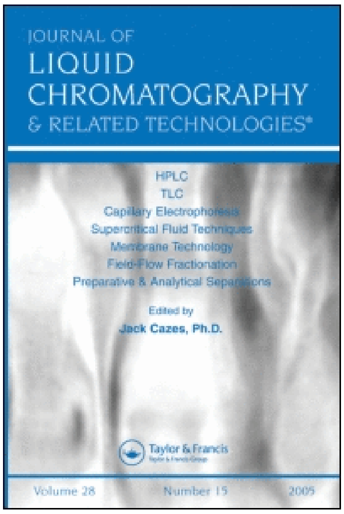

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# SEPARATION OF LABILE TERPENOIDS BY LOW TEMPERATURE HPLC

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## ABSTRACT

Semipreparative low temperature HPLC was used to isolate two sesquiterpene hydrocarbons, i. e. anastreptene and ß-barbatene, that could not be separated at ambient temperature. A linear relationship was observed between the logarithm of capacity factor and the reciprocal of column temperature. This was consistent with previous reports (4,5,8). It could be demonstrated that differences in enthalpy and entropy may cause a temperature-dependent resolution for both compounds. Low temperature $^1$H-NMR revealed that separation was not due to a fixation of conformation.

## INTRODUCTION

Recently we isolated several volatile sesquiterpene hydrocarbons from the German liverwort Sphenolobus minutus. The detailed isolation and characterization of these compounds will be described elsewhere. Because of the thermal lability of some naturally occuring terpenoids we avoided the use of preparative GLC. Furthermore preparative chromatography on $AgNO_3$-impregnated silica gel could cause isomerization of double bonds and loss of

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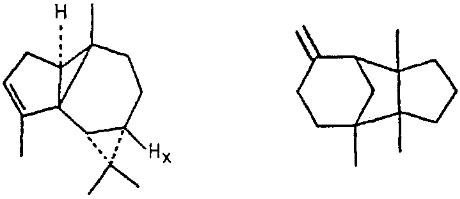

Anastreptene ß-Barbatene

FIGURE 1

substance. The two model compounds, anastreptene and ß-barbatene (figure 1) could not be separated at all on HPLC using silica gel at ambient temperature.

Temperature as a chromatographic parameter may be varied to improve resolution when conventional methods are unsuccessful (1-13). The subject has been reviewed recently by Henderson and O'Connor (14). Chang (1) investigated silica gel in the range of -55°C to +200°C and Stahl (2) employed TLC at -15°C for the separation of monoterpene hydrocarbons. Schwanbeck (3) isolated some monoterpene hydrocarbons with low temperature HPLC on silica gel at -15°C. He also tried to separate sesquiterpene hydrocarbons on the same conditions and reported that the selectivity had not been sufficient to achieve pure compounds.

Our own experiments with HPLC at low temperature however showed that separation of anastreptene/ß-barbatene in semipreparative scale was only possible at temperatures below -35°C. Through this method we obtained considerable amounts of both compounds in pure state. The results prompted us to investigate the chromatographic behavior of the compounds systematically in the range of -55°C to +65°C.

## MATERIALS AND METHODS

**Instrumentation.** HPLC (LDC/Milton Roy): two pumps constametric I and III, injector Rheodyne Model 7125, 20 µl injection loop, variable UV-detector spectro monitor D set at 208 nm and a data system MP 3000 were used. The column was 25 cm x 8 mm i. d. containing 5 µm Spherisorb SI (Merck). Temperature was set with a commercial temperature controlled bath cooler. The mobile phase was precooled before passing the pumps by the same bath cooler.

**Solvent.** A mixture of 80% n-pentane and 20% n-hexane was employed at low temperature. N-hexane was used at temperatures above 20°C. Both solvents were of HPLC grade.

