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Effect of carbon dioxide flow-rate on the separation of triacylglycerols by capillary supercritical fluid chromatography

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ABSTRACT

Triacylglycerols (TAGs) were separated using capillary supercritical fluid chromatography at various carbon dioxide densities and flow-rates at 150°C. The flow-rate was controlled with integral restrictors made directly at the end of the column. With a 50 μm I.D., d_f 0.20 μm, DB-5 [(95%)dimethyl(5%) diphenylpolysiloxane] column, separation numbers (*Tz*) of up to one per one triacylglycerol carbon number difference per metre were achieved at a practical optimum carrier flow-rate at 0.3–0.4 cm/s. Height equivalent to an effective theoretical plate–average linear carrier velocity–triacylglycerol carbon number three-dimensional plots at different pressures are presented.

INTRODUCTION

Capillary supercritical fluid chromatography (SFC) is a method that is often compared with capillary gas–liquid chromatography (GLC) with regard to efficiency. In practice, GLC always gives a better resolution per unit time than does SFC. In many laboratories the slowness of the latter method has led to the use of high carrier flow-rates in order to increase the speed of the analyses [1]. However, this approach brings about a reduced number of effective chromatographic plates.

Capillary SFC is applied to the analysis of sensitive and labile natural compounds in order to avoid thermal decomposition. As a tool in bioscience fields such as food and clinical chemistry, all the resolution and selectivity that an optimized capillary SFC analysis can provide are usually required. This is the case even at the expense of an increased duration of the analyses. Another limitation to increasing the flow-rate is a reduction in sensitivity and ionization accuracy in mass spectrometric analyses. For this reason, high carrier flow-rates cannot be recommended.

Most calculations concerning the number of theoretical plates (*n*) and the height equivalent to a theoretical plate (HETP) in SFC follow the Golay equation for non-adjusted retention times [2,3]. The height equivalent to a plate is the length of the column occupied by one theoretical plate (HETP) or by one effective theoretical plate (HEETP). This definition is always of primary importance when comparing experimental results with those reported in the literature.

The number of theoretical plates and the relative retentions of analyte mixtures in SFC are more difficult to predict according to theoretical definitions than in GLC. This is due in part to the solubility factor, which changes in relation to the density of the carrier fluid. The term "solubility" includes all the factors that affect the interactions between a solute and the mobile phase and is difficult to define accurately with a universal equation.

Increasing the carrier pressure at constant temperature results in a decrease in the k' values and resolution [4–6], even though the correlation is in practice never really smooth and regular, as was shown, *e.g.*, by Klesper [7] with naphthalene, anthracene, pyrene and chrysene with *n*-pentane as the mobile phase. At a constant pressure, k' has a maximum at a certain temperature. The shape of this curve can be explained on thermodynamic principles [8] and is independent of the mobile and stationary phases [9].

This optimization work was done to improve the mass spectrometric analysis of butter fat and fish oil triacylglycerols (TAGs). Electron impact mass spectrometry (EI-MS) in the single-ion monitoring (SIM) mode has been used in order to determine the level of unsaturation of TAGs having the same carbon numbers [10]. The main goal, however, was the determination of the positional isomers of the fatty acids in individual TAGs of natural origin. Flow-rates far above the optimum are not recommended because of the distinct decrease in sensitivity in the EI-MS analyses. For positional isomer analyses, sufficiently large α-values between the TAGs eluting close to each other are needed, although complete resolution is unnecessary. The minimum requirement is the possibility of discriminating the SIM signals of adjacent compounds from each other according to their different retentions.

The aim of this research was to optimize the size of the integral restrictor and the linear flow-rate of the supercritical carbon dioxide carrier at a constant temperature for the separation of triacylglycerols by capillary SFC using a flame ionization detector.

EXPERIMENTAL

Saturated monoacid triacylglycerols of even-numbered fatty acids from trioctanoylglycerol (3 × 8:0) to trioctadecanoylglycerol (3 × 18:0) were subjected to capillary SFC. The chromatographic system was as described previously [10]. The only difference was the 100-nl loop injector with a pneumatic helium actuator. The mobile phase was SFC-grade carbon dioxide. The column temperature was kept at 150°C and that of the detector at 320°C. The TAG samples dissolved in tricholoromethane were introduced into the fused-silica capillary column (DB-5, 3.8 m × 50 μm I.D., d_f 0.20 μm) (J&W Scientific) and a linear capillary split with a flow ratio of *ca.* 1:15 measured at a 24.1-MPa inlet pressure was used.

