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DEGRADATION OF TRIGLYCERIDES IN GAS CHROMATOGRAPHIC CAPILLARIES: STUDIES BY REVERSING THE COLUMN

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SUMMARY

The losses of triglycerides that occur in the column during gas chromatographic (GC) analyses were studied by analysis of the degradation products. For this purpose the exit end section of a glass capillary column was kept at ambient temperature, trapping degradation products and unchanged sample (triolein). The trapped material was analysed by reversing the column, connecting the former exit to the injector. The major breakdown products of triolein were found to be free oleic acid and a number of unidentified compounds of diolein-like structure. The further reaction of diolein in the column produced mainly oleic acid and triolein plus a trace amount of the monooleins. For diolein about 90% of the reaction products were recovered. For triolein the recovery is assumed to be very high, but calculations were restricted owing to the unknown correction factors for the (labile) diolein-like compounds. A sample of triolein in a vacuum-sealed tube was heated in a GC oven at the same time as an injected sample was "baking" in the capillary. Losses in the tubing and in the capillary were found to be similar, indicating that there is little or no catalytic contribution from the (persilanized) capillary column to the degradation of the triglyceride tested.

INTRODUCTION

Most workers reporting on the analysis of triglycerides emphasize that gas chromatographic (GC) conditions have to be selected carefully if an acceptably accurate quantitative result is to be achieved (*e.g.*, refs. 1–3), otherwise great losses of the high-molecular-weight species occur in the column. However, opinions differed on how these triglycerides were retained, either by some sort of an irreversible absorption of the molecule itself[4] or by reduced volatility due to polymerization[5]. Although this question has been discussed by many workers, little experimental evidence is available. Breckenridge and Kuksis[6] demonstrated, by using labelled compounds, that parts of the triglycerides remained inside the packed column. Surprisingly, they found that after extracting the column the lost radioactivity was still integrated in intact triglyceride molecules. In order to minimize these effects it has generally been recommended

that low elution temperatures (usually increasing absorption) and low retention times are used. For capillary GC this implied the use of columns only 4–6 m long[7].

We became interested in these losses during experiments on different injection techniques for triglycerides[8]. Even when using cold on-column injection[9,10] the peak areas of the heavier triglycerides were 5–10% lower than those calculated using estimated response factors (flame-ionization detector, FID). Several reasons lead to the assumption that this difference could not be due to the injection technique. A part of the losses could be traced to drifting FID sensitivity caused by the decreasing carrier gas (hydrogen) flow-rate during the temperature programme[7,11], but for most of the missing material it still remained to be proved that the losses were due to the column.

Another reason for starting this investigation was our doubt about the widely accepted opinion that materials (e.g., triglycerides) may disappear in capillary columns as such, i.e., simply because of low volatility (excluding adsorption due to polarity, acid–base effects or degradation), as was suggested by Breckenridge and Kuksis[6]. For alkanes we found that the losses could always be traced to the injection technique or to a decrease in FID sensitivity. The proportion of alkanes lost in the column was negligibly small.

The analysis of the breakdown products originating from a labile compound during GC usually requires a second chromatographic run, because the conditions of the degradation process seldom allow these products to form a sharp starting band. They are formed during an extended period of time. As they are usually considerably more volatile than the parent compound, they pass through the system as soon as they are generated, usually increasing the baseline to only a negligible extent. This is in contrast to degradation products formed during the injection. However, as the samples were always introduced by cold on-column injection, such degradation was precluded. Hence the breakdown products generated in the column have to be reconcentrated and analysed by a second separation process. This can be achieved by using a column-switching type of configuration with two columns. However, whenever the degradation products are eluted only at column temperatures of 150°C or higher, there is a simpler technique. During the first run (producing the degradation products) the exit end section of the column is kept at room temperature outside the GC oven, serving as a trap for the undegraded sample and all the new products formed during the chromatography. Then the column is reversed and the former exit is connected to the injector whereas the inlet is fixed to the FID. During the analysis all of the trapped materials are moved backwards through the column.

