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QUANTITATIVE CAPILLARY GAS–LIQUID CHROMATOGRAPHY OF TRIGLYCERIDES ON A FUSED-SILICA COLUMN WITH A CHEMICALLY BONDED STATIONARY PHASE

P. MAREŠ*
Lipid Metabolism Laboratory, Faculty of Medicine, Charles University, Prague (Czechoslovakia)
and
P. HUŠEK
Research Institute for Endocrinology, Prague (Czechoslovakia)
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SUMMARY

The effect of the sample injection technique, carrier gas flow-rate, amount and molecular weight of substance and temperature programming on the values of the weight correction factor, f_w, were studied for model samples consisting of identical amounts of saturated triglycerides with carbon numbers of 30, 36, 42, 48, 54 and 60. Measurements were carried out on two different capillary columns 5 m × 0.31 or 0.32 mm I.D., containing OV-1 chemically bonded stationary phase. The dependence of f_w on the sample composition and overloading of the column and the reproducibility of the analytical results for model samples of various amounts were studied on both columns. Differences were found between the two columns. The limit for quantitative analysis of triarachidin under optimum experimental conditions is one order of magnitude lower than that on packed columns. Very good reproducibility was achieved for the results of quantitative analysis on one capillary column, measured in terms of the series relative standard deviation, which has a value of less than 1.4% ($n = 5$) for analysis of a sample consisting of 10 ng of each of the above triglycerides. The mechanisms of loss of higher triglycerides during capillary gas chromatography are also discussed.

INTRODUCTION

In recent years, ever increasing use has been made of capillary gas chromatography (GC) in the analysis of triglycerides or complex mixtures containing triglycerides[1–8]. However, none of these studies provides a sufficiently extensive treatment of the quantitative analysis of these substances and the dependence of the recovery on the experimental conditions. Monseigny *et al.*[9] published an extensive study of the quantitative GC of triglycerides using glass capillaries. The authors discussed the dependence of the results on various parameters, such as the injector temperature, the type of carrier gas and its flow-rate through the column, the column length and

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temperature program, but completely omitted the mass of the studied substance. They stated that the optimum sample weight was between 100 and 200 ng, but apparently only in connection with the capacity of the column used, with a thin film of the stationary phase, rather than from the point of view of quantitative analysis.

In 1975 it was found that the weight correction factor, f_w, defined as the ratio of the relative weight of a given component of the sample to the relative area of the corresponding peak, is dependent, above a variable limit, on the absolute weight of the analyzed sample[10]. This is very important, especially in the analysis of samples containing higher triglyceride fractions. Thus, such a sample can be correctly analyzed quantitatively only when a number of parameters is carefully controlled and a non-linear calibration dependence is used[11]. We recently considered[12,13] the problems involved in the analytical application of the non-linear region of the calibration curve in the analysis of neutral human blood plasma lipids. These papers were based on earlier ones[10,14,15] in which it was found that the losses of the test substances during analysis under constant conditions are stable and sufficiently reproducible. The losses of triglycerides during analysis on packed[16] and capillary[17] columns were studied in greater detail and a hypothesis was proposed for the mechanism involved. The greatest losses appear to occur during sample injection[18]. The "cold on-column" type of injection was found to be optimal, but certain losses of the analyzed triglycerides were still observed[18]. In addition, this study employed only triglycerides with carbon numbers of $\leqslant 54$, while triglycerides with carbon numbers of $\geqslant 60$ were often encountered in practice and on published chromatograms.

The introduction in recent years of fused-silica capillary columns and progress in the technology of immobilization of the stationary phase has extended the possibilities for analysis of triglycerides and other natural substances with low volatility, as demonstrated by the work of Smith[19] and Myher and Kuksis[20]. The use of a fused-silica capillary with a chemically bonded stationary phase has permitted more sensitive determinations of a number of biologically important, poorly volatile substances[20], which could not be attained in the required range on packed columns.

An accurate quantitative analysis of all the components of a sample, including those present in units or even fractions of a nanogram requires knowledge of all the factors affecting the recovery of these substances in GC analysis. This work deals with the quantitative GC analysis of triglycerides on a fused-silica capillary column with a chemically bonded stationary phase, the factors that affect the recovery of the individual substances and the relationships among these factors.

EXPERIMENTAL

Apparatus

The analyses were carried out on an Hewlett-Packard 5730A gas chromatograph, equipped with two capillary injectors (cold on-column injector OCI-3, Scientific Glass Engineering, Kensington, Australia and split-splitless injector HP-18470B, Hewlett-Packard, Avondale, PA, U.S.A.), two flame ionization detectors and a linear temperature program. The capillary columns were a 5 m × 0.31 mm I.D. fused-silica column (1) with cross-linked silicone gum OV-1 (part of a 25-m column provided by Hewlett-Packard), coating thickness 0.17 μm, 2600 theoretical plates per metre at k = 7–9 and maximum operating temperature 350°C and a 5 m × 0.32 mm I.D.

fused-silica column (2) with chemically bonded OV-1 (part of a 25-m column CP-Sil 5CB provided by Chrompack, Middelburg, The Netherlands), coating thickness 0.12 μm, 3500 theoretical plates per metre at $k = 3.7$, coating efficiency 91% and maximum operating temperature 340°C. Prior to use, both the columns were stabilized, overnight at 300°C with a hydrogen flow-rate of 3 ml/min.

Chemicals

Triglycerides with carbon numbers (CN) of 30–60 (tridecanoin, CN = 30; trilaurin, CN = 36; trimyristin, CN = 42; tripalmitin, CN = 48; tristearin, CN = 54; triarachidin, CN = 60) were provided by Applied Science Europe (Oud-Beijerland, The Netherlands). *rac*-Glyceryl-1,3-stearate-2-palmitate, CN = 52 was obtained from Supelco (Bellefonte, PA, U.S.A.). All the triglycerides were at least 99% pure, as shown by GC analysis using a packed column. 99% pure undecane was provided by Sigma Chemicals (St. Louis, MO, U.S.A.); p.a. chloroform and toluene (Lachema, Brno, Czechoslovakia) were rectified on a 60-plate column immediately prior to use.

