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OPTIMIZATION OF GRADIENT ELUTION SEPARATION OF FATTY ACID PHENACYL ESTERS

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SUMMARY

The optimal conditions for the gradient elution separation of the phenacyl esters of saturated fatty acids were found to consist of an aqueous acetonitrile solvent in conjunction with a C_8 reversed phase (RP). The best results were achieved with a 70–100% linear gradient of acetonitrile. Although the highest relative retentions were obtained on a C_{18} RP, oleic and palmitic acids could not be separated. Therefore, a C_8 RP was used, which yielded a satisfactory separation.

It was demonstrated that if the gradient volume (the product of the flow-rate and solvent programme time) and also the initial and final eluent compositions were kept constant, each sample component is eluted at a certain solvent composition. The gradient volume should generally be about 20–60 ml for the usual column sizes (25–30 cm, 4 mm I.D.), although for multi-component analyses a volume of 100–200 ml may be required. Small gradient volumes enhance the peak height relative to the noise level, which is important for trace analysis. The minimal gradient volume should not be less than 2.5 times the eluent volume in the column.

INTRODUCTION

The separation of saturated and unsaturated fatty acids remains a significant problem in biological and industrial analysis. Samples usually contain a wide range of homologous straight-chain fatty acids (from C_4 to C_{22}) and also unsaturated acids varying in chain length and degree of unsaturation. For the separation of the members of a homologous series, non-polar stationary phases of the reversed-phase (RP) type are optimal[1]. As the retention volumes of fatty acids on an RP vary considerably under isocratic conditions, the use of a programming technique is essential. The most versatile programming mode in liquid chromatography is gradient elution[2]. Inasmuch as most fatty acids show no appreciable UV or visible-light absorption, of the commonly available detectors only the differential refractometer is suitable for monitoring them. This detector, however, is not compatible with gradient elution. Consequently,

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UV-absorbing derivatives of the fatty acids (esters, amides, etc.) must be prepared, which, moreover, exhibit a substantially enhanced detection sensitivity[3,4].

The derivatization of fatty acids as phenacyl esters[4] or anilides (*e.g.*, *p*-methoxyanilides[5]) is well known and has been shown to be quantitative, especially in the presence of such modern catalysts as the crown ethers[6]. Such derivatives have been separated on non-polar stationary phases by stepwise[4] or continuous[5] programming of the eluent concentration. However, pronounced differences in the relative retentions of certain pairs of acids were evident between the early- and late-eluting ones, in contrast to optimal gradient elution where the relative retentions of the members of a homologous series should be constant[7]. Such constancy facilitates the identification of the individual members and immediately reveals the presence of non-member components. To achieve such constant relative retentions the eluotropic strength of the eluent must be made to increase linearly, *i.e.*, a linear solvent strength (LSS) programme must be applied[7]. Because of the increasing molecular weights, a slight diminution in the relative retentions of the higher members can be expected[8].

In the last few years, many more or less general treatments of gradient elution have appeared[2,7–10]. Unfortunately, most of these approaches permit the calculation of sample retention times and optimal resolution only if the dependence of the sample retentions on the isocratic eluent composition is known. Therefore, the aim of this paper is to present a pragmatic approach for the selection of the optimal conditions for gradient elution. Phenacyl esters were selected as model compounds for demonstrating the advantages of LSS gradient elution with non-polar stationary phases for multi-component analysis.

EXPERIMENTAL

Apparatus

A liquid chromatograph (Model 244, Waters Assoc., Milford, Mass., U.S.A.) containing two pumps (Model 6000A), a solvent programmer (Model 660), a sampling system (U6K) and a UV detector (Model 440) operating at 254 nm was used. The dead volume between the reference valve of pump A, where the eluents were mixed, and column inlet was determined to be 4.0 ml. To calculate the delay of the effective gradient at the column exit, the mobile phase volume of the column (*ca.* 3.3 ml) had to be added to the dead volume. This means that at a flow-rate of 2 ml/min the arrival of eluent B at the column exit was delayed by at least 3.6 min.

Reagents

Methanol (analysed reagent grade, J. T. Baker, Phillipsburg, N.J., U.S.A.), acetonitrile (pro analysi grade, Merck, Darmstadt, G.F.R.) and distilled water were used exclusively as eluents.