EXPERIMENTAL

There are several ways to expose the column exit to ambient temperature, one possibility being to push it through an unheated detector block. The experiment described was carried out on a Model 2150 gas chromatograph (Carlo Erba, Milan, Italy) modified to take a cold on-column injector[9]. In the front door of this instrument there is a thermometer reaching into the oven, and this thermometer was removed so that the column could pass through the hole. A 15 m × 0.30 mm I.D. persilanized[12] column coated with 0.15 μm of SE-52 was used, having straightened end sections 18 cm long. One end section was connected to the on-column injector and the other

hung freely in the GC oven. After cleaning the column at 375°C for 30 min, the oven was cooled and the exit end section pushed through the hole in the oven door, exposing 10–12 cm of the column to ambient temperature. A 0.5-μl volume of a mixture containing 10 ppm of n-tetracontane (C_{40}) and 100 ppm of triolein in n-hexane was injected as described earlier[10] and chromatographed under conditions favouring degradation ("baking run"): 0.25 atm of hydrogen as the carrier gas and the column temperature programmed from 240 to 370°C at 3°C/min.

As soon as the triolein had eluted into the cold end section, heating of the oven was stopped. A rapid decrease in the column temperature was avoided because the contracting gases inside the column would have been sucking in air from the open end section. When cold, the column was reversed, the former inlet being connected to the FID. The column was kept at ambient temperature for a few minutes until the FID had stabilized, then the carrier gas inlet pressure was increased to 1.2 atm, and a temperature programme of 7°C/min from 80 to 370°C was started. After this "analysis run" the column exit was disconnected and the next trapping run could be started.

RESULTS AND DISCUSSION

The degradation of triglycerides was studied for tristearin and triolein, differences concerning their tendency to polymerize being expected. However, the results for the two triglycerides were so similar that the results for triolein can be used for both.

The mixture used contained triolein and n-tetracontane as a stable internal standard in the proportions 10:1. Considering the FID response, the triolein should produce almost nine times the peak area of the alkane. As shown in Fig. 1a, the peak area measured, using the column and the conditions described, was only 6.1 times the peak area for the alkane. The enhanced baseline in front of the peak (the shaded surface in Fig. 1a) is related to these losses, showing that at least part is caused by degradation, with the formation of more volatile products than triolein. The amounts of oleic acid and diolein as by-products of the mixture were negligible.

Fig. 1b shows the same mixture after a "baking run" with trapping of the eluted products, thus showing the volatile degradation products of the first (but not of the second) chromatographic run. The relative area for triolein decreased to 3.9 units. The new peaks are due to degradation products either of the triolein or of the stationary phase (bleeding). The trapped bleeding alone is shown in Fig. 1d and should be subtracted. The major peak originating from triolein was identified as oleic acid, representing a relative peak area of 1.3 units (area divided by the area of the n-tetracontane peak). A second, complex group of peaks eluted with a retention time close to that of diolein. However, the peak with the retention time of diolein was very small (indicated on the chromatogram as di-18:1). The major peak of the group, with a relative area of 0.55 unit, had a slightly shorter retention and was characterized by its peculiar broadened but tail-free shape. This peak was followed by another, even broader (shaded) peak with a number of small peaks on top of it. The sum of these areas was estimated to account for at least another 0.35 unit.

Considering the possible reaction mechanisms, it is unlikely that a diolein is formed by passage through a persilanized column. The concentration of free water or of silanols able to release water is too low to allow a hydrolysis reaction. Probably the

degradation occurs by a thermal fragmentation, producing oleic acid and an unsaturated product, one of the two possible enol esters. Enol esters, however, are reactive species, available for a number of reactions that might be partly reversible (including reactions with the support). We were not able to confirm such a hypothesis or that such enol esters produce broad "reaction peaks" as were observed.

