# Determination of bovine butterfat triacylglycerols by reversed-phase liquid chromatography and gas chromatography

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ABSTRACT

Triacylglycerols (TGs) from a sample of summer butterfat (bovine milk) were analysed and fractionated by reversed-phase liquid chromatography (RPLC). Fatty acid and TG compositions of each of the 47 RPLC fractions ranging from 0.1 tot 6.9% were determined by capillary gas chromatography. The data were used together to determine the quantitative composition of the molecular species of TGs. A large number of TG species, accounting for 80% of the total, could be unequivocally identified and individually determined. The combination of the chromatographic methods used proved to be a powerful and accurate approach for the determination of molecular species of TGs in a complex fat, but also a difficult and time-consuming task.

INTRODUCTION

Butterfat from bovine milk represents one of the most complex mixtures of natural triacylglycerols (TGs). The component fatty acids range from $C_2$ to $C_{26}$, including even and odd carbon numbers, straight and branched chains, numbers of double bonds from zero to six and *cis* and *trans* isomers. The fourteen even-carbon number, straight-chain fatty acids studied in this work, and commonly reported in a number of publications, comprise a total of 95 mol% [1]. About 40 minor fatty acids at levels ranging from 0.01 to 0.5%, except 15:0 (1.5%), are also present in butterfat [1–5].

Without considering the possible positional isomers of the three acyl chains within the molecular TG species the only $n = 14$ fatty acids distinguished in this present work can yield $(n + 3n + 2n)/6 = 560$ different TGs [6]. In the past, butterfat TGs have been resolved on the basis of carbon number and double bond number by packed [7] and capillary [8–12] gas chromatography (GC) and reversed-phase liquid

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chromatography (RPLC) [13–19] into a wide range of peaks from $C_{22}$ to $C_{54}$. However, most of the peaks contained several molecular species of TGs when total butterfat TGs were chromatographed, preventing the exact identification and determination of any TG species. A combination of two or more analytical techniques is therefore necessary.

Kalo et al. [11] fractionated butterfat TGs on the basis of degree of unsaturation by thin-layer chromatography (TLC) and analysed the fatty acids and TGs of each fraction by GC. However, they were not able to achieve the determination of any TG species. Myher et al. [1] combined silver nitrate TLC, capillary GC and mass spectrometry to analyse a volatile molecular distillate of butteroil, containing acetyl-diacylglycerols, and obtained the identities and amounts of many major TG species in the TLC fractions. By silver nitrate TLC, TGs are resolved according to chain length, number of double bonds and geometric configuration. As a result, overlapping between several bands is generally observed, and a complete extraction (without oxidation) of all the polyunsaturated TGs from the gel of minor bands is difficult.

An alternative method is the fractionation of total TGs by RPLC and analysis of the fatty acids and TGs of each fraction. Using the data obtained in this way, Weber et al. [20,21] could determine the proportions of 116 different molecular TG species in two butterfat samples, but the fatty acid and TG compositions of the RPLC fractions were not published. Barron et al. [22] also identified 116 molecular species of TGs in a sample of bovine butterfat by a combination of RPLC analysis of total TGs and GC analysis of fatty acids from each collected RPLC fraction and on the basis of a random TG composition calculated from the molar percentages of eleven main fatty acids present in the total milk fat. They considered a priori that the most probable molecular species were those with $>0.1\%$ random values. The way in which they combined experimental and theoretical data seemed to us open to criticism, because it is known that significant differences occur between experimental and random values in natural fats [23–25] and also that butyric acid in bovine butterfat TGs is specifically esterified at the sn-3 position [26] and not distributed randomly over the whole glycerol skeleton.

In this study, started before the publication of Weber et al.'s work, butterfat TGs were first analysed and fractionated by RPLC and each fraction was analysed for the fatty acid composition. The data were used for the identification and quantitative distribution of TG species, as has been done successfully for a natural long-chain TG mixture, namely peanut oil [27]. However, the fatty acid composition of TGs in most of the RPLC fractions appeared so complex, and the theoretical number of possible TG species so large, that extra data were required. Analysis of TGs by capillary GC was thought to provide sufficient data to determine the composition of the major TG species in butterfat. Some of the results are reported in this paper.

EXPERIMENTAL

*Materials*

The summer butterfat (bovine milk) was a sample from Union Laitière Normande (Condé-sur-Vire, France). Total lipids were extracted according to Delsal [28]. The pure TG fraction was isolated by silicic acid column chromatography [29] and its purity was checked by thin-layer chromatography. Synthetic TGs in an individual form and quantitative mixtures were obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.).

All the solvents were of pure grade and provided by SDS (Peypin, France), except *n*-butanol, obtained from Aldrich-France (Strasbourg, France).

*Reversed-phase liquid chromatography of triacylglycerols*

A Model 6000A solvent delivery system and a Model R401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.) were used. The 250 × 4 mm I.D. LiChroCART column packed with LiChrospher 100 RP-18 (4-*μ*m particles), protected by a LiChrosorb RP-18 precolumn, was purchased from Merck (Darmstadt, Germany). The column was maintained at a constant temperature using a thermostat described previously [30], which permitted reproducible analyses.

Analysis and fractionation of butterfat TGs were carried out on samples of 4 mg of TGs dissolved in 40 *μ*l of acetone under isocratic conditions in two steps. In the first step the column and injector were maintained at 40°C, avoiding possible precipitation of saturated long-chain compounds, and the mobile phase was acetone–acetonitrile (55:45, v/v) at 1 ml min$^{-1}$. The chromatographic profile showed 47 peaks but the first 27 peaks were poorly separated. The first 27 RPLC fractions were collected together and the next 20 RPLC fractions were collected individually at the outlet of the detector. In the second step, the first 27 RPLC fractions collected together were analysed at 30°C using acetone–acetonitrile (50:50, v/v) at 1 ml min$^{-1}$ and individually collected. The fractionation of butterfat TGs was repeated five times and the RPLC fractions collected were added. Each of the 47 collected RPLC fractions was purified by rechromatography under its own elution conditions to eliminate the contaminating adjacent RPLC fractions (essentially the preceding major peaks). The purified TG fractions were divided into two parts for GC of fatty acid butyl esters and TGs. During RPLC analyses of butterfat TGs, peak areas were measured by means of an ENICA 21 (Delsi, France) integrator–calculator.

Mixtures of standard saturated simple TGs were analysed under the same conditions to determine the theoretical partition number of the butterfat TGs in some RPLC fractions. The partition number (*PN*) of a TG is calculated from the total carbon number (*CN*) and total double-bond number (*DB*) of the three constituent acyl moieties using the equation $PN = CN - 2DB$, according to Litchfield [31].

*Gas chromatography of fatty acids*

Fatty acid methyl esters were prepared by instantaneous reaction by adding 0.1 ml of 0.5 *M* sodium methoxide to a 7-ml tube containing *ca.* 1 mg of a TG mixture dissolved in 1.8 ml of hexane at room temperature [3,32,33]. After centrifugation, the upper hexane phase containing methyl esters was not evaporated before injection so as to avoid any loss of short-chain fatty acids.

Fatty acid butyl esters were prepared in a 3-ml tube with a Teflon-lined screw-cap by heating *ca.* 1 mg of a TG mixture in the presence of 0.2 ml of acidic (2%, w/w, sulphuric acid) *n*-butanol at 100°C for 2.5 h. To the chilled mixture were added 1 ml of 5% (w/w) potassium carbonate in water and 1 ml of hexane and the tube was vigorously shaken. The clear butanol–hexane upper phase containing butyl esters was ready for injection. When the amounts of the collected RPLC fractions of butterfat TGs were less than 0.1 mg, butylation was carried out in 0.3-ml reaction vials (Reacti-Vials, Pierce, Rotterdam, The Netherlands) in the presence of 10-*μ*l of acidic *n*-butanol at 80°C for 2 h. Then 30 *μ*l of aqueous potassium carbonate and 90 *μ*l of hexane were added to the chilled mixture. The clear upper phase was injected directly.