**Solutes.** About 1 mg of pure anastreptene and ß-barbatene were combined and dissolved in 1 ml of the eluent.

**Procedures.** Flow rate was optimized for -40°C and determined to 1.0 ml/min. Because the precise determination of the dead time $t_0$ is very difficult (15) it was approximated by injecting n-hexane. Prior to the measurements the column was eluted with 300 ml of dichloromethane p. a. and then equilibrated by passing 300 ml of the original eluent at a flow rate of 5.0 ml/min at ambient temperature. The first sequence of measurements was carried out starting at 20°C. Subsequently, the column was cooled down in steps as given in table 1. The column was equilibrated to the set temperature for 10 minutes. The same sequence was repeated after the column had warmed up overnight proving the reproducibility of the system. Measurements at elevated temperatures followed in the same manner using n-hexane as eluent.

**$^1$H-NMR.** 250 MHz, $CDCl_3$, TMS int. stand., 293 K and 223 K.

**Calculations.** Values were calculated by means of the following formulae:

- number of theoretical plates n:

$$n = 16 \cdot \left(\frac{t_R}{w}\right)^2$$

- resolution R:

$$R = \frac{2 \cdot \Delta t_R}{w_1 + w_2}$$

- capacity factor k':

$$k' = \frac{t_R - t_0}{t_0}$$

- temperature dependence of capacity factor according to van't Hoff equation:

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi$$

$H$ : enthalpy change
$S^\circ$: entropy change
$\Phi$ : the ratio of surface area to the mobile phase volume

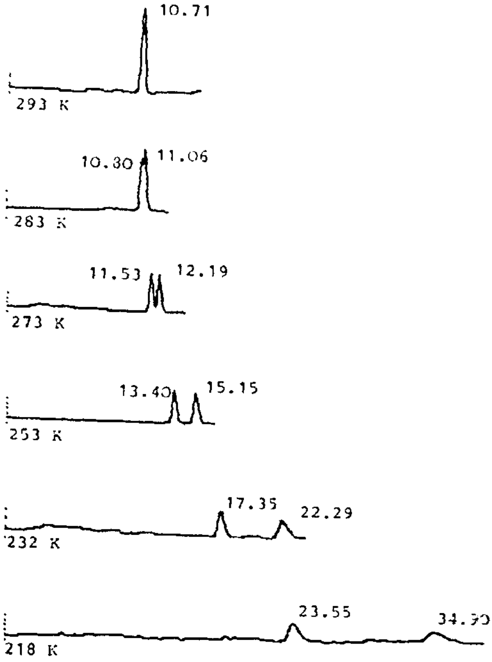

**FIGURE 2.** Temperature-dependence of the separation of anastreptene and ß-barbatene.

## RESULTS AND DISCUSSION

Due to their low polarity, both n-pentane and n-hexane appeared to be suitable solvents for the desired application. N-pentane as eluent seemed to be ideal because of its much lower viscosity (0.23 mPa·s at 20°C) compared to that of n-hexane (0.33 mPa·s at 20°C). Preliminary experiments revealed however that the use of pure n-pentane led to gas bubbles in the pump heads not adapted for external cooling. N-hexane caused unacceptable high pressure at temperatures below -25°C. Both problems could be overcome by employing a mixture of 80% n-pentane and 20% n-hexane. Chromatographical runs above +30°C were performed with 100% n-hexane due to the low b. p. of n-pentane (+36°C).