Fig. 1c shows the result of a differently heat-treated mixture of n-tetracontane–triolein (1:10). A 110-mg amount of the solvent-free mixture was kept in a small vacuum-sealed tube. The tube was heated inside the GC oven during a "baking run", exposing triolein to the same temperature as the chromatographed (and trapped) sample. The contents of the tube were then dissolved and analysed. As indicated in Fig. 1c, 3.5 area units of triolein and almost 0.9 units of oleic acid were recovered. Further, there was a peak identified as diolein (0.4 area unit). It has to be concluded that the moisture present in the tube was sufficient to allow the formation of a corresponding amount of diolein.
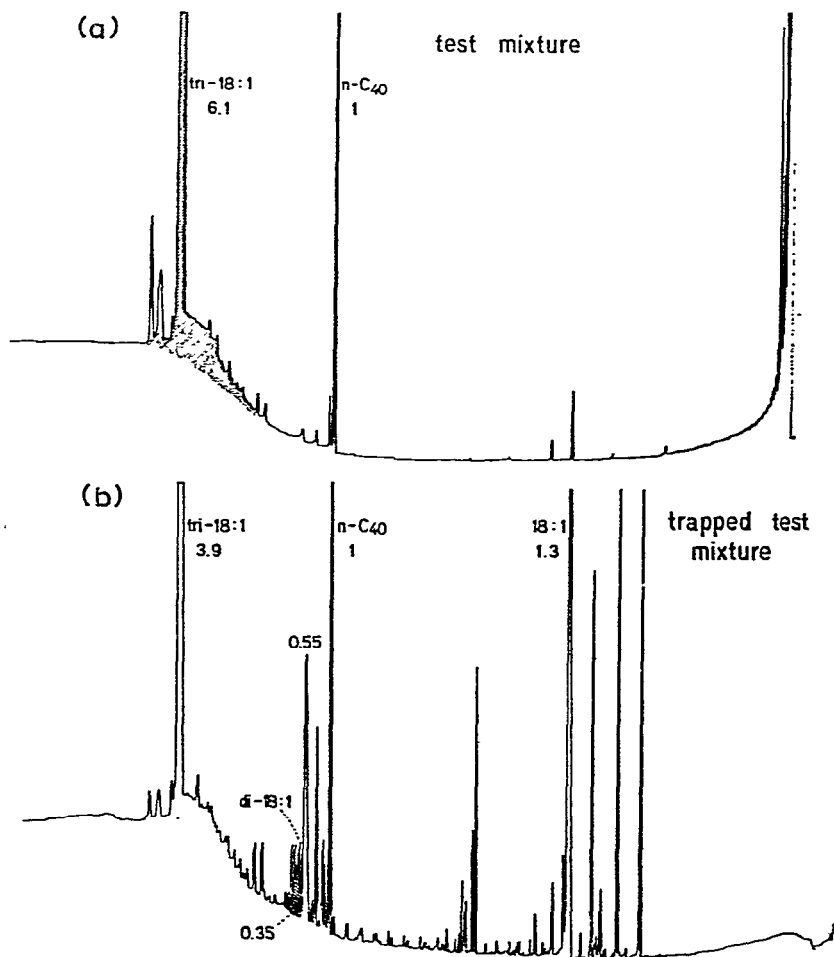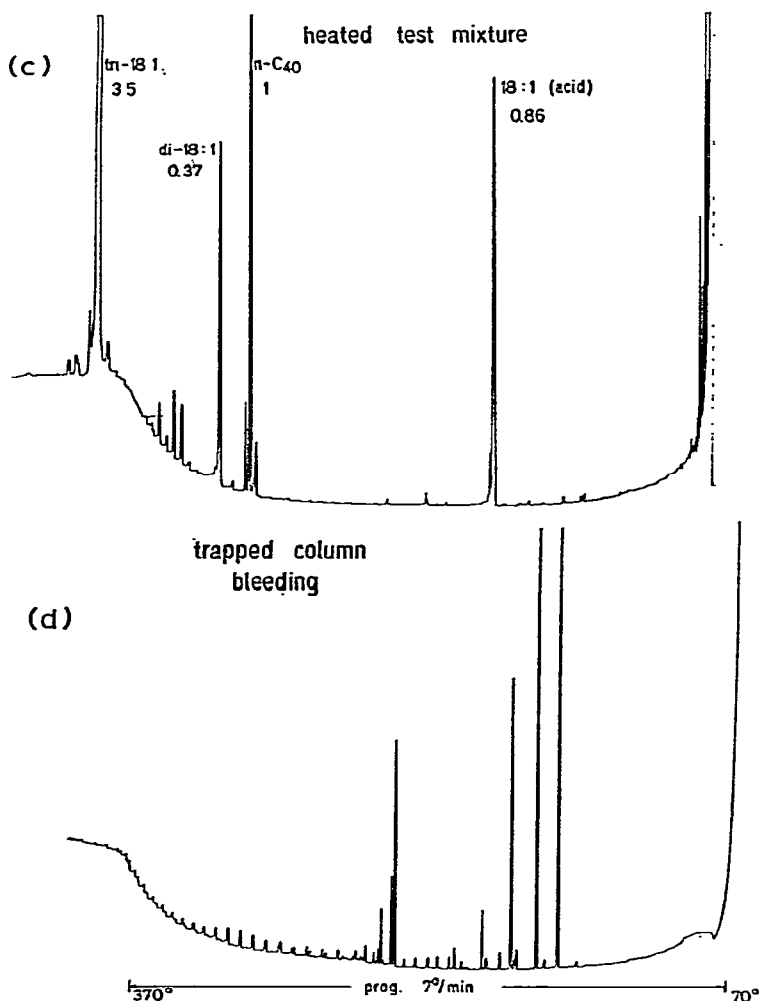