Preparation of analytical samples

Stock solutions (1 mg/ml) of the triglycerides were prepared in chloroform. The reproducibility of the sample weighing was better than ±2% (25 mg, $n = 10$). The analytical samples were prepared from the individual stock solutions by mixing appropriate volumes. Then chloroform was evaporated under a stream of nitrogen at 40°C and the sample was dissolved in the required volume of undecane–toluene (95:5, v/v). Toluene was necessary to dissolve saturated substances.

Dependence of f_w on the analytical conditions and on the amount and molecular weight of triglyceride

The dependence of f_w on the carrier gas flow-rate was measured in the hydrogen flow-rate range of 7–15 ml/min using samples containing the same weights of triglycerides with carbon numbers of 30, 36, 42, 48, 54 and 60. Similarly, the dependence on the mass of the analyzed substance was studied for samples containing 5–200 ng of each component. Each of the analyses yielded six values of f_w for the individual components of the sample with different molecular weights, permitting a study of the dependence of f_w on the molecular weight of each substance under various conditions. All of these measurements were made on column 1 using both splitless and cold on-column injection and on column 2 using only cold on-column injection.

The dependence of f_w on the temperature program was measured only on column 1 with splitless injection. The weights of the individual components in the sample were identical, 200 ng. All the samples were analyzed in duplicate and the arithmetical mean was taken of the results of the two analyses.

Effect of the sample composition on f_w

A pair of triglycerides with carbon numbers of 52 and 54, *rac*-glyceryl-1,3-stearate-2-palmitate and tristearin was employed. The experimental method has already been described for packed columns[12]. For capillary columns the procedure differed only in details. First, a sample containing 100 ng tridecanoin (internal stan-

dard) and 20 ng of the test triglyceride was injected. Then a concentrated stock solution of a second triglyceride was added to this sample so that the final concentration of this component in the analyzed sample equalled ten times that of the test triglyceride. The change in the volume of the sample as a result of addition of the concentrated stock solution equalled 2% and can be neglected for our purposes. In the next measurement, the order of addition (mixing) of the two triglycerides was reversed. All the samples were analyzed on both capillary columns using cold on-column injection. Analyses were carried out in triplicate and the arithmetical mean of all three measurements was evaluated. The carrier gas flow-rate was 10 ml/min, and the temperature was raised at 8 K/min.

Effect of column overloading on f_w in subsequent analyses

In experiments on packed columns it was found that overloading of the column with a sample containing several μg of higher triglycerides affects the recovery of these substances in subsequent analyses. This effect is utilized in practice to stabilize the recovery of triglycerides with carbon numbers of $\geqslant 54$ on new columns[11,13]. Overloading of the capillary column was achieved by injecting 2 μg of triarachidin. The measurement was carried out by cold on-column injection on both columns. The test sample was a mixture of triglycerides with carbon numbers of 30 (internal standard), 54 and 60. The sample contained 200 ng of each component. The analyses were carried out in duplicate before and after overloading. The carrier gas flow-rate was 10 ml/min and the temperature was raised at 8 K/min.

Evaluation of the separation efficiency

The separation efficiency for triglycerides under conditions of temperature programming is measured in terms of the ΔC value[14], defined as the minimum number of carbon atoms by which two consecutively eluted triglycerides must differ to achieve baseline separation. ΔC values were evaluated for the intervals C_{30}–C_{36}, C_{36}–C_{42}, C_{42}–C_{48}, C_{48}–C_{54} and C_{54}–C_{60} using the formula given previously[14].

Analytical conditions common to all analyses

The carrier gas was hydrogen; nitrogen was used as the make-up gas. The overall flow-rate of the two gases measured at the detector jet at the laboratory temperature was always 40 ml/min. The oven temperature was programmed from 180 to 340°C; initial time 0, final time 2 min. The injector temperature during splitless injection was 300°C, delay 30 s, and the exchangeable glass insert of the injector had a diameter of 1.5 or 2.5 mm. Cold on-column injection was carried out at an initial column temperature of 180°C. The volume of sample injected was always 1 μl. The detector temperature was 350°C.

Evaluation of results

The chromatograms were recorded on an Hewlett-Packard 3380A integrator. The recording sensitivity was in the range 1 × 2 to 1 × 64; the chart speed was 5 mm/min. The integrator evaluates the areas of the individual peaks which are then used as a basis for calculation of the corresponding f_w values for the individual components of the sample. Tridecanoin was used as an internal standard. The values of f_w^{30} were obtained from the ratios of the f_w values for the individual substances to

that for tridecanoin, and permitted a better description of the effect of the individual factors on the recovery of triglycerides in terms of the value of the weight correction factor, f_w.

The reproducibility of the results of quantitative analyses of triglycerides was expressed in terms of the relative standard deviation (R.S.D.) calculated from a series of five successive analyses of a single sample.

RESULTS

The effect of the technique of sample injection on the higher boiling component discrimination has already been described[18]. This work also considered the effect of the carrier gas flow-rate on the discrimination. Fig. 1 depicts the dependence of the f_w^{30} values on the carrier gas flow-rate and on the molecular weight of the triglycerides measured on capillary column 1 using cold on-column and splitless injection. Rather surprising results (Fig. 2) were obtained upon comparing the dependences for cold on-column injection on columns 1 and 2.

The chromatograms in Fig. 3a and b indicate the differences in the recoveries of the individual triglycerides from the two capillaries under identical conditions.