Methyl or butyl esters were analysed using a Packard Model 438A chromatograph including a cold on-column injector, a 30 m × 0.32 mm I.D. fused-silica column coated with Carbowax 20M (AML Chromato, Limoges, France) and a flame ionization detector maintained at 240°C. The carrier gas was hydrogen at a flow-rate of 1 ml min$^{-1}$. When methyl esters were analysed the oven temperature was programmed as follows: 35°C for 1.5 min after injection, then increased to 155°C at 30°C min$^{-1}$ and finally to 220°C at 4°C min$^{-1}$. When butyl esters were injected, the temperature was initially maintained at 35°C for 4.5 min instead of 1.5 min. Approximately 0.4 $\mu$l of fatty acid ester solution (0.5–1 $\mu$g $\mu$l$^{-1}$ of total esters) was injected. Peak areas were measured by means of a Shimadzu C-R3A integrator–calculator.

In preliminary experiments, methyl and butyl esters of a wide range of fatty acids (saturated and unsaturated, even fatty acids from $C_4$ to $C_{18}$) were prepared from a quantitative mixture of synthetic simple TGs approximating the composition of butterfat TGs. Methyl and butyl esters were used for comparison in the determination of fatty acids by GC and for the determination of calibration factors (Table I).

The possible loss of short-chain compounds during the preparation of butter fatty acid esters, especially methyl esters, was thoroughly examined. First, the response factors determined for methyl esters were found to vary little, from 1.15 (8:0) to 0.93 (unsaturated long chains), except for 6:0 (1.34) and 4:0 (1.67). When standard quantitative mixtures of methyl esters (Nu-Chek-Prep) were injected directly into the chromatograph, the calibration factors (results not shown) were not different from those obtained for the methyl esters prepared from the mixture of simple TGs. This

TABLE I

MOLAR RESPONSE FACTORS[a] FOR ACID METHYL AND BUTYL ESTERS DETERMINED FROM A STANDARD TG MIXTURE AND COMPOSITION (mol%)[a] OF MAJOR FATTY ACIDS OF TOTAL BUTTERFAT TGs ANALYSED AS BUTYL ESTERS

| Fatty acid | Response factor | | Total butterfat TGs |
|---|---|---|---|
| | Methyl esters | Butyl esters | |
| 4:0 | 1.669 ± 0.018 | 1.116 ± 0.010 | 9.61 ± 0.05 |
| 6:0 | 1.341 ± 0.017 | 1.155 ± 0.018 | 4.85 ± 0.04 |
| 8:0 | 1.147 ± 0.013 | 1.061 ± 0.012 | 2.17 ± 0.01 |
| 10:0 | 1.052 ± 0.011 | 1.031 ± 0.005 | 3.87 ± 0.01 |
| 12:0 | 0.998 ± 0.004 | 0.994 ± 0.003 | 3.71 ± 0.01 |
| 14:0 | 0.973 ± 0.006 | 0.984 ± 0.003 | 11.37 ± 0.02 |
| 14:1 | 0.985 ± 0.004 | 1.007 ± 0.004 | 1.08 ± 0.01 |
| 16:0 | 0.952 ± 0.008 | 0.969 ± 0.004 | 24.78 ± 0.03 |
| 16:1 | 0.966 ± 0.002 | 0.996 ± 0.002 | 1.51 ± 0.02 |
| 18:0 | 0.988 ± 0.011 | 1.028 ± 0.007 | 11.36 ± 0.02 |
| 18:1 | 0.929 ± 0.008 | 0.960 ± 0.006 | 23.02 ± 0.03 |
| 18:2 | 0.919 ± 0.007 | 0.949 ± 0.002 | 1.70 ± 0.04 |
| 18:3 | 0.942 ± 0.015 | 0.970 ± 0.010 | 0.83 ± 0.01 |
| 20:0 | 1.015 ± 0.014 | 1.050 ± 0.012 | 0.14 ± 0.01 |

[a] The values are means ± standard errors of the means (S.E.M.) of six GC analyses.

demonstrated the absence of losses of short-chain compounds during the preparation of methyl esters. The correction factors for short-chain fatty acid butyl esters determined from the known mixture of TGs were lower than those of the corresponding methyl esters (Table I) because of the large differences in molecular weights. When methyl and butyl esters were prepared from the same sample of butterfat TGs and analysed by GC, and when the appropriate response factors were applied, the same quantitative results were obtained. However, butyl esters were preferred to methyl esters for the following reasons. A loss of short-chain methyl esters was still possible for the smallest butterfat RPLC fractions. Moreover, the determination of short-chain methyl esters was less accurate than that of the corresponding butyl esters because of the higher response factors and the varying amounts of an impurity with a retention time very close to that of butyric acid methyl ester (shouldering). This impurity could not be entirely removed by distillation of methanol and the other solvents.

The composition of fatty acid butyl esters of each RPLC fraction was determined more accurately by subtracting a blank obtained under the same experimental conditions.

*Gas chromatography of triacylglycerols*

TGs of the butterfat RPLC fractions and standard mixtures (Nu-Chek-Prep) were analysed by GC using a Packard Model 419 chromatograph equipped with a Ros injector maintained at 330°C, a laboratory-made 10 m × 0.3 mm I.D. glass capillary column coated with SE-30 silicone phase and a flame ionization detector at 330°C. The chromatograph was connected to an ENICA 21 integrator–calculator. The column temperature was programmed from 200 to 300°C at 6°C min$^{-1}$. The carrier gas was hydrogen at a flow-rate of 3 ml min$^{-1}$.

RESULTS AND DISCUSSION

*RPLC resolution of butterfat triacylglycerols*

Among animal fats, bovine butterfat shows one of the broadest TG and fatty acid spectra. Gaining good resolution of a wide range of TG species in an RPLC run under isocratic conditions is a particularly difficult task. One possibility is the use of a temperature programme for the column and Fig. 1 shows a chromatogram obtained when temperature was programmed from 10 to 55°C at 1°C min$^{-1}$. More than 50 peaks were eluted within 70 min but some were only partially separated. Such an RPLC analysis of butterfat TGs using temperature programming was also reported by Weber *et al.* [20] under similar conditions. However, the chromatographic profile obtained in this work does not resemble theirs.