Fig. 1.

Fig. 1. Studies on the thermostability of triolein. (a) Analysis of triolein–*n*-tetracontane (*n*-C$_{40}$) (10:1) on a persilanized glass capillary column (15 m × 0.30 mm I.D.) coated with 0.15 μm of SE-52; hydrogen at 1.2 atm as carrier gas; temperature programmed at 7°C/min from 70 to 370°C. Peak areas are normalized on *n*-C$_{40}$ (set at 1). Part of the triolein is degraded, producing more volatile products as shown by the shaded surface in front of the triolein peak. (b) The same mixture re-chromatographed after a "baking run" with trapping of the eluted products in the cold exit end section of the column. Column reversed and the trapped material (unchanged sample, degradation products and column bleeding) analysed under the same conditions as in (a). Column bleeding alone: see (d). The major degradation products detected were free oleic acid (18:1) and a group of substances forming peaks close to diolein (di-18:1), eluting just after *n*-C$_{40}$, the major ones being broadened "reaction" (?) peaks (the second of them shaded). (c) same mixture "baked" on a preparative scale in a vacuum-sealed tube in the GC oven during the "baking run" for (b), then dissolved and analysed under the same conditions as in (a). As triolein is degraded to a similar extent as during the "baking" in the capillary, it is concluded that catalytic activity of the capillary is negligible.

The peak area detected for diolein has to be considerably corrected as diolein is a very labile compound. The response for pure diolein (or distearin) under the conditions of the "analysis run" was only between 15 and 28 % of that for n-tetracontane, averaging 20 % (six analyses). Estimating a response of 0.9 for diolein on the FID, compared with n-tetracontane, the area of the detected diolein has to be multiplied by a factor of 4.5. Hence after heat treating triolein in the vacuum-sealed tube, diolein was present in an amount equivalent to 1.8 area units. Including the recovered oleic acid, 2.7 area units of the lost triolein were recovered, compared with 4.4 area units of missing triolein. About 1.7 area units, representing 19 % of the original triolein, disappeared, possibly as a result of polymerization.

The method used to study the losses of triglycerides can also be applied to diolein. Again n-tetracontane was used as the internal standard (diolein:n-tetracontane = 10:1). After a "baking run" 1.9 area units were recovered as oleic acid and 3.2 units as triolein. Another two peaks had the same retentions as the monooleins, but the recovered area was small. The area of the triolein has to be corrected for losses during the analysis run, resulting in 4.3 units. The sum of these areas totalled 6.3 units, representing 82 % of the lost diolein. Inter-esterification, producing oleic acid and triolein, also liberated C_3 molecules from the glyceride moiety, but under the conditions used such compounds were either not trapped owing to their excessive volatility or they were not eluted from the column because of too high adsorptivity. It should be noted that the results for the thermal reactions of diolein were found to be strongly dependent on the amount of diolein injected. This is not surprising when it is considered that the formation of triolein is a bimolecular reaction, whereas the decay of triolein is not dependent on local concentrations in the stationary phase.