Columns

Columns of dimensions 30 cm × 4.2 mm I.D. were packed with RP prepared from LiChrosorb SI 100 (Merck, Darmstadt, G.F.R.) and trichlorooctadecylsilane [17% (w/w) carbon bonded] or dichloromethyloctylsilane [10% (w/w) carbon

bonded][11]. The columns were packed with *ca.* 10-μm particles by a modified viscosity slurry packing technique[12].

Gradient operation

The columns were regenerated after each run by switching to the starting eluent and pumping it through the column at 4 ml/min for 10 min. The solvent programme was started simultaneously with the injection.

Saponification of fats[13]

Approximately 3 g of the fat were refluxed for 2 h with 2 *N* sodium hydroxide solution. The cooled mixture was acidified with 2 *N* sulphuric acid and the liberated fatty acids were extracted with dichloromethane after saturating the aqueous phase with sodium sulphate. The organic phase was dried over sodium sulphate and the solvent carefully removed by evaporation.

Preparation of phenacyl esters[14]

About 200 mg of the fatty acids, 4 ml of a 5% solution of triethylamine in acetone and 6 ml of a solution of 5 g of recrystallized ω-bromoacetophenone in 150 ml of acetone were mixed. After a reaction time of at least 30 min, 5 μl of this solution were injected directly on to the column. The reagents were eluted unretained in the systems used.

RESULTS AND DISCUSSION

Selection of eluent

Methanol and acetonitrile and their aqueous mixtures are commonly used eluents in separations on non-polar stationary phases. Both eluents can be readily purified for gradient elution in order to reduce the appearance of ghost peaks resulting from impurities collected on the column[15]. With both solvents and their mixtures, plots of log k' *versus* solvent composition approach linearity, at least in the region of 60–100% of the organic component. Hence, in this region a linear gradient programme should produce linear solvent strength gradient elution.

Fig. 1 shows a comparison of the separation of the phenacyl esters of even-numbered saturated fatty acids with water–methanol and water–acetonitrile gradients under otherwise identical conditions. With the water–methanol gradient, the resolution of the acids eluted after the C_8 compound diminishes more markedly than with the water–acetonitrile, so that in the important C_{12}–C_{20} region, where most of the common unsaturated fatty acids will appear, the former gradient achieves only about half the resolution of the latter.

This poorer resolution may be attributed to the anomalous behaviour of methanol–water mixtures in the elution of aliphatic acid derivatives. Usually methanol and its aqueous mixtures exhibit lower elution strengths in RP chromatography than the corresponding acetonitrile mixtures[16]. For the acid derivatives, however, this is only true up to 80% (v/v) of methanol or acetonitrile. Hence pure methanol is a much stronger eluent for these substances than acetonitrile, whereas the addition of 10% of water to both yields eluents with the same strength. Plots of log k' *versus* eluent composition show a higher slope for water–methanol than for acetonitrile–water