The dependence of the f_w^{30} values on the amount and molecular weights of the

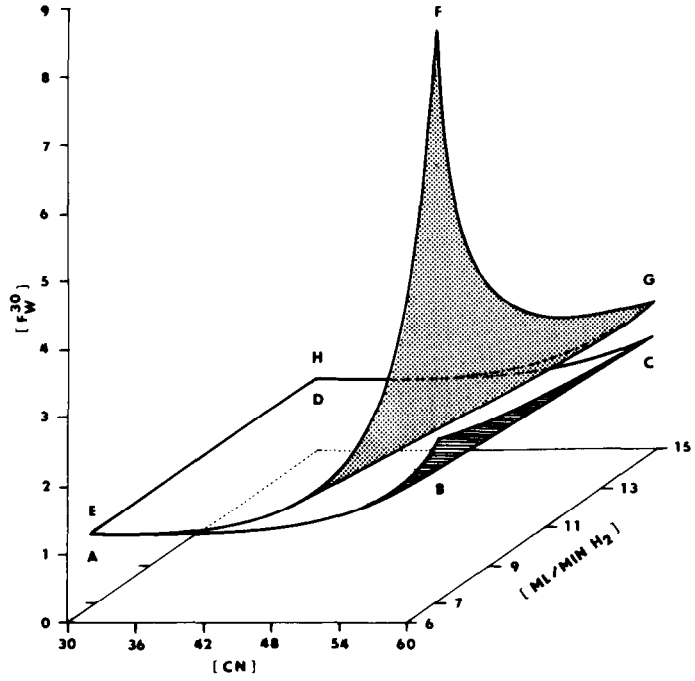

Fig. 1. Dependence of the values of the relative weight correction factor, f_w^{30}, on the carbon number (CN) and hydrogen carrier gas flow-rate (ml/min) for splitless (E,F,G,H) and cold on-column injection (A,B,C,D) into the same capillary. Column 1, sample 1 μl containing 200 ng of each triglyceride with carbon numbers of 30, 36, 42, 48, 54 and 60, program 8 K/min, remaining conditions as in the text. Coordinates x (CN), y (f_w^{30}), z (ml/min) are as follows: A 30, 1.0, 7.0; B 60, 2.36, 7.0; C 60, 1.66, 15.0; D 30, 1.0, 15.0; E 30, 1.0, 7.0; F 60, 8.32, 7.0; G 60, 2.16, 15.0; H 30, 1.0, 15.0.

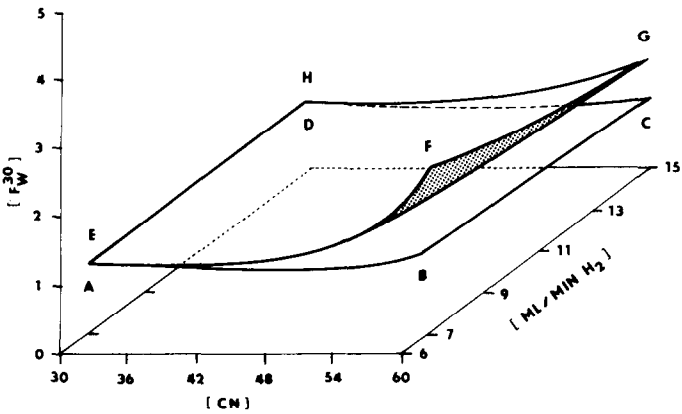

Fig. 2. Dependence as in Fig. 1 for injection of a single sample type by cold on-column injection into two different capillaries: column 2 area A,B,C,D, column 1, area E,F,G,H. Coordinates x (CN), y (f_w^{30}), z (ml/min) are as follows: A 30, 1.0, 7.0; B 60, 1.21, 7.0; C 60, 1.03, 15.0; D 30, 1.0, 15.0; E 30, 1.0, 7.0; F 60, 2.36, 7.0; G 60, 1.66, 15.0; H 30, 1.0, 15.0.

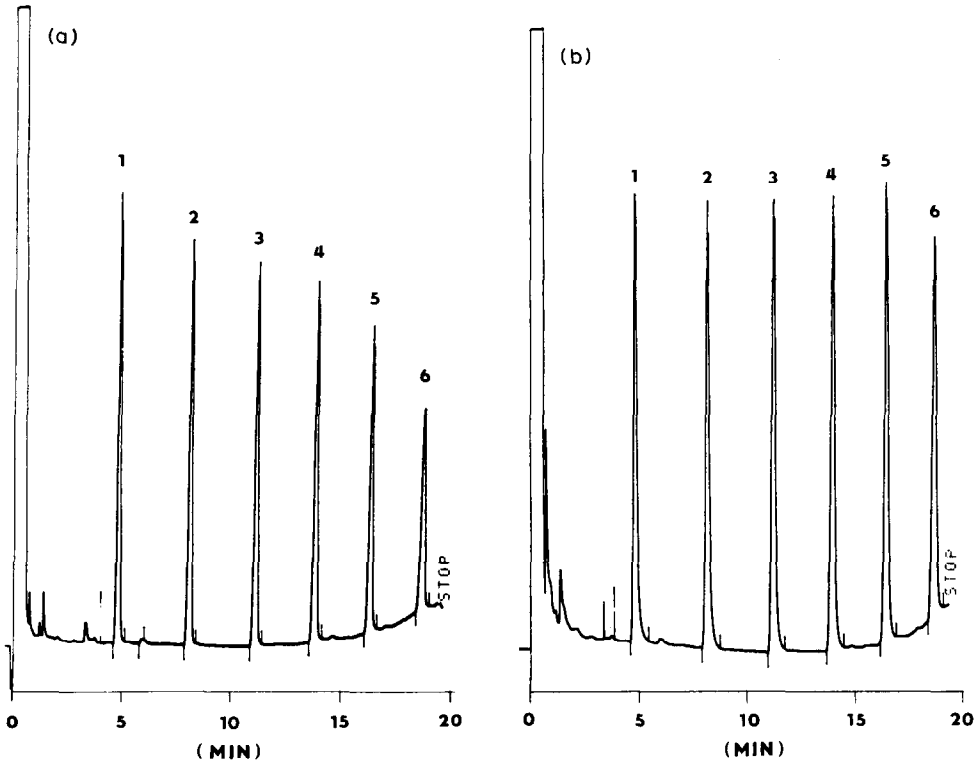

Fig. 3. Comparison of chromatograms for a single sample on columns 1 (a) and 2 (b) under the same analytical conditions. Samples as in Fig. 1, carrier gas flow-rate 15 ml/min, program 8 K/min; other conditions are as in the text. Peaks: 1 = tridecanoin; 2 = trilaurin; 3 = trimyristin; 4 = tripalmitin; 5 = tristearin; 6 = triarachidin. The baseline drift was not compensated.