Separation characteristics are listed in table 1. Figure 2 demonstrates the development of separation with decreasing temperature. Solute retention was affected most dramatically by temper-

TABLE 1

| | Anastreptene | | | | ß-Barbatene | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| T (K) | $t_R$ (min) | $w_1$* (min) | k' | n/m* | $t_R$ (min) | $w_2$* (min) | k' | n/m* | $\Delta t_R$ (min) | R* |
| 218 | 34.90 | 3.02 | 3.23 | 8847 | 23.55 | 1.77 | 1.85 | 11330 | 11.35 | 4.74 |
| 223 | 28.82 | 2.28 | 2.49 | 10226 | 20.56 | 1.41 | 1.49 | 13608 | 8.26 | 4.48 |
| 232 | 22.29 | 1.49 | 1.70 | 14323 | 17.35 | 1.01 | 1.10 | 18886 | 4.94 | 3.95 |
| 235 | 20.63 | 1.49 | 1.50 | 12269 | 16.44 | 1.12 | 0.99 | 13789 | 4.19 | 3.21 |
| 238 | 19.99 | 1.33 | 1.42 | 14458 | 16.30 | 0.96 | 0.98 | 18451 | 3.69 | 3.22 |
| 241 | 18.99 | 1.17 | 1.30 | 16860 | 15.69 | 0.85 | 0.90 | 19334 | 3.30 | 3.27 |
| 244 | 18.04 | 1.09 | 1.19 | 17531 | 15.12 | 0.84 | 0.83 | 19134 | 2.92 | 3.07 |
| 247 | 16.80 | 1.01 | 1.04 | 17707 | 14.41 | 0.85 | 0.75 | 18051 | 2.39 | 2.57 |
| 250 | 15.91 | 0.89 | 0.93 | 20452 | 13.86 | 0.74 | 0.68 | 21452 | 2.05 | 2.52 |
| 253 | 15.15 | 0.82 | 0.84 | 21846 | 13.40 | 0.69 | 0.62 | 22992 | 1.75 | 2.32 |
| 263 | 13.43 | 0.75 | 0.63 | 20522 | 12.36 | 0.64 | 0.50 | 22196 | 1.07 | 1.54 |
| 273 | 12.19 | 0.63 | 0.48 | 23961 | 11.53 | 0.58 | 0.40 | 24627 | 0.66 | 1.09 |
| 283 | 11.06 | 0.60 | 0.34 | 21746 | 10.80 | 0.55 | 0.31 | 24678 | 0.26 | 0.45 |
| 293 | 10.71 | | 0.30 | | 10.71 | | 0.30 | | 0.00 | 0.00 |
| 303 | 10.05 | | 0.22 | | 10.05 | | 0.22 | | 0.00 | 0.00 |
| 313 | 9.48 | | 0.15 | | 9.48 | | 0.15 | | 0.00 | 0.00 |
| 323 | 9.23 | | 0.12 | | 9.23 | | 0.12 | | 0.00 | 0.00 |
| 333 | 9.01 | | 0.09 | | 9.01 | | 0.09 | | 0.00 | 0.00 |
| 338 | 8.89 | | 0.08 | | 8.89 | | 0.08 | | 0.00 | 0.00 |

*Values are calculated according to the given formula although the peaks at very low temperatures exhibited remarkable tailing. Where no values are given calculation was impossible due to unresolved peaks.

ature. At 20°C/293 K no separation of both compounds was obtained, at 10°C/283 K the beginning of separation was observed and at -55°C/218 K the difference of retention time was 11.35 minutes. A linear relationship was observed between the logarithm of capacity factor and the reciprocal of column temperature for both compounds (figure 3). The linearity indicated a constant sorption mechanism for silica gel within the investigated range of temperature according to results of Jinno and Hirata (4,5). This behavior was also in agreement to van't Hoff equation. Linear regression of the obtained data (ln k' vs. 1/T) resulted in correlation coefficients of 0.997 for anastreptene and 0.993 for ß-barbatene. Errors in the dead time measurements could be responsible for the observed non linearity at small k'. Another reason for this might be certain changes in water-content of the stationary phase at elevated