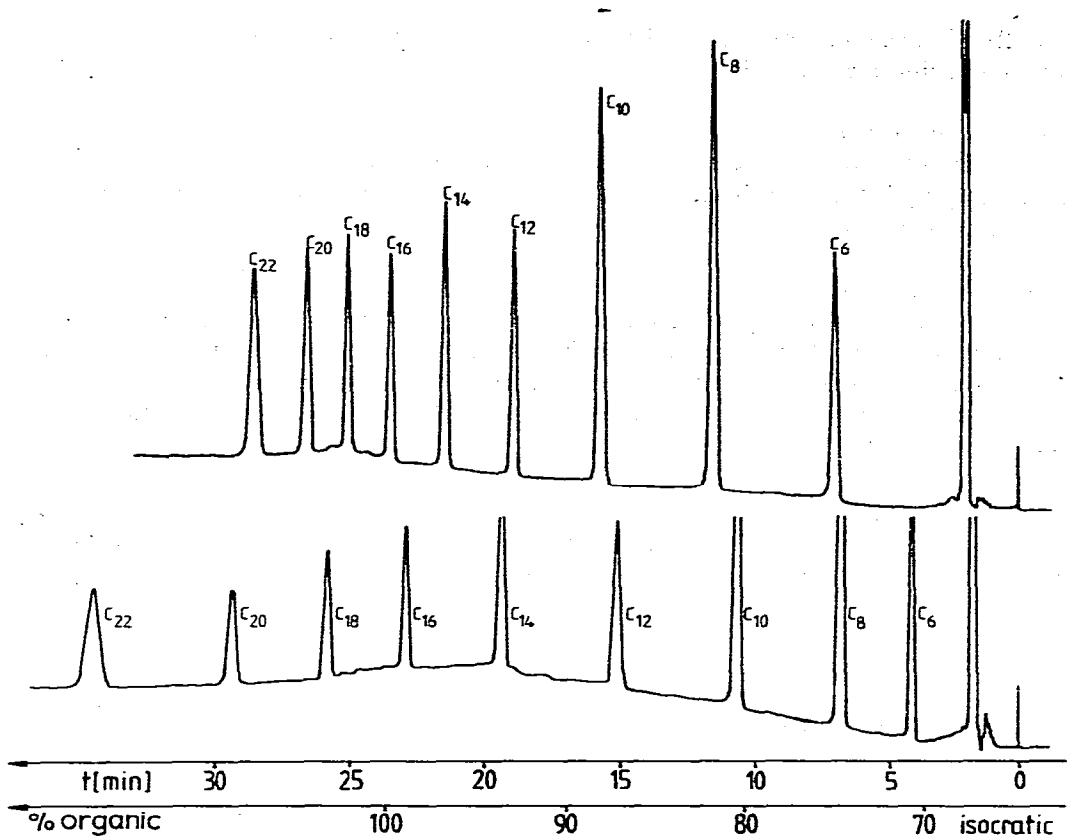

Fig. 1. Gradient elution of saturated fatty acid phenacyl esters. Comparison of methanol and acetonitrile. Samples: phenacyl esters of even-numbered fatty acids between C_6 (capronic acid) and C_{22} (behenic acid). Column, 30 cm × 4.2 mm I.D.; stationary phase, SI 100 C_{18} RP; flow-rate, 2 ml/min; gradient, 20 min, linear. Upper curve: starting eluent 70% methanol in water, gradient to 100% methanol. Lower curve: starting eluent 70% acetonitrile in water, gradient to 100% acetonitrile.

mixtures. Similar anomalous behaviour was observed for other compounds of homologous series containing long aliphatic chains, such as the phenylalkanes[17].

Because of its superior resolution of the higher-molecular-weight acid derivatives, only the water–acetonitrile gradient was investigated further. An initial gradient consisting of 70% acetonitrile was chosen, with which the C_6 acid derivative is eluted at about the solvent front. A gradient with a lower acetonitrile content (*e.g.*, 50%) allows an improvement only for the $< C_6$ derivatives and does not alter the resolution of the higher members. In this instance the analysis time is increased and expensive eluent is wasted. Fig. 2 shows that the C_6 derivative is eluted at the acetonitrile concentration used in the initial gradient. If only the C_{12}–C_{20} acids are of interest, a gradient of 90–100% acetonitrile can be employed, but in this instance isocratic analysis is probably equally effective and less cumbersome. The unsaturated acid derivatives were included in the sample but their resolution was not optimized.

At low initial acetonitrile concentrations another problem common to gradient elution arises, namely the collection of solvent impurities on the stationary phase