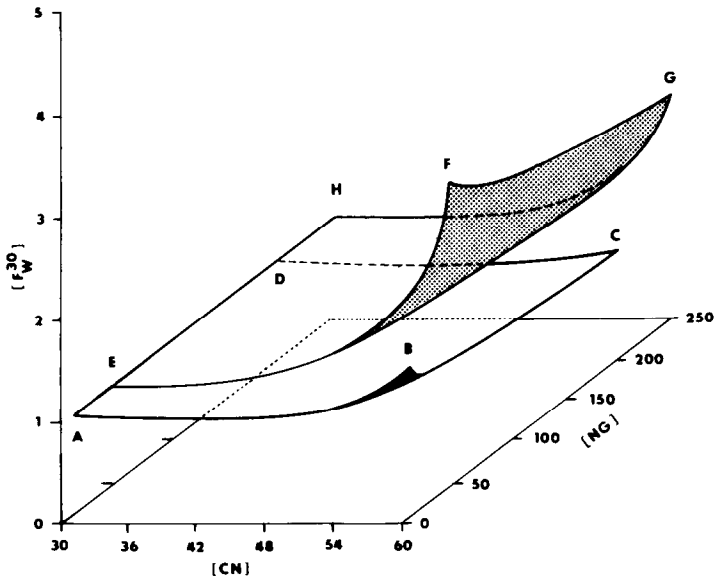

Fig. 4. Dependence of f_w^{30} on the carbon number (CN) and on the amount of triglyceride (ng) on packed (E,F,G,H) and capillary columns (A,B,C,D). Packed column: 0.5 m × 1.75 mm I.D., 1.5% OV-1 on Gas-Chrom Q (120–140 mesh). Analytical conditions: capillary column, cold on-column injection, hydrogen carrier gas flow-rate 15 ml/min, temperature program 8 K/min, remaining conditions as in the text; packed column, injection into the heated part of the column at 300°C, helium carrier gas flow-rate 55 ml/min, Perkin-Elmer F-30 instrument. Temperature program: 180–350°C, 8 K/min. Flame ionization detector; temperature 350°C. Coordinates x (CN), y (f_w^{30}), z (ng) are as follows: A 30, 1.0, 10; B 60, 1.41, 10; C 60, 1.03, 200; D 30, 1.0, 200; E 30, 1.0, 50; F 60, 2.90, 50; G 60, 2.15, 250; H 30, 1.0, 250.

triglycerides with carbon numbers of 30–60 measured on capillary column 2 with cold on-column injection are given in Fig. 4. For comparison of the basic difference between the packed and capillary columns for the analysis of small amounts of triglycerides, this figure also gives an analogous dependence measured on a packed column.

In the study of the effect of the amount of the given substance and the carrier gas flow-rate on the f_w^{30} values for the individual components of the test mixture on capillary column 2 with cold on-column injection dependence was found only for the heaviest triglycerides, tristearin and triarachidin. The dependence for the latter substance is depicted in Fig. 5.

In addition to the carrier gas flow-rate, the f_w value on the fused-silica capillaries with a chemically bonded stationary phase is also affected by the temperature programming, as indicated in Table I.

The effect of the sample composition on the f_w values is greatest for substances eluted close together, especially in the presence of a several-fold excess of one of these substances[10,12]. Table II gives the results obtained on the two capillary columns with cold on-column injection.

The reproducibility of the f_w value in replicate analyses, and the effect of column overloading by a sample containing higher triglycerides, was also tested for comparison with the results obtained on packed columns[13]. The results for the capillary columns are given in Table III.

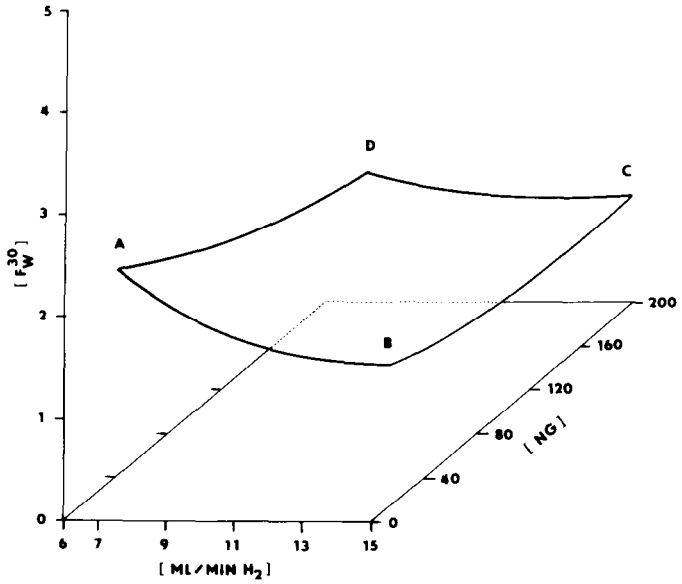

Fig. 5. Dependence of f_w^{30} for triarachidin on the hydrogen carrier gas flow-rate (ml/min) and on the amount of substance (ng). Column 2, cold on-column injection, program 8 K/min, remaining conditions as in the text. Coordinates x (ml/min), y (f_w^{30}), z (ng) are as follows: A 7.0, 2.33, 10; B 15.0, 1.41, 10; C 15.0, 1.03, 200; D 7.0, 1.23, 200.