The carbon dioxide flow-rate was regulated with integral restrictors [11] attached directly to the end of the column. The mixture of the homologous series of TAGs was analysed at carrier inlet pressures of 24.1, 26.2, 28.3, 30.3, 32.4 and 34.4 MPa at least three times at each pressure. The analyses were carried out with seven restrictors of various sizes at each pressure. The restrictors resulted in average linear flow-rates of carbon dioxide varying from 0.34 to 1.70 cm/s at the highest column inlet pressure of 34.4 MPa.

The column hold-up time (t_0) was measured at the front slope of the solvent, trichloromethane. Overloading of the column with the solvent resulted in a t_0 value very close to the theoretical hold-up time.

RESULTS AND DISCUSSION

Optimization of the carrier flow-rate in order to reach the minimum height equivalent to a chromatographic plate was achieved by selecting the size and construction of the restrictor. The integral restrictors were attached directly to the end of the fused-silica capillary column, thus the evident dead volumes caused by the capillary–capillary connectors could be avoided. The extremely rapid pressure drop in the short restriction area also prevented the solutes from precipitating prior to the detector flame. Some clogging problems occurred, however, with very narrow pinholes yielding linear flow-rates less than the optimum on the van Deemter curve. The results presented in this paper concern conditions at and slightly above the optimum flow-rate.

All the analyses were performed isothermally at 150°C. This fairly low temperature was chosen as preliminary studies showed only a slight increase in resolution at temperatures above 150°C. The conditions were safe for TAGs, as was also shown by Proot *et al.* [1].

The splitting ratio of the carrier flow between the column and the exit was on average only 1:15. The injector device was located outside the oven and the supercritical conditions chosen for the analyses were only reached after the splitting point. The injection system with the subsequent splitting area was responsible, at least in part, for a reduced separation efficiency in comparison with the theoretical column efficiency [12].

When starting up a "cold" chromatographic system, which had been at ambient pressure and temperature with the pumps depressurized, it could take as long as 1 h before acceptable analyses were obtained. During the first run(s) the k' values of the TAG solutes were higher than those with continuously repeated, reproducible analyses.

With tight restrictors the pressure drop along the column is minimized. In this work the widest hole used generated an average linear flow-rate of 1.57 cm/s at 24.1 MPa pressure and 1.70 cm/s at 34.4 MPa. The correlation between the inlet pressure and the average linear flow-rate (measured at six pressures) was highly linear (r = 0.989) and the increase in the flow-rate was only 7.8% when the pressure was increased from 24.1 to 34.4 MPa. The observed strong influence of the fluid density on linear velocity [13,14] can, in practice, be reduced by optimizing the flow-rate of the capillary column with a tight restrictor.

Height equivalent to an effective theoretical plate (HEETP)–average linear carrier velocity ($\bar{\mu}$)–triacylglycerol carbon number (CN) three-dimensional plots at different pressures are presented in Fig. 1. When the TAG size increased, the HEETP values decreased at constant pressure. The same phenomenon was clearly shown, *e.g.* by Leyendecker *et al.* [9] with polyaromatic hydrocarbons by using *n*-pentane as the mobile phase. On the other hand, the HETP values increased with increasing triacylglycerol carbon number in isobaric runs (Fig. 2). The increase in plate height with increasing fluid velocity can be seen clearly in Figs. 1 and 2. The same effect was