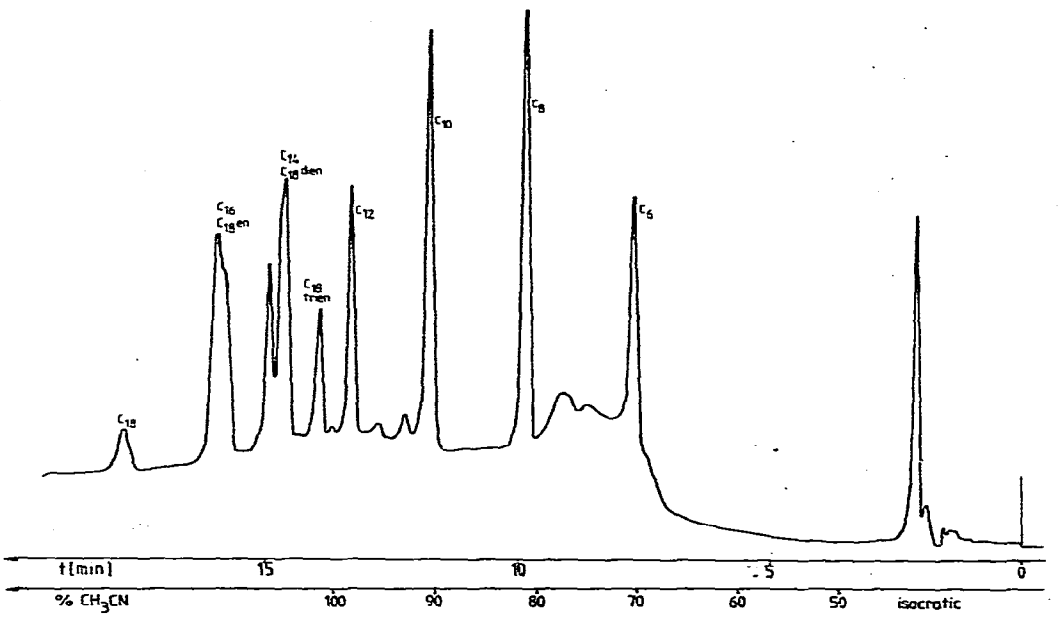

Fig. 2. Gradient elution of saturated and unsaturated fatty acid phenacyl esters. Samples: phenacyl esters of even-numbered fatty acids between C_6 (capronic acid) and C_{18} (stearic acid); unsaturated C_{18} acids (oleic, elaidic, linoleic, linolenic). Column and stationary phase as in Fig. 1. Flow-rate, 2 ml/min; gradient, 10 min, linear. Starting eluent: 50% acetonitrile in water, gradient to 100% acetonitrile.

and their subsequent elution as the proportion of acetonitrile increases[15]. The amount of these impurities concentrated on the column is sometimes surprisingly high. In Fig. 2 this phenomenon is shown by the rising baseline after 5 min and the humps under the C_6 and between the C_6 and C_8 esters. However, such interferences can be avoided by starting with a 70% or greater concentration of acetonitrile. It is necessary to standardize the equilibration time before each analysis; it is also recommended that a blank gradient run be carried out after changing the batch of eluent to check for possible impurities concentrated on the column.

Correlation of gradients; gradient volume

In gradient elution, the sample components may be characterized by the gradient composition at which they are eluted. At the same initial and final gradient compositions and with otherwise constant conditions the sample retention times depend on the flow-rate and the duration of the gradient programme.

In Figs. 3 and 4 the same separation is compared under different gradient conditions. In the first instance the programme time was 10 min at a flow-rate of 2 ml/min and in the second the programme time was increased to 20 min and the flow-rate was reduced to 1 ml/min. It is evident that the eluent composition at which each component elutes is characteristic of it. This characteristic composition remains valid even if the gradient conditions are altered, provided that the product of the volume flow-rate and the duration of the gradient programme (gradient time) is kept constant. This

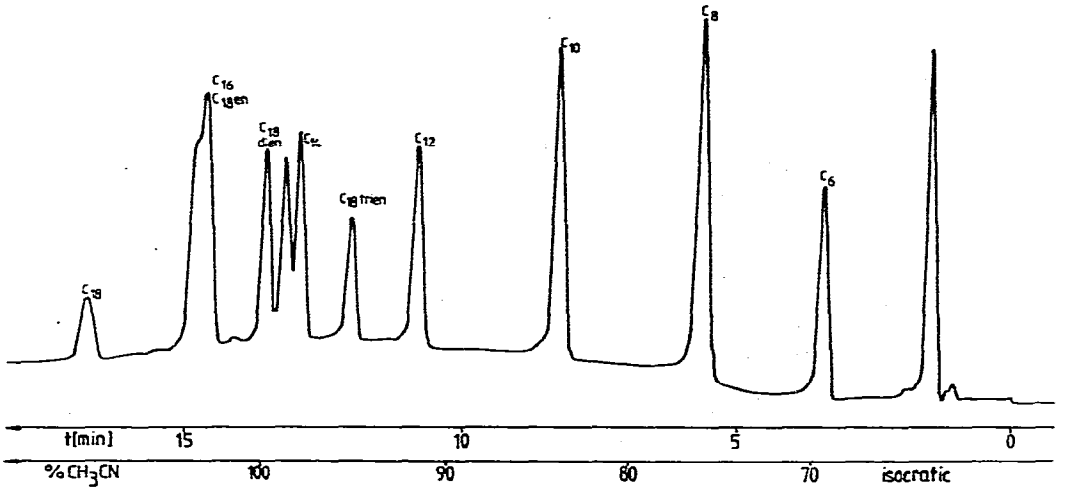

Fig. 3. Gradient elution of fatty acid phenacyl esters. Gradient volume, 20 ml; flow-rate, 2 ml/min; gradient, 10 min, linear. Starting eluent: 70% acetonitrile in water, gradient to 100% acetonitrile. Samples, column and stationary phases as in Fig. 2.