Quantitative analysis requires not only independence of the f_w value on the sample composition and size, but also reproducibility of the measurement, especially with very small triglyceride samples. The results obtained on the two capillary columns under different conditions are given in Table IV.

It has been found[9,13,14] that an increase in the carrier gas flow-rate leads to an increase in the recovery of the analyzed triglyceride, but that the separation efficiency decreases. The results measured on the two capillaries under various conditions are given in Fig. 6. However, the separation efficiency is dependent not only on the carrier gas flow-rate but also on the temperature programming, as shown in Table V for measurements on column 1.

TABLE I

EFFECT OF THE TEMPERATURE PROGRAM ON f_w FOR SELECTED TRIGLYCERIDES

Column 1, splitless injection, carrier gas flow-rate 10 ml/min.

f_w	*Temperature program (K/min)*		
	2	*4*	*8*
42	0.95	0.93	0.92
48	0.97	0.95	0.93
54	1.22	1.30	1.43
60	2.14	2.40	2.83

TABLE II

EFFECT OF THE SAMPLE COMPOSITION ON f_w FOR HIGHER TRIGLYCERIDES

*Tested substance**	*Added substance**	*Column 1***		*Column 2***	
		Before	*After*	*Before*	*After*
20/52	200/54	2.05	1.22	1.01	0.97
20/54	200/52	2.83	1.44	1.03	0.97

* The amount (ng) of the given triglyceride is given first followed by its carbon number.

** The f_w value of the tested substance alone, and that for the tested substance after addition of a 10-fold excess of the other triglyceride are given.

TABLE III

EFFECT OF COLUMN OVERLOADING ON f_w FOR TRISTEARIN AND TRIARACHIDIN

f_{w54} = Weight correction factor for tristearin; f_{w60} = weight correction factor for triarachidin.

Measurement	*Column 1*		*Column 2*	
	f_{w54}	f_{w60}	f_{w54}	f_{w60}
Before overloading	1.06	1.42	0.96	1.08
After overloading	1.05	1.40	0.95	1.06

Fig. 7 depicts a practical application of a fused-silica capillary with a chemically bonded non-polar stationary phase for the analysis of the neutral lipid profile in human blood plasma after administration of cod-liver oil; the triglyceride spectrum contains fractions with low levels of carbon numbers 56–62.

TABLE IV

REPRODUCIBILITY OF THE QUANTITATIVE ANALYSIS OF TRIGLYCERIDES WITH CARBON NUMBERS OF 30–60

Carrier gas flow-rate: 15 ml/min. Temperature program: 8 K/min.

Column No.	*Carbon number*					
	30	*36*	*42*	*48*	*54*	*60*
1*	2.52	2.93	2.18	4.31	4.32	6.51
1**	2.12	1.69	1.37	1.71	1.67	2.25
1***	2.32	2.42	2.10	1.99	3.62	7.28
2**	0.72	0.32	0.46	0.41	0.43	0.81
2***	0.91	0.43	0.48	0.51	0.53	1.40

* Splitless injection, injector insert 1.5 mm I.D.; the sample contained 200 ng of each triglyceride.

** Cold on-column injection; the sample contained 200 ng of each triglyceride.

*** Cold on-column injection; the sample contained only 10 ng of each triglyceride.

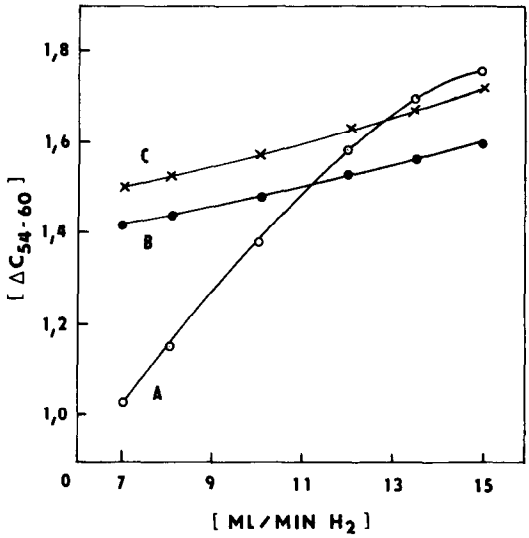

Fig. 6. Dependence of the separation efficiency in the carbon number interval 54–60, ΔC_{54-60}, on the hydrogen carrier gas flow-rate (ml/min) for various capillaries with splitless and on-column injection. Curves: A, column 1, splitless; B, column 1, cold on-column injection; C, column 2, cold on-column injection. Sample as in Fig. 1, program 8 K/min, remaining conditions as in the text.

DISCUSSION

For greater lucidity, the dependences found are presented in the form of three-dimensional graphs. From a mathematical point of view, the dependences in Figs. 1, 2 and 4 are not defined as continuous surfaces as the molecular weight of the triglycerides plotted on the x axis can only attain certain values. This is, however, not true for the dependence depicted in Fig. 5, where all the quantities, f_w^{30}, carrier gas flow-rate and amount of substance, can assume an infinite number of values. However, a simplification of the graphical representation of the results in Figs. 1, 2 and 4 provides a better approximation to the relationships between the variables. These dependences are characteristic and have been repeatedly verified on capillaries and, in some cases, also on packed columns.

TABLE V

DEPENDENCE OF ΔC_{48-54} ON THE TEMPERATURE PROGRAM

Sample: triglycerides with carbon numbers of 30–60, each 200 ng. Carrier gas flow-rate: 10 ml/min. Cold on-column injection.

