four isomers is possible. This may be due to

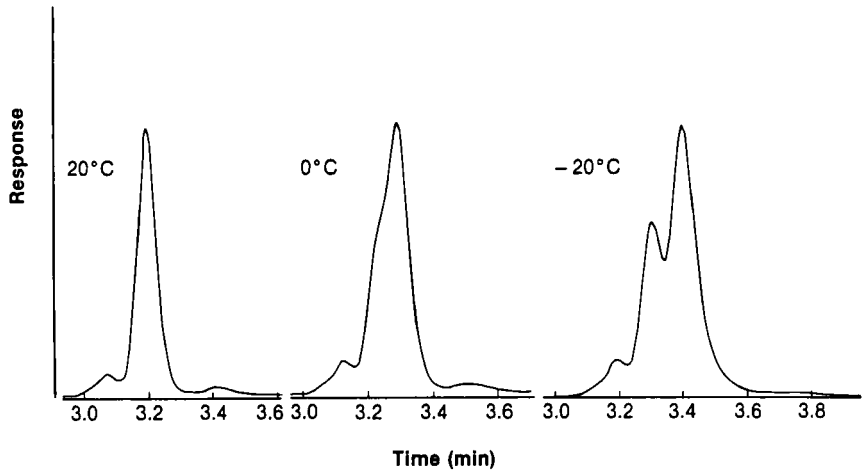


Figure 9. Chromatograms of the separation of the syn and anti isomers of 2,6-dimethyldinitrosopiperazine. Experimental conditions as in Figure 6.

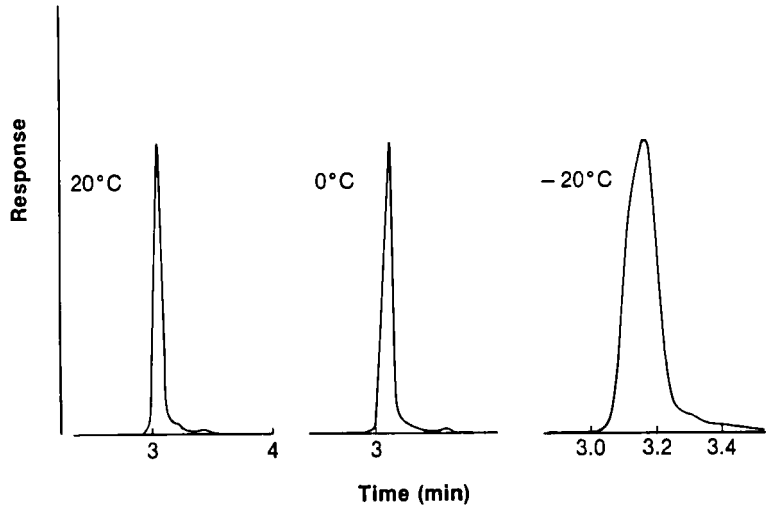


Figure 10. Chromatograms of the separation of the syn and anti isomers of 2,5-dimethyldinitrosopiperazine. Experimental conditions as in Figure 6.

the fact that the N-O of the NNO group at the 4-position is free to rotate, and so it is possible to resolve two isomers at above 0°C column temperature. On the other hand when the two N-O groups of the 2,5-DMDNP are restricted, a sub-zero temperature is required to achieve the separation of only two of the three conformers observed as only a shoulder at -20°C. However, in the case of the 2,6-DMDNP where one of N-O groups is free to rotate while the other is restricted by the two methyl groups, a shoulder and separation of the two conformers is observed at 0°C and -20°C, respectively. A small peak, an impurity, is observed at the beginning of the chromatogram.

The mobile phase used for the separation of TNHH, 2MDNP, 2,5-DMDNP and 2,6-DMDNP was tetrahydrofuran (THF). Using other conventional solvents such as methanol/TEAA or acetonitrile/TEAA with widely different proportions, these compounds eluted with the column's void volume. They also eluted faster when TEAA solution was added to THF.

CONCLUSION

The separation of the conformers of six membered N-nitrosamino acids is possible using a 90% acetonitrile/TEAA mobile phase, a 15 cm column packed with α-cyclodextrin bonded silica gel at sub-zero temperatures. The results show that while the conformers of a five membered ring nitrosamino acid (NTCA) and an aliphatic nitrosamino acid (NSAR) were resolved at room temperature, the 6 membered ring N-nitrosamino acid conformers were not.

Also, the separation of conformers of other 6 membered tri- and di-substituted nitrosamines required a sub-zero column temperature, a longer (25 cm) α-cyclodextrin bonded silica gel column and a mobile phase of pure THF.

It was observed in the case of the piperazines, that both the number of methyl groups and their position on the ring affected the temperature required for the resolution of the syn and anti isomers.

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