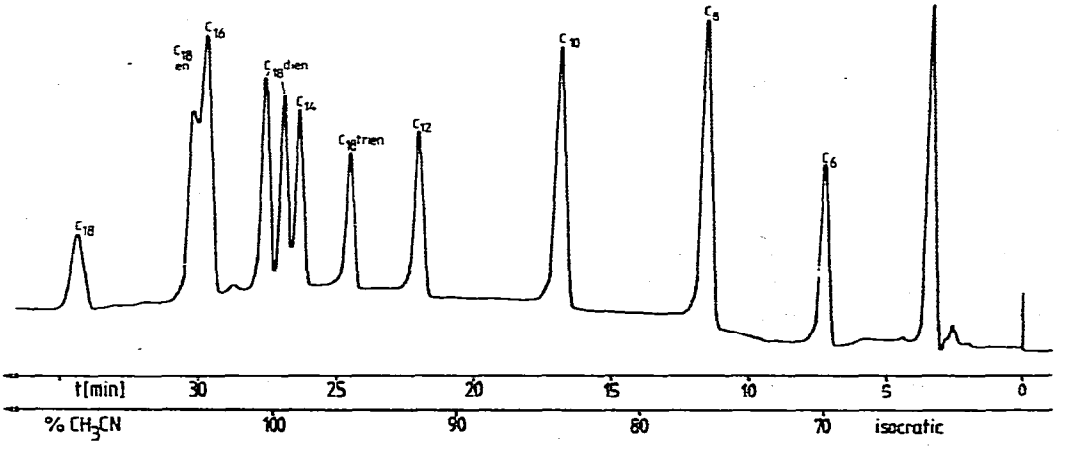

Fig. 4. Gradient elution of fatty acid phenacyl esters. Gradient volume, 20 ml; flow-rate, 1 ml/min; gradient, 20 min, linear. Sample, column, stationary phase and eluent composition as in Fig. 3.

product can be defined as the gradient volume[18] and corresponds to the volume of eluent passed through the column during a gradient run.

In Fig. 3, with the higher flow-rate, the analysis requires about 18 min, but the resolution of the saturated C_{14} and C_{16} derivatives from the C_{18} singly and doubly unsaturated derivatives is unsatisfactory. In Fig. 4, the analysis time is doubled because of the slower flow-rate but the amount of solvent needed is the same. However, the lower eluent velocity results in an improved resolution owing to the decreased peak volume analogous to decreasing H values under isocratic conditions.

Lower peak volumes are also obtained if the flow-rate is maintained constant but the gradient volume is reduced. This is particularly significant in trace analysis

where the peak heights are frequently difficult to distinguish from the noise. Hence, by utilizing a small gradient volume a greater sensitivity can be achieved than under isocratic conditions.

However, the gradient volume should not be less than 2.5 times the mobile phase volume. For the usual high-performance liquid chromatographic columns (30 cm × 4 mm I.D.) the minimum volume should be about 10 ml, and a volume of 20–60 ml is commonly used. Greater volumes are required when the number of components increases, and in multi-component analysis volumes of 100–200 ml may be necessary. The maximum amount of sample separable by gradient elution is a function of the gradient volume and the average peak volume in which the components are contained. The ratio of these two parameters can be called the gradient peak capacity[18]. Ideally, the peak volume in gradient elution should be constant over the entire range, but owing to the anomalous viscosity behaviour of aqueous methanol and acetonitrile solutions, the peak volume is a function of the eluent composition in RP gradient elution. The peak volume also varies directly with the flow-rate. A decrease in the flow-rate from 2 to 1 ml/min increases the peak capacity by about 10% but doubles the analysis time. Below 1 ml/min, however, additional instrumental problems are encountered.

The dependence of the elution behaviour on the gradient volume can be seen by comparing Figs. 3 and 5, where the flow-rates were identical but the gradient volume was double in the latter instance. It is significant that although the volume was doubled, the analysis time was increased by a smaller factor because the components emerged at a solvent composition corresponding to a lower elution strength; the resolution was substantially improved.