Fig. 2 gives the chromatograms obtained during the two-step RPLC resolution of the same sample of butterfat TGs without temperature programming under slightly different conditions. The first 27 peaks, which were not sufficiently separated in the first step at 40°C (Fig. 2A) were collected together and rerun at 30°C (Fig. 2B). The elution pattern of the 47 peaks is very similar to that obtained by Weber *et al.* [20] with temperature programming and by other workers [14,17,19] at constant temperature. The RPLC profile of butterfat TGs is generally characterized by a series of four-peak groups (quartets), such as the groups 28–31, 32–35, 36–39, 40–43 and 44–47 in Fig. 2A.
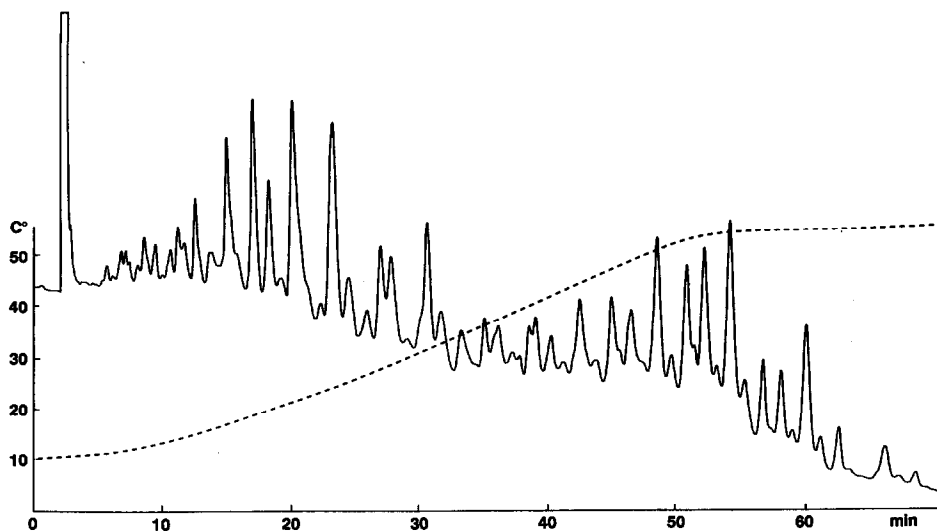
Fig. 1. RPLC profile of total butterfat TGs using temperature programming. RPLC conditions: column, 250 × 4 mm I.D. LiChrospher 100 RP-18 (Merck); eluent, acetone–acetonitrile (50:50, v/v) at 1 ml min$^{-1}$; column temperature, programmed (— — — —) at 1°C min$^{-1}$ from 10 to 55°C; injection, 4 mg of TGs in 40 μl of acetone.

Butterfat TGs have also been eluted with a solvent gradient and then detected by means of either a light-scattering detector [13,15,16], or a UV detector at 220 nm [22], or a flame ionization detector [34]. However, the chromatographic profile characterized by quartets was not always found under such RPLC conditions [13,15,16, 18,22].

Although TGs seemed to be less well resolved at constant temperature (Fig. 2) than using a temperature programme (Fig. 1), the two-step procedure was preferred in this study for the following reasons. The present results can be easily compared with those of other authors [14,17, 19–21] who obtained a similar RPLC profile of butterfat TGs. Further, when a run was started at low temperature (10°C), the risk of making long-chain saturated TGs insoluble could not be entirely ruled out.

A mixture of standard simple TGs made up of saturated or unsaturated even-carbon number fatty acids ($C_{24}$ to $C_{56}$) was injected along with total butterfat TGs and their retention times were determined. As expected, the caption in Fig. 2 indicates that the standard saturated TGs were resolved on the basis of their increasing total carbon number and eluted later than the corresponding unsaturated TGs, according to the general rules of RPLC resolution of TGs [35,36]. Further, TGs were partially resolved on the basis of the chain length of each of the three constituent acyl moieties. Fig. 3 clearly shows that TGs containing one or two butyric acids were eluted later than long-chain TGs. In contrast, the two TGs 12:0, 12:0, 16:0 and 12:0, 14:0, 14:0 having the same carbon number ($CN = 40$) and double bond number ($DB = 0$) were not resolved under our chromatographic conditions.
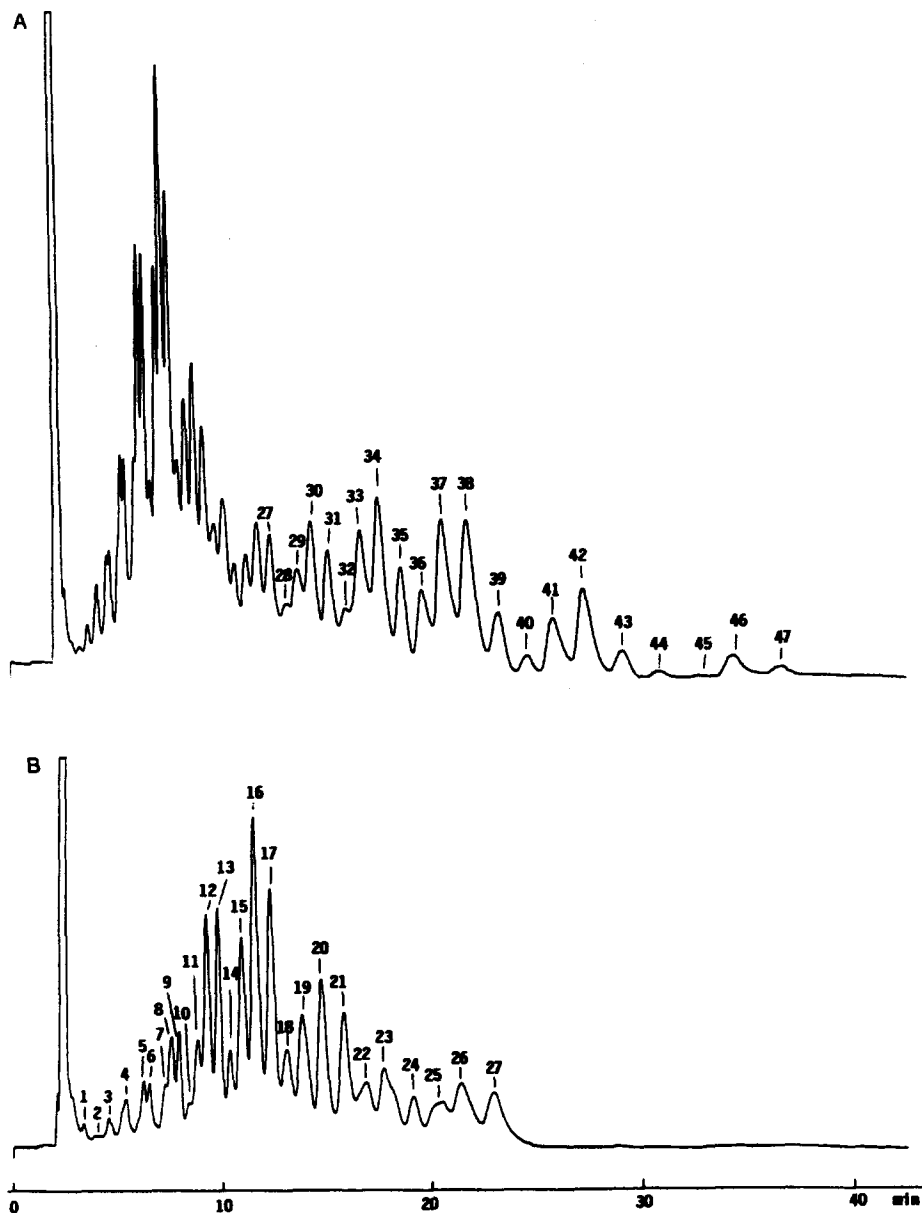
Fig. 2. Two-step RPLC analysis of butterfat TGs: (A) analysis at 40°C using acetone–acetonitrile (55:45, v/v); (B) analysis at 30°C of the first 27 peaks collected in (A) using acetone–acetonitrile (50:50, v/v). Other conditions as in Fig. 1. The retention time of trioctanoylglycerol was identical with that of peak 2. Such an identity of retention times was also observed for the following TGs and RPLC peaks: tridecanoylglycerol (peak 5), trilinolenoylglycerol (peak 12), trilauroylglycerol (peak 15), trilinoleoylglycerol (peak 23), tripalmitoleoylglycerol (peak 24), trimyristoylglycerol (peak 27), trioleoylglycerol (peak 36), tripalmitoyl-glycerol (peak 39).
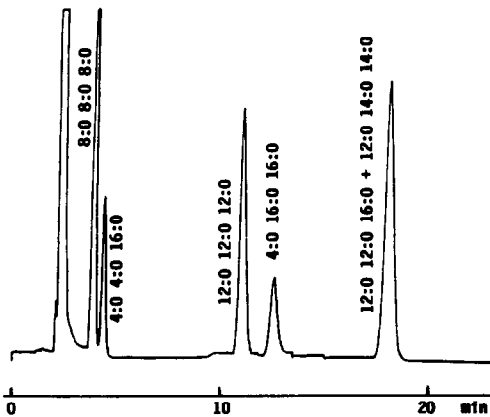
Fig. 3. RPLC analysis of a mixture of standard saturated TGs. RPLC conditions as in Fig. 2B. The retention times of 8:0 8:0 8:0 + 4:0 4:0 16:0, 12:0 12:0 12:0, 4:0 16:0 16:0 and 12:0 12:0 16:0 + 12:0 14:0 14:0 correspond to those of peaks 2, 15, 17 and 23 in Fig. 2B, respectively.