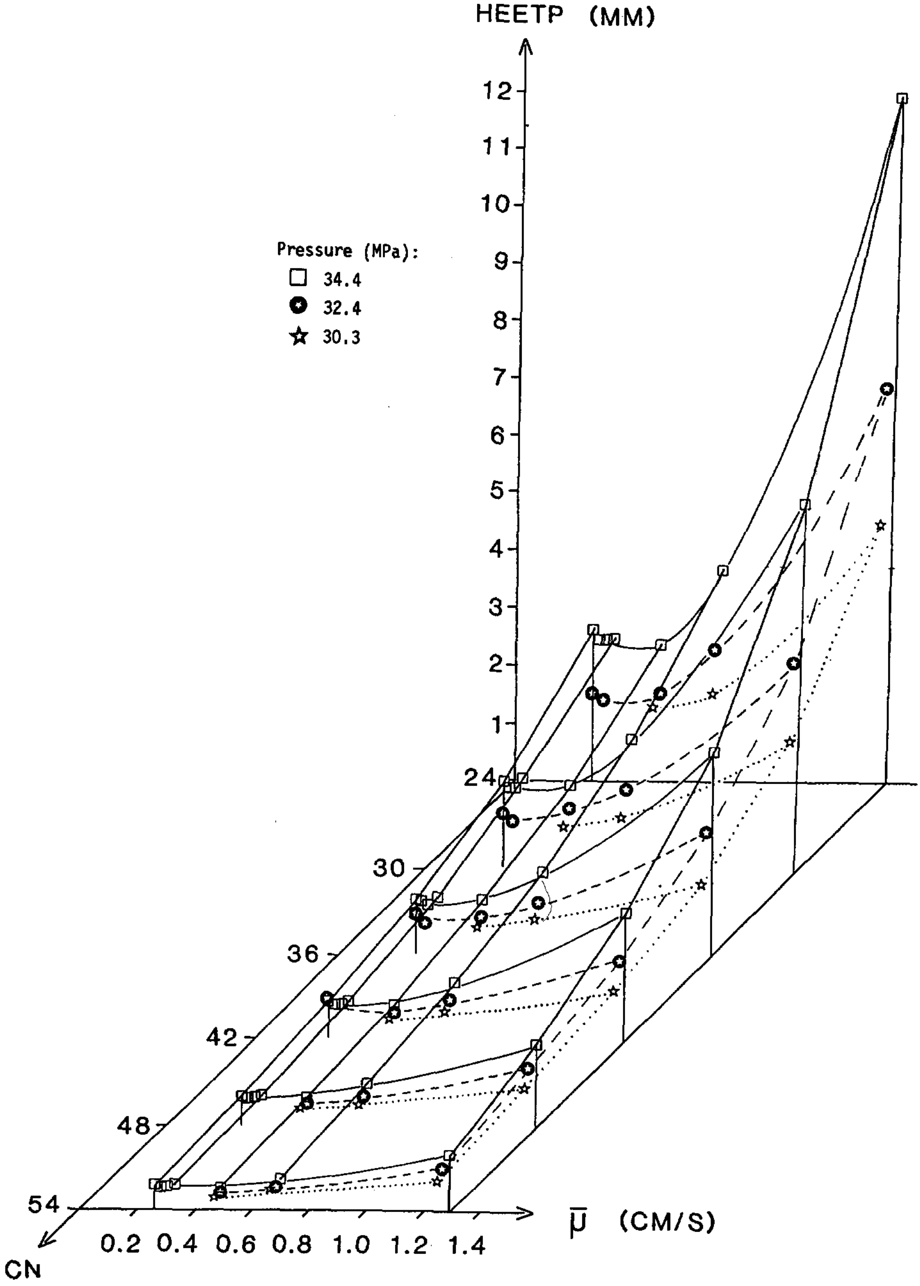

Fig. 1. Isothermal (150°C) plot of the height equivalent to an effective theoretical plate (HEETP) for saturated monoacid triacylglycerols (TAGs) containing fatty acids with an even number of carbon atoms *versus* average linear CO_2 carrier flow-rate ($\bar{\mu}$) and triacylglycerol carbon number (CN). Column, 3.8 m × 50 μm I.D., d_f 0.20 μm, DB-5.