Reducing the gradient volume has, of course, the opposite effect. As this volume is decreased the advantage of a shorter analysis for gradient elution diminishes

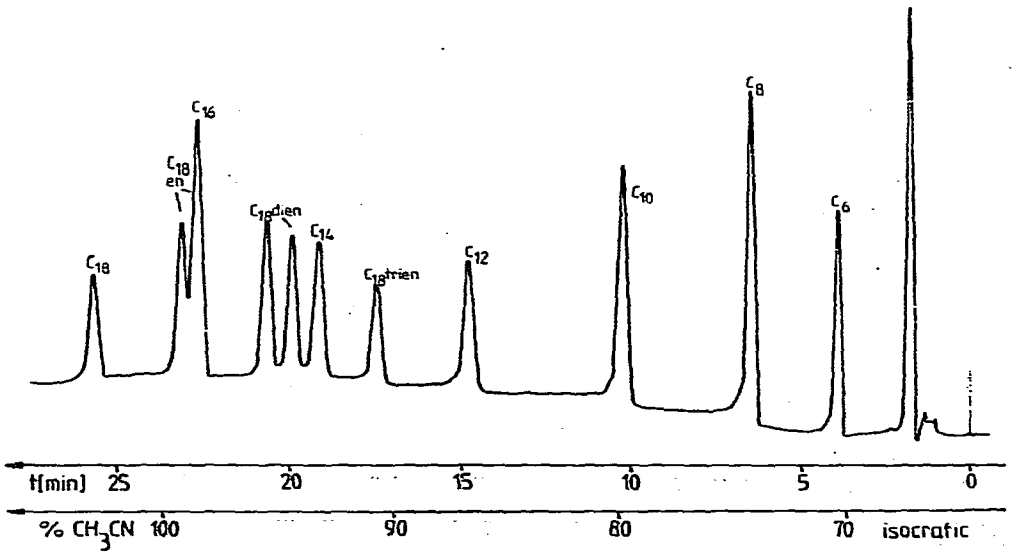

Fig. 5. Gradient elution of fatty acid phenacyl esters. Gradient volume, 40 ml; flow-rate, 2 ml/min; gradient, 20 min, linear. Sample, column, stationary phase and eluent composition as in Fig. 3.

because of the constant amount of time required for column regeneration. For this reason, it is impractical to employ a gradient volume of less than 10 ml.

Gradient elution is frequently used to establish quickly the optimum eluent composition for isocratic analysis. For gradient volumes of 20–40 ml with the usual chromatographic columns the following general rule holds: the k' values of a sample in isocratic analysis will be about 1–2 if the solvent composition at which it is eluted is used. Changing the gradient volume alters the k' values correspondingly.

Optimization of the stationary phase

The initial work was performed with a C_{18} RP because the relative retentions increase with increasing length of the bonded alkyl chain. However, it was found that the important palmitic and oleic acid derivatives could not be separated on this RP as the introduction of one double bond coincidentally had the same effect on retention as the removal of two methylene groups. Similar behaviour was reported for the methyl esters of fatty acids[19]. The derivative of the *trans*-acid (elaidic acid) was more strongly retained than the *cis*-isomer (oleic acid) and could be separated from it satisfactorily. The introduction of a second double bond caused a further, but smaller, reduction in retention so that linoleic acid derivatives were eluted between the myristic