## RPLC determination of butterfat triacylglycerols

The measurement of peak areas and the calculation of the relative proportions of peaks were performed automatically during the two analysis steps. The results given in Table I were established on the basis of peak 27 common to both chromatograms. Because of the complexity of the TG mixture in each RPLC fraction, it was not possible to determine useful response factors using standard simple TGs and assuming that the response factors of the constituent fatty acids are additive, as proposed by Goiffon *et al.* [37]. The molar percentage of a peak was therefore calculated directly from the peak area and the average molecular weight of TGs in the peak, calculated from $MW = 134 + 14CN - 2DB$. The average number of double bonds of TGs was calculated from the fatty acid composition of the RPLC fraction concerned (see Table II). The average carbon number of TGs was calculated not from the fatty acid composition but from the TG composition of the RPLC fraction (see Table III). The CN data in Table III were more accurate than those in Table II because they were calculated from all the TGs, *i.e.*, even- and odd-carbon number TGs, whereas in Table II only the distribution of the listed even-carbon number fatty acids was used for calculation.

As most of the RPLC peaks were not completely separated (no return to the baseline), the reproducibility of peak-area measurements was checked. The relative standard deviation ranged from 1.7 to 13.6% (5.6% on average) when considering the peaks $\geq 1\%$.

Tabel I shows that only two peaks represented slightly more than 5%, namely peaks 16 (6.9%) and 17 (5.6%). The percentages of most of the peaks (34 peaks) ranged from 1.0 to 5.0% and amounted to 80% of the total. Our results are similar to those of Weber *et al.* [20,21], although the bovine butterfat samples studied were different. In the butterfat there is, therefore, no really major TG species that could be made up of three major fatty acids, such as butyric, palmitic and oleic acids. In other words, the numerous short- and long-chain fatty acids in butterfat are largely interesterified.

TABLE II

QUANTITATIVE DISTRIBUTION[a] OF 47 FRACTIONS OF BUTTERFAT TRIACYLGLYCEROLS OBTAINED BY RPLC AND FATTY ACID COMPOSITIONS (mol %) OF THE RPLC FRACTIONS ANALYSED BY GC

| Fatty acids[b] | RPLC fraction No. and mol % | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 0.13 ±0.02 | 2 0.11 ±0.01 | 3 0.46 ±0.03 | 4 1.13 ±0.03 | 5 1.02 ±0.03 | 6 1.05 ±0.03 | 7 0.90 ±0.02 | 8 1.64 ±0.04 | 9 1.71 ±0.04 | 10 0.67 ±0.03 | 11 1.65 ±0.04 | 12 4.15 ±0.10 | 13 3.97 ±0.10 |
| 4:0 | 22.5 | 24.7 | 26.0 | 23.6 | 16.7 | 25.1 | 15.1 | 24.0 | 27.6 | 12.6 | 15.2 | 25.0 | 27.7 |
| 6:0 | 5.9 | 13.4 | 16.5 | 11.3 | 14.5 | 4.4 | 12.9 | 9.5 | 1.5 | 9.6 | 11.7 | 7.5 | 1.0 |
| 8:0 | 2.9 | 7.6 | 9.9 | 12.5 | 7.3 | 4.3 | 7.2 | 3.1 | 0.6 | 5.0 | 4.3 | 1.2 | 0.2 |
| 10:0 | 3.7 | 5.9 | 9.8 | 12.2 | 14.2 | 17.0 | 14.7 | 6.1 | 3.5 | 8.1 | 7.6 | 1.6 | 0.4 |
| 12:0 | 3.3 | 8.6 | 7.9 | 5.5 | 6.1 | 9.6 | 9.3 | 13.0 | 15.8 | 7.7 | 7.8 | 3.6 | 3.1 |
| 14:0 | 16.0 | 10.0 | 9.2 | 10.9 | 9.5 | 10.5 | 8.6 | 9.7 | 21.4 | 17.5 | 11.1 | 19.8 | 28.7 |
| 14:1 | 1.2 | 1.0 | 1.0 | 1.1 | 2.1 | 1.3 | 4.0 | 3.4 | 1.5 | 4.0 | 2.5 | 0.6 | 0.4 |
| 16:0 | 21.3 | 9.5 | 7.6 | 8.6 | 6.3 | 16.8 | 6.2 | 9.5 | 18.9 | 10.6 | 6.4 | 12.9 | 27.3 |
| 16:1 | 2.1 | 1.6 | 0.7 | 0.9 | 1.7 | 0.6 | 2.7 | 1.9 | 1.0 | 3.1 | 4.3 | 3.4 | 1.4 |
| 18:0 | 2.7 | 2.7 | 0.8 | 2.1 | 3.1 | 4.1 | 1.6 | 1.0 | 3.9 | 2.3 | 0.4 | 1.8 | 3.5 |
| 18:1 | 13.5 | 10.9 | 8.6 | 8.8 | 13.0 | 4.3 | 10.2 | 12.6 | 2.9 | 11.4 | 19.0 | 19.2 | 5.7 |
| 18:2 | 3.5 | 1.8 | 1.0 | 1.1 | 2.4 | 0.6 | 3.2 | 2.5 | 0.4 | 3.7 | 7.2 | 3.4 | 0.4 |
| 18:3 | 1.4 | 2.3 | 1.0 | 1.4 | 3.1 | 1.3 | 4.3 | 3.5 | 1.0 | 4.2 | 2.3 | – | 0.2 |
| 20:0 | – | – | – | – | – | 0.1 | – | 0.2 | – | 0.2 | 0.2 | – | – |
| CN[c] | 36.60 | 31.57 | 28.67 | 30.34 | 32.20 | 32.32 | 33.61 | 33.52 | 34.06 | 37.14 | 36.62 | 35.98 | 36.08 |
| DB[d] | 0.84 | 0.72 | 0.46 | 0.52 | 0.83 | 0.34 | 1.09 | 1.00 | 0.28 | 1.16 | 1.41 | 0.90 | 0.27 |
| PN[e] | 34.92 | 30.13 | 27.73 | 29.30 | 30.54 | 31.64 | 31.43 | 31.52 | 33.50 | 34.82 | 33.80 | 34.18 | 35.54 |