Temperature program (K/min)	ΔC_{48-54}
2	0.89
4	1.13
8	1.38

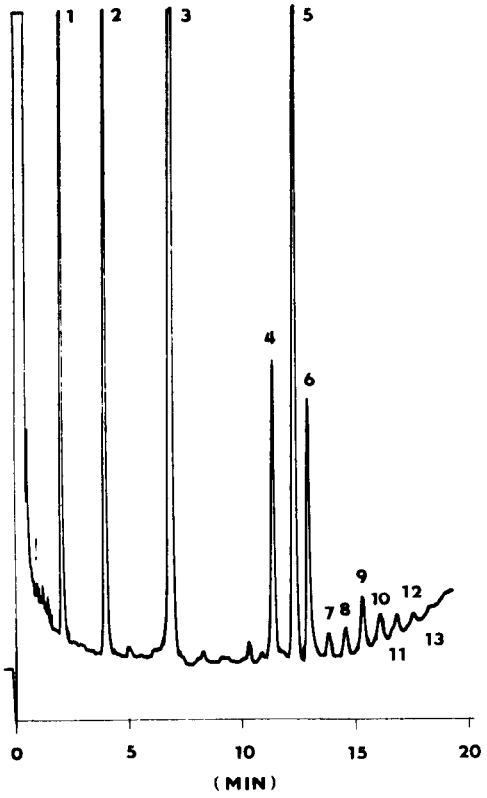

Fig. 7. Analysis of the profile of neutral lipids in human blood plasma after the administration of cod-liver oil. Column 1, cold on-column injection, hydrogen carrier gas flow-rate 15 ml/min, program 8 K/min, sample 1 μl dissolved in undecane–toluene (95:5, v/v), other conditions as in the text. Peaks: 1 = cholesterol; 2 = cholesteryl butyrate (internal standard I); 3 = tridecanoin (internal standard II); 4–6 = cholesterol esters with carbon numbers of 43, 45 and 47; 7–13 = triglycerides with carbon numbers of 48, 50, 52, 54, 56, 58 and 60. A triglyceride fraction with carbon number 62 can also be seen on the chromatogram; the baseline drift was not compensated.

Effect of various factors on f_w

From the results given here and in earlier papers[9–14] it is seen that the recovery of a given triglyceride and thus f_w depend on several factors. For capillary columns, these are primarily the injection technique, column quality, carrier gas flow-rate and the amount and molecular weight of the substance. The latter two factors are given in a particular case by the composition and size of the analyzed sample. The other factors must be optimized for each case. It should be realized that the individual optimized factors change dynamically even during a single analysis.

It is known, for example, that the carrier gas flow-rate through the column decreases with increasing temperature in analyses carried out on common contemporary gas chromatographs[21]. This leads to a change in the composition of the gas flowing through the flame ionization detector and thus also in the response. The concept of "column quality" includes not only the amount of stationary phase and coverage of the internal surface of the column, but also the homogeneity of the

stationary phase film and the inertness of the internal capillary surface. Ageing of the column changes its properties not only through a decrease in the content of the stationary phase by thinning of the film, but also in an increase in the number of bare patches on the inner surface of the column. In addition, the stationary phase may become contaminated by residues of analyzed samples. All these factors affect the interaction of a given substance with the column and with the stationary phase, which can appear as a change in the recovery of the substance compared with the value obtained on a new column, even when all the important parameters remain constant.

An interaction of a dynamic character apparently also exists between the factors affecting the f_w value. Effects of this type are probably important as ragards the reproducibility of f_w in time. This problem will be considered later.

It is apparent from Fig. 1 that the carrier gas flow-rate affects the f_w^{30} value for both types of injection, splitless and cold on-column. When using splitless injection, the discrimination of the components with higher carbon number of the sample is apparently affected primarily by the linear velocity of the carrier gas in the injector. This is in agreement with the results obtained at the same carrier gas flow-rate, but with two different diameters of the exchangeable glass insert of the injector (1.5 and 2.5 mm I.D.). Less discrimination of the higher components of the sample was attained using an insert with an internal diameter of 1.5 mm. However, the carrier gas flow-rate is also important for cold on-column injection, as is seen from Figs. 1 and 2. Probably the carrier gas flow-rate or, more precisely, its linear velocity in the column, affects the interactions between the substance and the column. Both the layer thickness and thus also the amount of the stationary phase, as well as the column quality, *i.e.*, inertness of the inner surface of the capillary and coverage with the stationary phase, and the homogeneity of the layer probably play a role. These concepts are in accord with the results published by Grob[17,18] and with our previously published results obtained on packed columns[12,13]. The ratio of losses as a result of retention of the substance in the stationary phase and degradation or sorption during contact with the column surface cannot be determined on the basis of these experiments.

It is, however, important that, above a certain variable limit, the f_w value on capillary columns, as for packed columns, is dependent on the amount of the analyzed triglyceride. The results obtained for various triglycerides on column 2 using cold on-column injection are depicted in Fig. 4. For comparison, the dependence of the f_w^{30} values on the sample amount and on the molecular weight of the triglyceride, measured using samples of identical composition on a packed column, is also given. Fig. 4 also depicts the difference between capillary and packed columns, especially for the analysis of small amounts of higher triglycerides. The results are in agreement with data published previously[10,11,15]. Papers describing problems in quantitative analysis using capillary columns have paid insufficient atttention to the dependence of f_w on the amount of the analyzed triglyceride. Neglect of this factor can lead to erroneous results, especially for the higher components of the sample.

The results depicted in Fig. 5 are also in agreement with previous results[10,14] and complement information on the dependence of f_w^{30} on other factors given in Figs. 1, 2 and 4. This dependence is also affected by the loss of high-molecular-weight triglycerides in the stationary phase and irreversible interactions with the column,

especially at low carrier gas flow-rates. The injection effect is practically eliminated as the measurements were based on cold on-column injection. If a different type of injection were used (split, splitless), the f_w^{30} values would certainly be far more dependent on both parameters, even for substances with lower carbon numbers.