Returning to the losses of triolein in the column, one should attempt to make a balance between losses and the recovered degradation products. Using the percentage of the triolein lost during the "analysis run", extrapolation indicated that the amount of triolein present at the end of the "baking run" corresponded to 5.1 area units (confirmed by an analysis using the conditions of the "baking run"). Thus, the losses amounted to 3.9 area units. Some 1.3 area units (33 %) were recovered as oleic acid and 0.9 units (23 %) as diolein-like substances. The area representing oleic acid does not require a correction. However, there remain the poorly explained fragments with retentions close to that of diolein, which should be multiplied by an unknown, but certainly large correction factor. For diolein the correction factor was determined as 4.5, whereas here a factor of less than 3 would be sufficient for a full explanation of the lost triolein.

The fact that the recovered oleic acid corresponded just to one third of the losses may lead to a simplified interpretation of the results, $i.e.$, that the triolein decays into a diolein-like fragment and oleic acid. Although this appears to be the first step of the reaction, the diglyceride derivative will again be subject to chemical reactions. The reactions of diolein have been considered above. However, the reaction conditions are different to those in the degradation experiment with diolein. The diolein-like fragment is formed at column temperatures considerably exceeding the elution temperature of diolein (350–370°C). On the other hand its retention, $i.e.$, the time it spends in the liquid stationary phase or on the support surface, is accordingly short also. Further, this diolein-type compound is formed during a period of several minutes, keeping its local concentration at a low level. This reduces the probability that it may build up

triolein as observed in the experiment with diolein. This may be considered to be fortunate as it blocks a possible transesterification reaction on the column, which would render the results of triglyceride analyses dubious. In fact, when injecting triolein and tristearin as a mixture of standards, it was never observed that mixed triglycerides were formed, e.g., by a reaction of oleic acid with a distearin-type diglyceride.

CONCLUSION

Losses of triglycerides in capillary columns occur mainly, if not exclusively, as a result of degradation processes, rather than polymerization or losses of intact triglyceride material. This is in contrast to the findings of Breckenridge and Kuksis[6] for packed columns. Although the values given depend on a number of parameters, the results allow the following three conclusions to be drawn: (1) the detection of most of the degradation products confirms our previous assumption[8] that the discrimination of the heaviest triglycerides is not due to the injection technique when using cold on-column sampling; (2) there is still no upper limit for the boiling point of substances amenable to capillary GC, in contrast to packed-column GC (?) and we still could not find an example of compounds lost in the column due to low volatility; and (3) when compounds are degraded in a chromatographic column it is of interest to know if the reaction has been catalysed by the column or if the compound reacted with a functional group present in the column. If the degradation could be shown to be due to poor column quality there would be a need to improve the results by better deactivation of the column.

For triolein the comparison of the degradation rates of the triglyceride passing through the column and that heated in the vacuum-sealed tube provided some information. The triolein in the tube was shown to be modified to a greater extent than the triolein passing through column (losses of 50 % compared with 43 %). However, for about 20 % of the losses in the tube no degradation products were found, so that the material may have been polymerized. On the other hand, polymerization in the column seems to be negligible. Hence degradation occurred by similar rates, which precludes a high activity of the chromatographic column. It should be mentioned that this was found for persilanized[12] columns. Columns of different types may be much more active, as is known, e.g., for many of the barium carbonate[13] columns.

In practice the results only confirm what has been recommended by other workers. If a suitable type of capillary column (without catalytic activity) is found, the chromatographic process has to be optimized to expose the triglycerides to a minimum of thermal stress. The elution temperature is the most important parameter to be controlled. As shown previously[14], the elution temperature decreases by 15°C when the film thickness of the stationary phase is halved. Another cheap measure (as it does not decrease the separation efficiency) is the choice of hydrogen as the carrier gas (halving the retention time for a given efficiency in comparison with helium). If the gas flow-rates into the FID are chosen carefully, the drifting sensitivity does not cause problems (this is the reason why Monseigny et al.[7] prefered helium). On the other hand, the increase in the carrier gas flow-rate above the optimum and the reduction in the column length lead to reduced column efficiency.

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