| Fatty acids[b] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14 1.54 ±0.02 | 15 4.11 ±0.06 | 16 6.86 ±0.17 | 17 5.61 ±0.16 | 18 2.31 ±0.11 | 19 3.65 ±0.06 | 20 4.05 ±0.04 | 21 3.42 ±0.06 | 22 2.10 ±0.04 | 23 3.01 ±0.03 | 24 1.42 ±0.09 | 25 1.87 ±0.13 | 26 2.71 ±0.14 |
| 4:0 | 17.0 | 15.3 | 21.5 | 30.2 | 8.3 | 3.1 | 9.7 | 20.0 | 2.7 | 0.2 | – | 9.5 | 0.8 |
| 6:0 | 9.0 | 11.9 | 7.9 | 1.9 | 12.6 | 18.6 | 15.8 | 2.2 | 1.9 | 6.0 | 22.8 | 1.2 | 0.3 |
| 8:0 | 4.4 | 3.2 | 0.6 | 0.6 | 6.2 | 5.5 | 1.1 | 2.5 | 9.3 | 8.3 | 2.2 | 1.0 | 2.3 |
| 10:0 | 6.3 | 4.9 | 0.4 | 0.8 | 6.5 | 5.5 | 0.7 | 2.7 | 13.7 | 11.7 | 4.4 | 11.9 | 16.5 |
| 12:0 | 4.6 | 3.8 | 1.0 | 0.6 | 4.1 | 3.9 | 0.5 | 1.6 | 5.2 | 5.7 | 1.8 | 4.3 | 2.8 |
| 14:0 | 11.3 | 12.3 | 7.5 | 8.2 | 7.7 | 9.2 | 5.6 | 1.8 | 12.7 | 15.6 | 3.2 | 7.9 | 13.1 |
| 14:1 | 0.7 | 0.6 | – | 0.4 | 1.4 | 0.2 | 0.2 | 1.1 | 2.3 | – | 1.6 | 3.8 | 3.9 |
| 16:0 | 17.4 | 10.6 | 28.2 | 42.3 | 15.4 | 27.0 | 28.8 | 28.2 | 18.1 | 27.5 | 27.3 | 7.1 | 22.6 |
| 16:1 | 3.3 | 1.7 | 1.0 | 0.4 | 1.7 | 0.9 | 0.2 | 0.9 | 2.1 | – | 0.9 | 2.1 | 2.1 |

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TABLE II (continued)

| Fatty acids[b] | RPLC fraction No. and mol % | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 14<br>1.54<br>±0.02 | 15<br>4.11<br>±0.06 | 16<br>6.86<br>±0.17 | 17<br>5.61<br>±0.16 | 18<br>2.31<br>±0.11 | 19<br>3.65<br>±0.06 | 20<br>4.05<br>±0.04 | 21<br>3.42<br>±0.06 | 22<br>2.10<br>±0.04 | 23<br>3.01<br>±0.03 | 24<br>1.42<br>±0.09 | 25<br>1.87<br>±0.13 | 26<br>2.71<br>±0.14 |
| 18:0 | 1.9 | 1.1 | 2.9 | 8.0 | 4.8 | 4.8 | 20.4 | 27.4 | 6.3 | 14.0 | 27.7 | 18.8 | 6.9 |
| 18:1 | 18.6 | 32.6 | 28.4 | 5.8 | 28.8 | 20.6 | 15.9 | 9.7 | 23.2 | 11.0 | 6.0 | 25.6 | 25.9 |
| 18:2 | 4.0 | 1.5 | 0.6 | 0.6 | 1.4 | 0.7 | 0.5 | 1.1 | 1.4 | – | 1.2 | 4.0 | 1.5 |
| 18:3 | 1.3 | 0.4 | – | 0.2 | 1.1 | – | 0.2 | 0.7 | 0.9 | – | 0.9 | 2.6 | 1.3 |
| 20:0 | 0.2 | 0.1 | – | – | – | – | 0.4 | 0.1 | 0.2 | – | – | 0.2 | – |
| *CN*[c] | 37.19 | 38.13 | 39.02 | 36.56 | 39.70 | 39.53 | 41.24 | 41.03 | 42.17 | 41.91 | 41.48 | 43.70 | 44.88 |
| *DB*[d] | 1.04 | 1.17 | 0.88 | 0.25 | 1.14 | 0.69 | 0.54 | 0.48 | 0.99 | 0.33 | 0.41 | 1.42 | 1.16 |
| *PN*[e] | 35.11 | 35.79 | 37.26 | 36.06 | 37.42 | 38.15 | 40.16 | 40.07 | 40.19 | 41.25 | 40.66 | 40.86 | 42.56 |

| Fatty acids[b] | 27<br>2.59<br>±0.18 | 28<br>0.93<br>±0.07 | 29<br>1.85<br>±0.02 | 30<br>2.92<br>±0.02 | 31<br>2.18<br>±0.03 | 32<br>0.74<br>±0.01 | 33<br>3.41<br>±0.05 | 34<br>4.01<br>±0.06 | 35<br>2.08<br>±0.02 | 36<br>1.79<br>±0.05 | 37<br>3.97<br>±0.03 | 38<br>4.24<br>±0.03 | 39<br>1.51<br>±0.02 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4:0 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 6:0 | – | 3.8 | 1.5 | – | – | – | – | – | – | – | – | – | – |
| 8:0 | 2.3 | 4.3 | – | – | 0.4 | – | – | – | – | – | – | – | – |
| 10:0 | 17.9 | 5.3 | 1.8 | 4.1 | 7.0 | 2.4 | – | – | 1.5 | – | – | – | – |
| 12:0 | 11.0 | 4.8 | 8.7 | 10.9 | 10.8 | 3.5 | – | 3.1 | 5.6 | – | – | – | – |
| 14:0 | 17.6 | 7.6 | 12.8 | 19.0 | 21.2 | 10.7 | 15.9 | 22.3 | 23.0 | 5.4 | 1.6 | 9.4 | 17.3 |
| 14:1 | 0.7 | 3.8 | 5.5 | 3.8 | – | – | – | 1.1 | – | 1.5 | – | – | – |
| 16:0 | 34.8 | 15.8 | 15.8 | 23.1 | 32.7 | 15.4 | 17.5 | 29.6 | 37.9 | 14.2 | 28.0 | 38.1 | 41.4 |
| 16:1 | 0.7 | 3.0 | 3.9 | 2.6 | 0.1 | 6.0 | 4.9 | 2.6 | – | – | 1.6 | 1.3 | – |
| 18:0 | 8.9 | 16.9 | 5.7 | 7.1 | 13.0 | 8.3 | 4.9 | 7.9 | 14.3 | 5.8 | 7.0 | 16.4 | 26.9 |
| 18:1 | 5.4 | 24.0 | 33.0 | 24.9 | 11.5 | 39.5 | 46.9 | 30.3 | 17.7 | 69.8 | 58.4 | 34.8 | 14.4 |
| 18:2 | 0.5 | 7.1 | 7.4 | 3.0 | 3.0 | 11.0 | 6.7 | 3.1 | – | 3.3 | 3.4 | – | – |
| 18:3 | 0.2 | 3.3 | 3.9 | 1.5 | 0.3 | 3.2 | 3.2 | – | – | – | – | – | – |
| 20:0 | – | 0.3 | – | – | – | – | – | – | – | – | – | – | – |
| *CN*[c] | 42.71 | 46.72 | 48.01 | 46.00 | 46.37 | 50.22 | 50.75 | 48.70 | 47.60 | 52.32 | 52.03 | 50.54 | 49.44 |
| *DB*[d] | 0.25 | 1.65 | 2.07 | 1.25 | 0.56 | 2.31 | 2.24 | 1.21 | 0.53 | 2.34 | 2.00 | 1.06 | 0.43 |
| *PN*[e] | 42.21 | 43.42 | 43.87 | 43.50 | 45.25 | 45.60 | 46.27 | 46.28 | 46.54 | 47.64 | 48.03 | 48.42 | 48.58 |