The dependence of f_w on the temperature program rate will be considered later.

Reproducibility of f_w

Capillary column 2 yielded f_w values that were practically independent of the sample composition, but capillary column 1 behaved similarly to a packed column[12], as is seen from Table II. This behaviour is primarily a result of the column quality, as there is very little difference in the contents of the stationary phase between the two capillaries. Thus an explanation for the different behaviours of the two capillaries must be sought in the inertness of the column material and the homogeneity of the coating with the stationary phase. Although column 1 behaved similarly to a packed column in the study of the dependence of f_w on the sample composition, the dependences on sample overloading are not similar for these two types of columns, as is seen in Table III. The different behaviours of the capillary columns can be explained in terms of the difference (about one order of magnitude) in the ratio of the amount of the stationary phase to that of the sample, compared with the situation with the packed column. This ratio was 3500–5000:1 for a 5-m capillary with an internal diameter of 0.31 or 0.32 mm and a stationary phase thickness of 0.17 or 0.12 μm, for a sample containing 1.2 μg triglycerides. It attained values of 30 000–50 000:1 for a 0.5-m packed column (1.75 mm I.D.) packed with 1% stationary phase on Gas-Chrom Q (100–120 mesh) for a sample containing 3.0 μg triglycerides.

The behaviour of the capillary columns documented in Table III is in agreement with the previously stated assumption of competitive saturation of the stationary phase by higher components of the sample[12]. The results of Grob[17] and of Breckenridge and Kuksis[16] are also in accord.

If a factor that can be termed the column quality is analyzed in the light of all the results published so far, it follows that several physico-chemical phenomena are important. Our results obtained on capillaries with a chemically bonded stationary phase and the data published by Grob[17] indicate that the losses of the analyzed substances when using capillaries are primarily a result of interactions between the sample and the column, and that interactions with the stationary phase are not decisive. The opposite behaviour can be expected for packed columns; this reasoning explains the difference between the results of Grob[17] obtained on capillaries and those of Breckenridge and Kuksis[16] on packed columns. This explanation is in accord with our previously published suggestion for the mechanism of losses of higher triglycerides on packed columns[12]. Thus, the main difference between capillary and packed columns for the quantitative analysis of triglycerides will probably lie in the ratio of the masses of the sample and the stationary phase, leading to suppression of losses resulting from competitive saturation of the stationary phase. However, an exact experimental verification of these ideas is difficult to achieve. From a practical point of view, it is, however, very important that reproducible f_w values were obtained under optimum conditions, even for higher triglycerides, using capillaries with a thin film of the stationary phase; these values were practically independent of the sample composition and of the size and composition of previously analyzed samples.

TABLE VI

DEPENDENCE OF THE ELUTION TEMPERATURES AND SEPARATION EFFICIENCY FOR TRIGLYCERIDES ON THE CARRIER GAS FLOW-RATE

The elution temperatures were calculated from the elution times for the individual triglycerides recorded by an integrator. Measurements were made on column 2 with cold on-column injection. The sample contained the same amounts (200 ng) of each triglyceride. Temperature program: 8 K/min.

Carbon number	*Elution temperature (°C)*		*Difference between elution temperatures (K)*		*ΔC*	
					10	*15*
	*10**	*15*	*10*	*15*		
30	224.5	219.6	27.3	27.2	0.87	1.04
36	251.8	246.8	24.6	24.6	1.03	1.21
42	276.4	271.4	22.2	22.1	1.22	1.46
48	298.6	293.5	20.0	19.9	1.43	1.66
54	318.6	313.4	18.4	18.3	1.62	1.92
60	337.0	331.7				

* Carrier gas flow-rate in ml/min.

Separation efficiency, its dependence on the analytical conditions and relationship with f_w

The denominator in the expression for the calculation of ΔC contains the difference in the elution times or temperatures, and thus the ΔC values increase, *i.e.*, the separation efficiency decreases, with a decrease in this difference. It follows from Table VI that the difference in the elution temperatures of the triglycerides decreases with increasing molecular weight, so that, under constant analytical conditions, the separation efficiency decreases from the lower to the higher homologues. Thus the range of carbon numbers for which the ΔC value was calculated must always be given. In order to attain a single ΔC value over a wide range of carbon numbers, a non-linear or multilinear temperature program must be used.

In addition to the above dependence, the separation efficiency in a given carbon number range is also dependent on the experimental conditions. It decreases with increasing carrier gas flow-rate and with increasing rate of temperature change. The recovery of the analyzed substance increases with increasing carrier gas flow-rate and decreases with increasing rate of temperature change. An increase in the carrier gas flow-rate leads to a decrease in the elution temperature for the triglycerides, but the differences between these temperatures remain practically unchanged, as is seen in Table VI. Thus a decrease in the column efficiency at higher linear carrier gas velocities leads to a decrease in the separation efficiency.

The rate of change of temperature (Table VII) has the opposite effect to the carrier gas flow-rate. The difference between the elution temperatures again varies very little.

A change in the rate of temperature change also affects the recovery of triglycerides, as is seen in Table I. A similar dependence was obtained on packed columns[13]. On the other hand, Monseigny *et al.*[9] did not observe this effect for glass capillaries. The values given here were measured with splitless injection, however, the same, although less marked effect of the rate of temperature change on f_w could be

expected for cold on-column injection. This could be one reason why Monseigny *et al.* did not find such a dependence. Another reason could be that they used substances with lower molecular weights; the highest carbon number was 54.