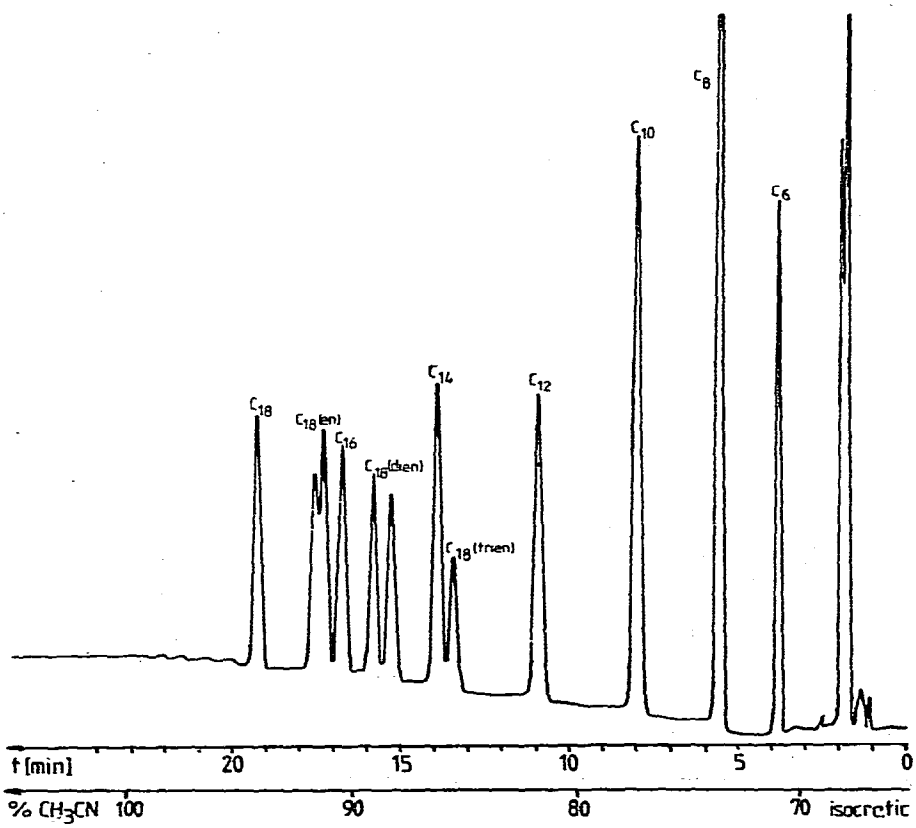

Fig. 6. Gradient elution of fatty acid phenacyl esters. Stationary phase: C_8 RP on SI 100. Other conditions as in Fig. 5.

(C_{14}) and palmitic (C_{16}) acid esters. Analogous behaviour was observed with the linolenic acid derivatives.

On an RP with a shorter alkyl chain (C_8 instead of C_{18}) the decrease in retention on introduction of a double bond is less pronounced and the palmitic, oleic and elaidic acid derivatives can be separated (Fig. 6). However, the resolution between oleic and elaidic acid esters is smaller than on the C_{18} RP, using the same gradient conditions (*cf.*, Figs. 5 and 6). The shortening of the bonded carbon chain length results in an overall decrease in the retention so that the derivatives appear at a solvent composition of lower elution strength. The relative and absolute retentions are smaller on the C_8 than the C_{18} RP.

Applications

Because the reagents and solvents used in the esterification of the acids are eluted in front of the derivatives of interest, the reaction mixture could be injected directly. Fig. 7 shows the distribution of fatty acids in butter, obtained by direct injection of 5 μl of the solution after a 30-min reaction time. The derivatives were monitored with a UV detector at 254 nm. The sensitivity for the upper track was 0.1 a.u.f.s. (absorption units full-scale) and for the lower 2.0 a.u.f.s. The principal peaks corresponded to myristic, palmitic, oleic and stearic acids. The butyric acid peak was eluted together with the most strongly retained reagent peak. Its determi-