| | 40 0.34 ±0.01 | 41 1.44 ±0.02 | 42 2.29 ±0.08 | 43 0.63 ±0.06 | 44 0.19 ±0.02 | 45 0.14 ±0.02 | 46 0.88 ±0.05 | 47 0.47 ±0.04 | Total TGs[f] |
|---|---|---|---|---|---|---|---|---|---|
| 4:0 | – | – | – | – | – | – | – | – | 10.07 |
| 6:0 | – | – | – | – | – | – | – | – | 4.93 |
| 8:0 | – | – | – | – | – | – | – | – | 2.02 |
| 10:0 | – | – | – | – | – | – | – | – | 4.16 |
| 12:0 | – | – | – | – | – | – | – | – | 3.71 |
| 14:0 | 4.9 | 0.8 | 0.6 | 9.2 | – | 4.7 | – | – | 11.72 |
| 14:1 | – | – | – | – | – | – | – | – | 1.05 |
| 16:0 | 32.7 | 5.4 | 32.0 | 40.4 | 23.6 | 22.2 | 3.1 | 34.3 | 24.05 |
| 16:1 | – | – | 0.6 | – | – | – | – | – | 1.52 |
| 18:0 | 31.6 | 34.5 | 34.6 | 42.9 | 57.8 | 42.3 | 63.6 | 49.7 | 10.66 |
| 18:1 | 30.8 | 57.6 | 32.2 | 7.0 | 18.6 | 24.7 | 32.7 | 12.8 | 23.33 |
| 18:2 | – | 1.7 | – | – | – | – | – | – | 1.93 |
| 18:3 | – | – | – | – | – | – | – | – | 0.77 |
| 20:0 | – | – | – | 0.5 | – | 6.1 | 0.6 | 3.2 | 0.08 |
| $CN$[c] | 51.45 | 53.26 | 51.97 | 50.50 | 52.58 | 82.47 | 53.79 | 52.13 | 43.87 |
| $DB$[d] | 0.92 | 1.83 | 0.98 | 0.21 | 0.56 | 0.74 | 0.98 | 0.38 | 0.96 |
| $PN$[e] | 49.61 | 49.60 | 50.01 | 50.08 | 51.46 | 50.99 | 51.83 | 51.37 | 41.95 |

[a] The values (mol%) are the means ± S.E.M. of five RPLC analyses. Two minor fractions (48, 0.10% and 51, 0.05%) were eluted later than fraction 47, but their acid composition was not determined.

[b] Isomers $n$-7 and $n$-9 were added for 16:1 and 18:1. 18:2 included 18:2 $n$-6 and 18:2 conjugated. 18:3 was the isomer 18:3 $n$-3.

[c] $CN$ = average total acyl carbon number of TGs calculated from the constituent fatty acids.

[d] $DB$ = average total number of double bonds of TGs calculated from the fatty acid compositions.

[e] $PN$ = partition number of TGs ($PN = CN - 2DB$).

[f] The values (mol%) are calculated from the proportions of the RPLC fractions in total butter TGs and the fatty acid compositions of the RPLC fractions.

TABLE III

TG COMPOSITIONS (mol %) OF 46 BUTTERFAT FRACTIONS OBTAINED BY RPLC AND ANALYSED BY CAPILLARY GC

| Carbon number | RPLC fraction No. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| 24 | 62.4 | 1.4 | — | — | — | — | — | — | — | — | — | — | — |
| 26 | 27.9 | 58.7 | 2.1 | — | — | — | — | — | — | — | — | — | — |
| 27 | — | — | 2.1 | — | — | — | — | — | — | — | — | — | — |
| 28 | 6.4 | 32.6 | 54.8 | 3.5 | — | — | — | — | — | — | — | — | — |
| 29 | — | — | 1.6 | 5.3 | — | — | — | — | — | — | — | — | — |
| 30 | 3.3 | 4.0 | 28.5 | 24.6 | 75.2 | 3.7 | — | — | — | — | — | — | — |
| 31 | — | — | — | — | 3.2 | 11.7 | — | — | — | — | — | — | — |
| 32 | — | 3.3 | 7.4 | 43.4 | 16.5 | 11.3 | 23.1 | 75.6 | 10.9 | — | — | — | — |
| 33 | — | — | — | — | 1.4 | 3.2 | — | 1.2 | 32.3 | 12.1 | 1.0 | — | — |
| 34 | — | — | 2.5 | 11.5 | 0.8 | 29.9 | 43.5 | 15.5 | 1.4 | 10.5 | 19.4 | 71.5 | 6.5 |
| 35 | — | — | — | — | — | — | — | 1.0 | 13.3 | 3.4 | — | 2.3 | 31.1 |
| 36 | — | — | 0.6 | 8.4 | 1.5 | 15.1 | 18.0 | 1.9 | 9.4 | 28.2 | 54.5 | 14.1 | 0.8 |
| 37 | — | — | — | — | — | 0.9 | — | 1.3 | 4.9 | 1.6 | — | 6.4 | 23.1 |
| 38 | — | — | 0.2 | 1.8 | 0.8 | 9.1 | 9.4 | 1.8 | 12.4 | 18.5 | 18.9 | 2.7 | 13.2 |
| 39 | — | — | — | — | — | — | — | 0.5 | — | — | — | 2.2 | 2.0 |
| 40 | — | — | 0.2 | 1.5 | 0.5 | 13.4 | 5.7 | 0.2 | 6.4 | 22.7 | 5.9 | 0.3 | 11.4 |
| 42 | — | — | — | — | 0.1 | 1.5 | 0.3 | 1.0 | 8.4 | 2.7 | 0.1 | 0.2 | 11.0 |
| 44 | — | — | — | — | — | 0.2 | — | — | 0.6 | 0.3 | 0.2 | 0.3 | 0.9 |
| *CN*[a] | 25.01 | 26.98 | 29.06 | 32.00 | 30.65 | 34.88 | 34.64 | 32.75 | 35.52 | 36.87 | 36.22 | 34.78 | 37.30 |
| *PN*[b] | 23.57 | 26.06 | 28.02 | 30.34 | 29.97 | 32.70 | 32.64 | 32.19 | 33.20 | 34.07 | 34.42 | 34.24 | 35.22 |

|        | 15    | 16    | 17    | 18    | 19    | 20    | 21    | 22    | 23    | 24    | 25    | 26    | 27    |
|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 35     | 11.6  | 1.1   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| 36     | 11.9  | 22.2  | 71.2  | 3.4   | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| 37     | 3.1   | 0.4   | 3.3   | 29.0  | 8.6   | –     | –     | –     | –     | –     | –     | –     | –     |
| 38     | 30.1  | 62.7  | 11.6  | 0.8   | 24.9  | 52.9  | 67.7  | 2.9   | 2.8   | 0.6   | –     | –     | –     |
| 39     | –     | 1.0   | 6.1   | 8.0   | 0.9   | 1.9   | 6.4   | 11.1  | 54.9  | 78.5  | 28.6  | 1.9   | –     |
| 40     | 40.6  | 10.8  | 0.6   | 25.6  | 57.7  | 39.4  | 6.3   | 1.6   | 0.6   | 4.6   | 14.6  | 2.2   | 1.1   |
| 41     | –     | 1.7   | 0.5   | –     | –     | 4.8   | 3.5   | 1.3   | 38.3  | 1.8   | –     | 2.1   | 81.1  |
| 42     | 2.7   | 0.1   | 4.7   | 31.0  | 7.9   | –     | –     | 60.4  | –     | 10.9  | 4.6   | –     | –     |
| 43     | –     | –     | –     | –     | –     | 1.0   | –     | –     | –     | –     | –     | 75.3  | –     |
| 44     | –     | –     | 1.5   | 2.2   | –     | –     | 10.5  | 18.8  | 1.8   | –     | –     | –     | 10.6  |
| 45     | –     | –     | –     | –     | –     | –     | –     | –     | 1.4   | 0.9   | –     | 14.8  | 4.7   |
| 46     | –     | –     | 0.5   | –     | –     | –     | 4.6   | 3.7   | –     | –     | 40.7  | –     | 0.5   |
| 47     | –     | –     | –     | –     | –     | –     | –     | –     | 0.2   | –     | –     | 3.7   | 2.0   |
| 48     | –     | –     | –     | –     | –     | –     | 1.0   | 0.2   | –     | 1.3   | 7.6   | –     | –     |
| 50     | –     | –     | –     | –     | –     | –     | –     | –     | –     | 0.7   | 3.9   | –     | –     |
| 52     | –     | –     | –     | –     | –     | –     | –     | –     | –     | 0.7   | –     | –     | –     |
| $CN^a$ | 38.30 | 37.80 | 36.81 | 39.61 | 39.39 | 39.00 | 39.53 | 42.04 | 40.86 | 40.79 | 43.72 | 44.26 | 42.46 |
| $PN^b$ | 35.96 | 36.04 | 36.30 | 37.33 | 38.01 | 37.92 | 38.57 | 40.06 | 40.20 | 39.97 | 40.88 | 41.94 | 41.96 |