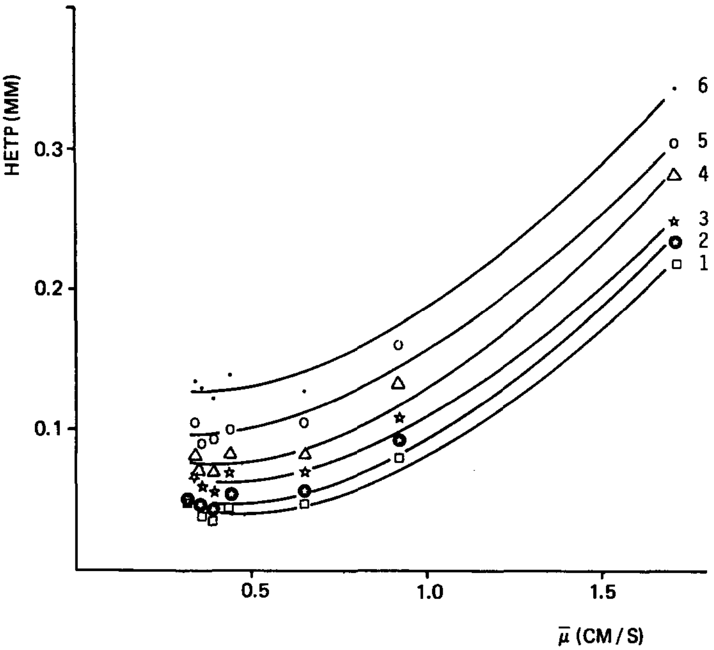

Fig. 2. Isothermal (150°C), isobaric (34.4 MPa) plot of the height equivalent to a theoretical plate (HETP) *versus* linear CO_2 carrier flow-rate ($\bar{\mu}$) for monoacid TAGs. Curves: 1 = 3 × 8:0; 2 = 3 × 10:0; 3 = 3 × 12:0; 4 = 3 × 14:0; 5 = 3 × 16:0; 6 = 3 × 18:0.

previously shown in practice by Sie and Rijnders in 1967 [15]. By increasing the average linear flow-rate of the supercritical carbon dioxide from 0.5 to 1.5 cm/s by changing the restrictor, increases in plate height of up to 3–5-fold were achieved. Losses in plate numbers were greater for the compounds having the lowest capacity factors (k'). An even steeper decline in the plate numbers per unit time was observed at flow-rate higher than 1.5 cm/s. In practice, this shows that when faster analyses are needed, shorter columns with optimized flow-rates are useful alternatives to longer columns run, *e.g.*, at ten times the optimum flow-rate. With packed fused-silica microcolumns the effect of flow-rate on efficiency is much less [5].

The approximated curves presented in Figs. 1 and 2 show reasonable symmetry and the coefficient of variation of the number of theoretical plates achieved with one restrictor was typically of the order of 5%. The HETP_{min} values (Fig. 2) increased at constant pressure with increasing capacity factors (k'), whereas the $\text{HEETP}_{\text{min}}$ values (Fig. 1) showed the opposite behaviour.

Isothermal (150°C) plots of $\text{HEETP}_{\text{min}}$ of TAGs as a function of the capacity factor k' and average linear velocity $\bar{\mu}$ are shown in Fig. 3. The experimental $\text{HEETP}_{\text{min}}$ values are compared with theoretical values calculated according to the equations

$$\text{HEETP}_{\text{min}} = \text{HETP}_{\text{min}} \, [(k' + 1) \,/\, k']^2$$

and

$$\text{HETP}_{\text{min}} = r \, [(1 + 6k' + 11k'^2) \,/\, 3(1 + k')^2]^{1/2}$$

where r is the radius of the column (25 μm).