So far, no mechanism has been given for the effect of the rate of temperature change on the recovery of the test substances. A decrease in the rate of temperature change leads to a marked decrease in the elution temperature of a given triglyceride. It has been found that the carrier gas flow-rate decreases with increasing temperature at constant inlet pressure[21]. Thus, a decrease in the elution temperature is associated with an increase in the carrier gas flow-rate, which can effect the f_w value for a given substance analyzed at different rates of temperature change. The dependence of f_w on the carrier gas flow-rate is also greater for higher-molecular-weight substances.

It follows from this short discussion of the effect of some analytical conditions on the ΔC and f_w values that it is necessary to optimize these factors with respect to the composition and amount of the sample and to the requirements and limitations of the analysis.

Reproducibility of the analytical results

The reproducibility of the results of quantitative analysis of triglycerides in series and in time is dependent on the constancy of the f_w value. The results given are basically in agreement with data published by Grob[18]. An increase in the relative standard deviation for analysis of a sample containing 10 ng of the individual triglycerides compared with the values obtained on the same column for a sample containing 200 ng of these substances with carbon numbers of 30–60 is seen in Table IV. The relative standard deviation values for triglycerides with carbon numbers of 30–48 are acceptable even for samples containing 10 ng of each substance. However, if the values obtained on column 2 for 200 ng and for 10 ng are compared with the corresponding values measured on column 1, the effect of the column on the series reproducibility of the results is apparent.

TABLE VII

EFFECT OF THE TEMPERATURE PROGRAM ON FACTORS AFFECTING THE SEPARATION EFFICIENCY FOR TRIGLYCERIDES

Carbon number	*2 K/min*			*4 K/min*			*8 K/min*		
	T_e*	ΔT_e**	W***	T_e	ΔT_e	W	T_e	ΔT_e	W
30	207.3			216.3			225.4		
36	232.8	25.5		243.0	26.7		252.8	27.4	
42	256.9	24.1		267.2	24.2		277.4	24.6	
48	278.4	21.5	26–34	289.1	21.9	14–20	299.5	22.1	8–12
54	298.3	19.9		308.8	19.7		319.5	20.0	
60	315.8	17.5		326.8	18.0		338.2	18.7	

* Elution temperature (°C) obtained as in Table VI.

** Difference (K) between the elution temperatures.

*** Peak width in seconds at half-height. The peaks for all the triglycerides are not of the same width, as is seen in Fig. 3. The lower values correspond to tridecanoin, the higher to triarachidin. The widths for the other triglycerides lie between these two values. Column 2, carrier gas flow-rate 10 ml/min, other conditions as in Table VI.

In addition to the series reproducibility, the reproducibility in time is also important. Although this problem was not studied in this work, the data in Tables II and III indicate the long-term reproducibility of the results obtained on both columns. Column 2 and other columns with similar characteristics can yield results with high reproducibility in time that are almost independent of the composition and of the sample size. In contrast, column 1 and other columns with similar characteristics will also yield results that are well reproducible in time, but will be far more sensitive to a change in the sample composition or a decrease in its amount.

Similar conclusions following from the results of analyses of test samples should always be considered in the application of capillary columns to the quantitative analysis of triglycerides. This permits suitable steps to be taken to ensure and maintain the required reproducibility.

Application of fused-silica capillary columns with a chemically bonded stationary phase

The relative standard deviations obtained on column 2 confirm the ability of this capillary column to analyse even very small samples of triglycerides and other lipids. The results obtained here are practically identical with those published by Myher and Kuksis[20]. In the quantitative analysis of minor fractions of higher triglycerides from human blood plasma, the problem of possible sample hydrogenation by hydrogen usually used as a carrier gas must be recognized. Triglycerides with carbon numbers of $\geqslant 56$ contain long-chain fatty acids with a large number of double bonds. Partial or complete hydrogenation would greatly decrease the chemical heterogeneity of these substances and incomplete hydrogenation could affect the reproducibility of the analysis of these triglycerides. The model samples used in this work consisted only of saturated substances, so that conclusions on the sample hydrogenation cannot be drawn from the analysis of these substances. The heterogeneity of the peaks of higher triglycerides partly visible in Fig. 7 also does not suggest hydrogenation, which has been described for capillary columns only by Smith[19]. Myher and Kuksis[20] and other workers who used hydrogen as a carrier gas in the analysis of natural triglycerides containing double bonds also do not mention sample hydrogenation[2,3,5,6]. It is thus possible that the hydrogenation of the cholesterol esters described by Smith, who also used hydrogen as a carrier gas, involves other factors and it cannot be excluded that impurities in the sample, in the stationary phase or on the surface of the column acted as catalysts. No evidence has been found for hydrogenation of triglycerides on fused-silica capillaries, but this possibility should be studied experimentally. Replacing hydrogen by helium as a carrier gas would solve the problem of hydrogenation, but would involve loss of a number of advantages[2]. The most important of these in the analysis of triglycerides is the higher sensitivity and lower elution temperatures.

CONCLUSIONS

Summarizing the results obtained:

(1) Use of cold on-column injection on a good quality column about 5 m long yields f_w values for triglycerides with carbon numbers of 30–54 that are practically independent of, and for triarachidin only slightly dependent on, the amount of analyzed triglyceride and the carrier gas flow-rate. The limit of high reproducibility of

quantitative analyses is 4–5 ng for tristearin and lower triglycerides and 10 ng for triarachidin.

(2) When a good-quality column is used, the effect of sample composition on the f_w values is minimal. The f_w values were practically independent of column overloading and time.

(3) This type of column does not require the quantitative stabilization described for packed columns[12,13].

All these properties make short-fused silica capillary columns with a chemically bonded stationary phase the best columns so far described for very accurate and reproducible quantitative analyses of down to nanogram amounts of triglycerides including minor fractions of these substances.The analysis of triglycerides on capillary columns is a typical example of the application of these columns at higher linear velocities of the carrier gas, where use is nor made of the high separation efficiency. However, the high quality of fused-silica capillaries, which has so far not been fully appreciated, is utilized.

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