**TABLE III** (*continued*)

| Carbon number | RPLC fraction No. | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| 42 | 49.2 | 3.6 | – | – | – | – | – | – | – | – | – | – | – |
| 43 | – | 11.1 | 1.4 | 1.7 | – | – | – | – | – | – | – | – | – |
| 44 | 1.8 | – | 1.8 | 65.8 | 16.2 | 0.7 | 0.6 | – | – | – | – | – | – |
| 45 | 15.3 | 4.3 | 0.7 | – | 20.2 | 7.4 | 1.4 | 67.4 | – | – | – | – | – |
| 46 | – | 2.5 | 67.8 | 10.4 | 26.2 | – | 0.6 | – | 2.4 | 4.5 | 1.3 | – | – |
| 47 | 2.3 | – | – | 8.1 | – | 5.9 | 68.5 | 6.9 | 13.3 | – | – | 2.9 | 1.7 |
| 48 | – | 41.4 | 22.9 | 2.3 | – | – | – | 14.4 | 1.0 | 2.2 | 1.0 | 66.4 | 32.5 |
| 49 | 0.5 | – | – | 5.4 | 8.2 | 0.3 | – | – | 23.9 | 2.3 | 79.9 | – | 4.0 |
| 50 | 17.0 | 24.0 | 5.4 | – | – | 51.4 | 24.5 | – | – | – | – | 6.9 | 58.8 |
| 51 | – | – | – | 1.2 | – | – | – | 11.3 | 4.5 | – | – | 16.0 | – |
| 52 | 6.3 | 10.4 | – | – | 13.8 | 28.3 | 4.4 | – | – | 76.4 | 17.5 | 0.6 | 3.0 |
| 53 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 54 | 7.6 | 2.7 | – | 5.1 | 15.4 | 6.0 | – | – | 54.9 | 14.6 | 0.3 | 7.2 | – |
| $CN^a$ | 45.55 | 48.11 | 46.59 | 45.39 | 47.90 | 50.21 | 48.61 | 47.14 | 51.49 | 51.96 | 50.31 | 49.05 | 50.32 |
| $PN^b$ | 42.25 | 43.97 | 44.09 | 44.27 | 43.28 | 45.73 | 46.20 | 46.08 | 46.81 | 47.96 | 48.19 | 48.22 | 48.48 |

| Carbon number | 41 | 42 | 43 | 44 | 45 | 46 | 47 |
|---|---|---|---|---|---|---|---|
| 48 | 1.2 | – | – | – | – | – | – |
| 49 | 7.5 | – | 8.4 | – | – | – | – |
| 50 | – | 1.0 | 76.7 | 2.5 | 11.2 | 0.6 | – |
| 51 | 2.3 | 3.3 | – | 41.3 | 72.8 | – | – |
| 52 | 2.6 | 91.9 | 12.8 | 4.2 | – | 1.7 | 95.8 |
| 53 | 2.3 | – | – | 49.9 | 7.9 | 5.1 | – |
| 54 | 84.1 | 3.8 | 2.1 | 2.1 | 8.1 | 92.6 | 4.2 |
| $CN^a$ | 53.41 | 52.02 | 50.26 | 52.08 | 51.29 | 53.89 | 52.08 |
| $PN^b$ | 49.75 | 50.06 | 49.84 | 50.96 | 49.81 | 51.93 | 51.32 |

[a] $CN$ = average total acyl carbon number of TGs calculated from the constituent TGs.

[b] $PN$ = Partition number of TGs calculated from $CN$ (data in this table) and from $DB$ determined from the constituent fatty acids (Table II).

*Fatty acid compositions of RPLC fractions*

Table I shows that butterfat contained a high proportion of short- and long-chain fatty acids, mainly butyric (10%), myristic (12%), palmitic (24%), stearic (10%) and oleic (23%) acids, in agreement with the data generally published. In addition to the even-carbon number saturated and unsaturated long-chain fatty acids and the saturated short-chain fatty acids listed in Table I, minor amounts of odd-carbon number saturated and monounsaturated acids and branched-chain acids were also seen but not reported here. In the whole butterfat TGs, odd-carbon fatty acids totalled 4.5%, and even-carbon number branched acids and monounsaturated medium acyl chains reached 0.5%. These percentages are not different from those reported elsewhere [1].

Butterfat TGs were fractionated by RPLC, and TGs of each RPLC fraction were purified by RPLC and analysed as butyl esters. Table II gives the fatty acid compositions determined for TGs of 47 RPLC fractions. As could be expected, the short-chain fatty acids were mainly found in the RPLC fractions eluted first (Fig. 2B), for example 4:0 in fractions 1–25 and 6:0 and 8:0 in fractions 1–28. However, saturated and unsaturated long-chain fatty acids, especially palmitic and oleic acids, were present in all the fractions. The fatty acid complexity of almost all the RPLC fractions confirms the large interesterification of fatty acids in butterfat.

Table II also shows that *CN* and *PN* roughly increased in order of increasing retention times. When the values for *CN, DB* and *PN* are examined within a four-peak group, such as the quartet 32–35 or 36–39, it can be seen that *PN* was fairly constant as a result of a simultaneous decrease in *CN* and *DB* in the four peaks of the quartet in order of increasing retention times. *CN* increased by 2 units on average from a quartet (*e.g.*, *CN* = 46.17 in group 32–35) to the following one (*CN* = 48.17 in group 36–39). TGs in the last peak of a quartet (peaks 27, 31, 35, 39, 43 and 47 in Fig. 2B) were mainly made up of saturated fatty acids. Indeed, the retention times of the standard saturated simple TGs in Fig. 2 correspond to these peaks (trimyristoylglycerol to peak 27 and tripalmitoylglycerol to peak 39). All these observations agree with the general rules of the resolution of TGs by RPLC according to their degree of unsaturation and chain length [35,36]. In the first two minor RPLC fractions 1 and 2, the values of *CN* and *PN* seem too high, and the fatty acid compositions are probably wrong, as a result of an insufficient subtraction of long-chain compounds via the blank. In fact, on the basis of the RPLC resolution of long-chain TGs, a theoretical *PN* can be assigned to TGs of the RPLC fractions showing a retention time identical with that of at least one known standard TG. Thus, the retention time of peak 2 corresponds to that of trioctanoyl-glycerol (Figs. 2 and 3), *i.e.*, *PN* = 24, whereas the experimental value was 31.6. In the same way, the theoretical *PN* of TGs in RPLC fractions 5, 15, 24, 27, 36, 39 and 51 are 30, 36, 42, 42, 48, 48 and 54, respectively. The experimental values (Table II) are generally close to the theoretical values (the difference is 1.1% on average), except in RPLC fractions 24 and 25 where the experimental *PN* values (40.7 and 40.9, respectively) are lower than the theoretical *PN* (42). In this instance, the TG species 6:0 16:0 18:0 and 4:0 18:0 18:0 (theoretical *PN* = 40) were probably eluted not in fraction 23 as expected but later in fractions 24 and 25, respectively, because of the presence of a short-chain fatty acid (6:0 and 4:0) in the molecule, as demonstrated for 4:0 in Fig. 3.