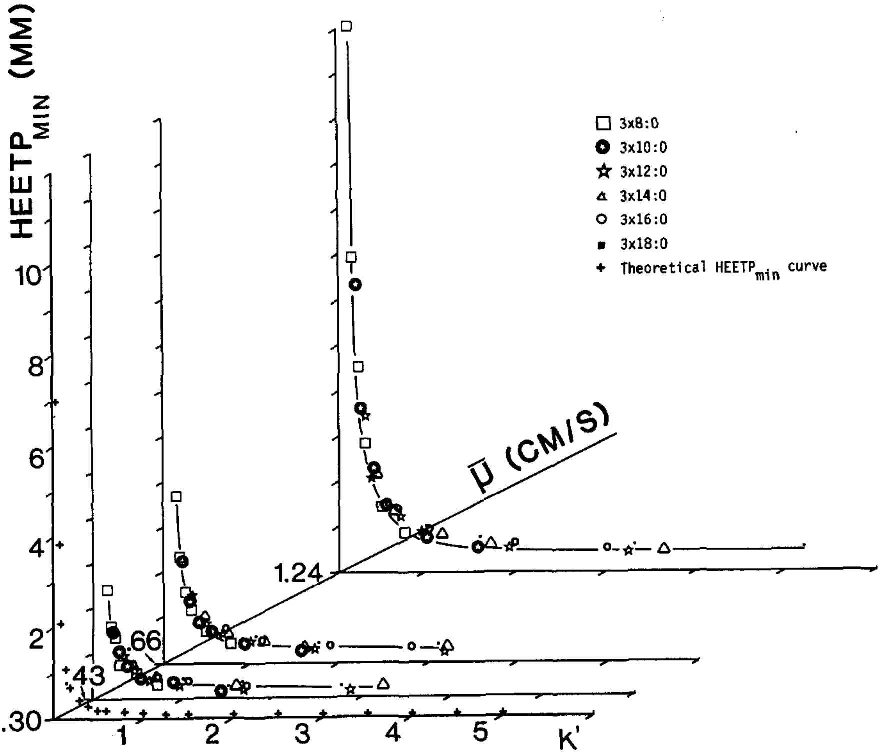

Fig. 3. Isothermal (150°C) plot of HEETP$_{min}$ of TAGs *versus* capacity factor (k') and $\bar{\mu}$.

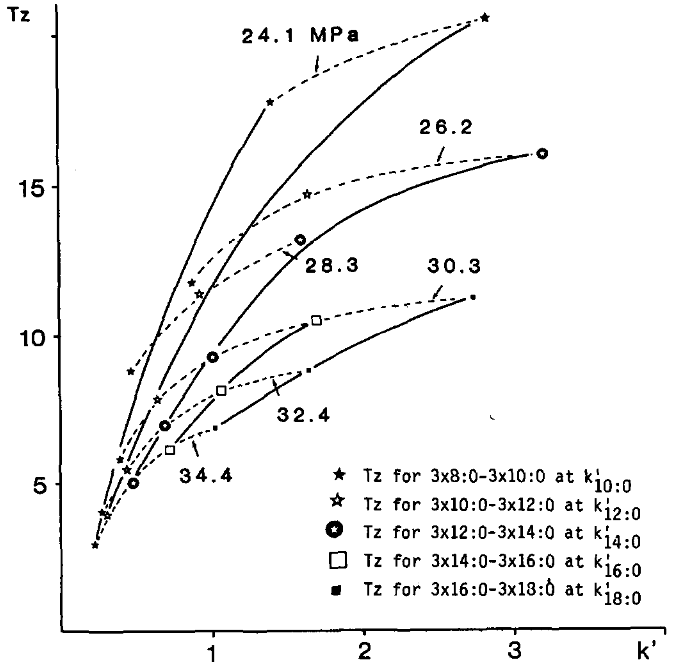

Fig. 4. Separation number (Tz) of adjacent TAGs as a function of capacity factor (k').

The plate heights obtained with the smallest TAG studied (trioctanoylglycerol) were 1.3–2 times the theoretical values, and this difference did not clearly depend on the pressure or k' value.

The plate numbers, or plate heights as such, do not yield sufficient information about the resolving power and usefulness of the column for TAG analysis. The separation number (Tz), which defines the number of average TAG peaks that can be placed between the adjacent compounds, is plotted against k' in Fig. 4. With high carbon dioxide density (pressure 34.4 MPa, $k' < 1$), the Tz between the smallest TAGs (CN = 24–30) was only 43% of the Tz of the largest compounds (CN = 48–54). With decreasing carbon dioxide density the relative difference between the Tz values of various TAG pairs decreased. At a pressure of 24.1 MPa, when the large molecules could no longer be analysed, the Tz of TAGs with CN = 30–36 exceeded 20.

Fig. 4 clearly shows that the smaller the compounds in a homologous series, the higher are the Tz values that can be generated. When increasing the capacity factor of each compound by decreasing the carbon dioxide density, one practical limitation will be the shape of the peak and the detection sensitivity lost when the peaks become broader. All the molecules are present but the misshaped peaks and the inaccuracy of the integration raise the standard deviations to unacceptable levels. At the same time, the improvement in the separation power by decreasing the fluid density becomes less and less in unit time. There are no empirical definitions to be followed when selecting the density or density programming of carbon dioxide for TAG analysis. According to our experience with the method used in this work, k' values higher than 3 cannot be recommended with the DB-5 column.

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