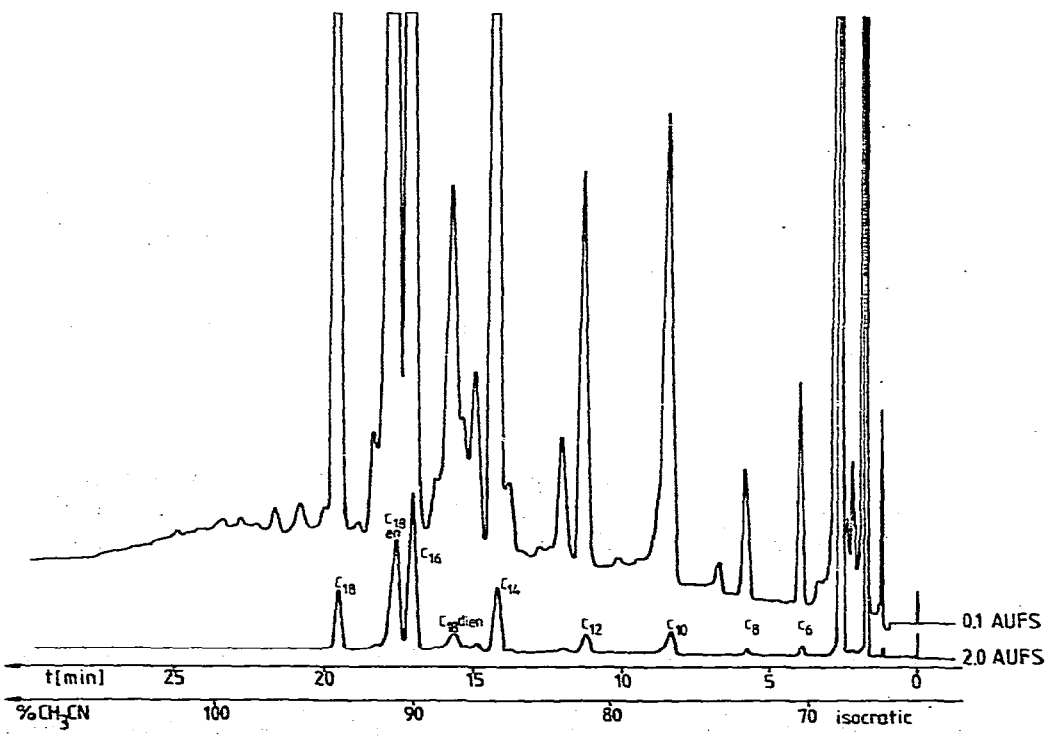

Fig. 7. Gradient elution of the fatty acid phenacyl esters from butter. Conditions as in Fig. 6.

nation would require either a preliminary clean-up procedure or an initial eluent richer in water to effect a better resolution of the weakly retained components.

Fig. 8 shows the separation of the phenacyl esters of a highly unsaturated natural fat (trade-name Becel). The principal constituents were found to be linoleic and oleic acids; only two saturated acids (palmitic and stearic) were present in appreciable concentrations. Quantitative analysis based on the peak heights, which is optimal for gradient analysis[20], indicates more than 85% of the fatty acids to be unsaturated.

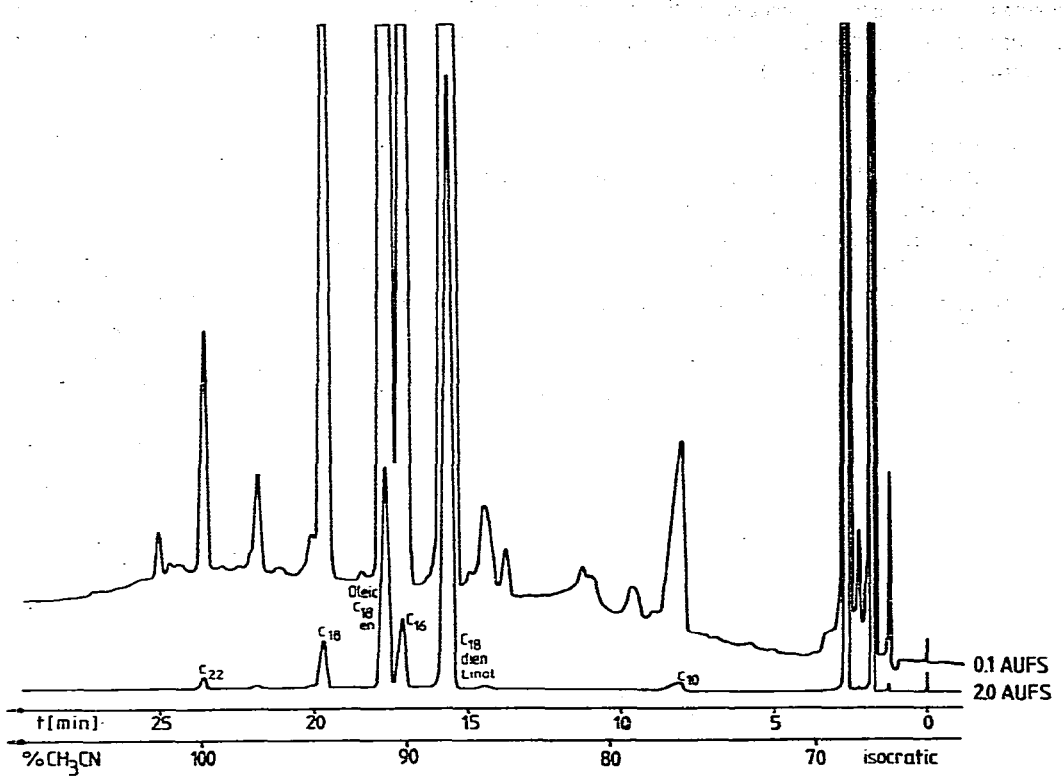

Fig. 8. Gradient elution of the fatty acid phenacyl esters from a highly unsaturated fat (Becel). Conditions as in Fig. 6.

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