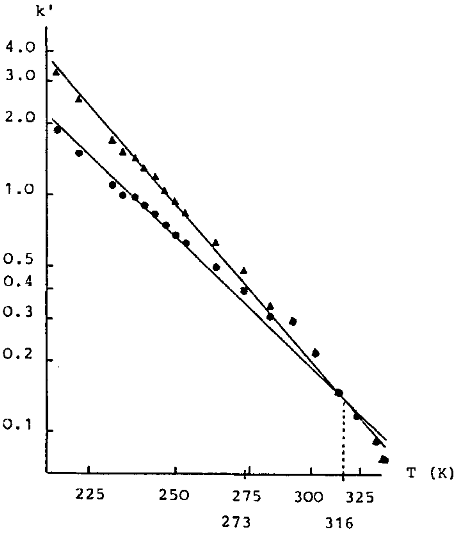

**FIGURE 3.** Plots of ln k' against 1/T. ▲ = anastreptene. ● = ß-barbatene.

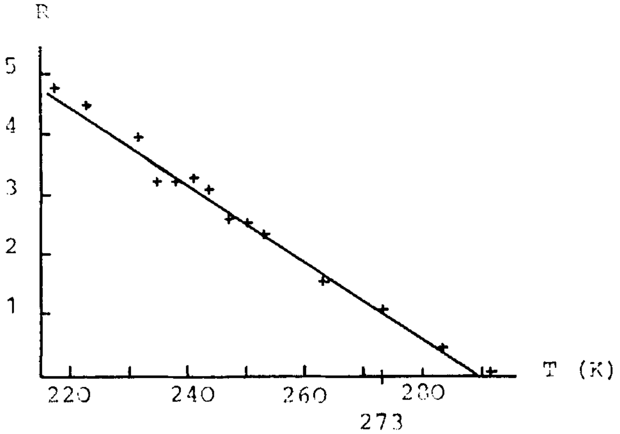

**FIGURE 4.** Plot of resolution R against T.

temperature whereas this influence seems to become unimportant at lower temperature (6).

Deep temperature $^1$H-NMR proved that separation was not induced by fixation of a conformation. Theoretically anastreptene (figure 1) could change its conformation by turning of the six-membered ring. The fixation of a thermodynamically favored conformation was disproved by measuring $^1$H-NMR at -50°C. In case of a fixed conformation a change in the coupling constants of $H_x$ and the adjacent methylene group would be expected. Such an effect was not visible with the well observable signal of $H_x$ (0.50 ppm, dt 9.5, 8.5 Hz).

Differences in conformation, anastreptene is a quite spherical molecule, might cause the differences in enthalpy and entropy of anastreptene and ß-barbatene. The possible influence of molecule shape on temperature-induced selectivity was first discussed for reversed phase chromatography by Snyder (7). Enthalpies were calculated from van't Hoff equation. $\Delta H$ of anastreptene was -18.3 $kJ \cdot mol^{-1}$ and $\Delta H$ of ß-barbatene was -15.3 $kJ \cdot mol^{-1}$. As shown in figure 3, there were also differences in entropy $\Delta S°$ which results in theoretical crossing of the two lines at 43°C/316 K. Practically no separation at elevated temperature was possible due to the very low capacity factors k'. Temperature-induced changes in the sequence of solute retention were observed by Chang (1) and recently by Takeuchi et al. (8).

Resolution R is the most important parameter for preparative chromatography. It was 0 for both solutes in the range of 20°C to +65°C but improved constantly with deeper temperature to reach the final value of 4.74 at -55°C/218 K (figure 4). Semipreparative runs with 6 mg mixture of both compounds still showed a resolution R of 1.44 at -40°C/232 K. In contrast to the improvement of R, a decrease in column temperature resulted in a reduction in number of total theoretical plates n, calculated per meter (n/m), probably due to the increase of viscosity of the mobile phase. This supports the theory that improved separation was caused by differences in enthalpy and entropy of both solutes.

## SUMMARY AND CONCLUSIONS

Temperature determined the solute retention, resolution and column efficiency. A linear dependence of ln k' on 1/T was observed for the two hydrocarbons. Their differences in enthalpy and entropy had induced separation at subambient temperature.

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