To check the accuracy of the relative proportions and fatty acid compositions of the 47 RPLC fractions, these results were used to calculate a fatty acid composition of

the total butterfat TGs. Table II shows that the calculated values are in close correlàtion with the experimental values (Table I) for all the fatty acids except for 20:0 because the minor RPLC fractions eluted later than fraction 47 were not studied.

*GC resolution of triacyglycerols*

Total butterfat TGs and aliquots of each RPLC-purified fraction were analysed directly by GC. Fig. 4 illustrates the GC profile of total butterfat TGs, which were essentially resolved on the basis of carbon number. Carbon numbers were identified by addition of the standard TGs already used for RPLC (see Figs. 2 and 3). Between the major even-carbon number peaks are seen minor peaks, which are due to odd-carbon number TGs arising from the substitution of one of the three-component even-carbon acyl moieties by an odd-carbon fatty acid, mainly 15:0 or 17:0. Identification of TGs in each RPLC fraction according to carbon number was realized by comparison of their relative retention times with those of the total butterfat TGs. Although GC analyses were performed using a relatively short capillary column (10 m) and a non-polar stationary phase (SE-30), the peaks did not appear homogeneous and parent TGs were partially resolved into several peaks within each carbon number, on the basis of the carbon and double bond numbers of each of the three acyl moieties.
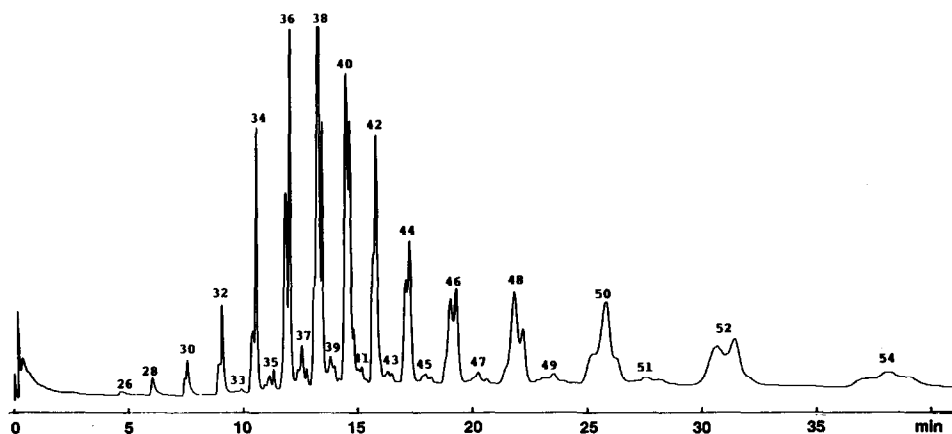


Fig. 4. Capillary GC profile of total butterfat TGs. Peaks are identified by their carbon number (*CN*). GC conditions: instrument, Packard Model 419; column, 10 m × 0.3 mm I.D. coated with SE-30; carrier gas, hydrogen at 3 ml min$^{-1}$; oven temperature, programmed from 200 to 300°C at 6°C min$^{-1}$.

Fig. 5 shows the segregation of some standard TGs under the same chromatographic conditions. It is seen clearly that butyrates (4 *X X*) were eluted later than the longer chain-length species of TGs within the range $C_{24}$–$C_{36}$. Such chain-length separations beyond the carbon number have also been observed by Myher *et al.* [1] on a polar capillary column. They demonstrated that within a given carbon number butyrates were preceded by caproates (6 *X X*), caprylates (8 *X X*) and mixtures of TGs containing exclusively longer chain-length fatty acids. They also found that for TG species within the same total carbon number, the shifts in equivalent carbon number decreased as the minimum chain length increased. As a result, the saturated TG species
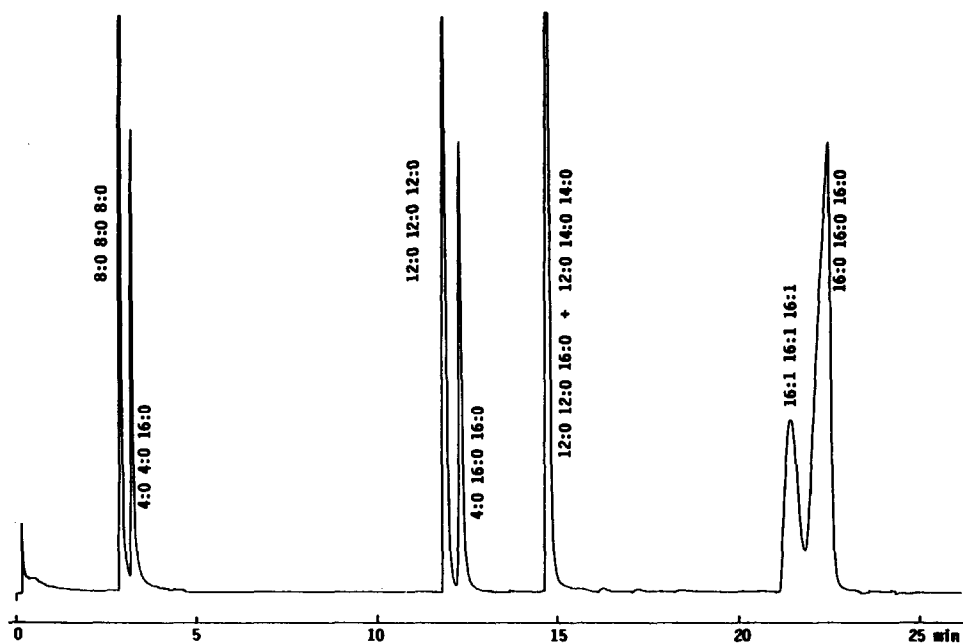
Fig. 5. Capillary GC profile of a mixture of standard TGs. GC conditions as in Fig. 4.

containing capric and longer fatty acids within a carbon number (*e.g.*, 10 16 18, 12 14 18, 12 16 16 and 14 14 16 in $C_{44}$) were not resolved, although Myher *et al.* used a 25-m capillary column. The non-resolution of 12 12 16 and 12 14 14 within carbon number $C_{40}$, as shown in Fig. 5, is in agreement with their findings. Thus, in the present study the resolution factors observed in GC for the saturated TGs within a carbon number are similar to those found in RPLC (see Fig. 3).

A partial resolution of TGs on the basis of double bound number was also obtained as tripalmitoleoylglycerol was separated from tripalmitoylglycerol (Fig. 5). However, unsaturated TGs were eluted ahead on our apolar capillary column, whereas saturated TGs preceded unsaturated TGs when using a polar capillary column [1]. It is possible that the actual segregations in our GC profiles of TGs were more complex and other factors, such as the presence of small amounts of acetates (2 *X X*) and *trans* and branched isomers or the positional placement of the fatty acids on the glycerol skeleton, could intervene, as described in detail by Myher *et al.* [1]. Under our experimental conditions, the GC resolution of TGs within a carbon number was generally too poor to be useful for identification and determination of the parent species. Further, fractionation of butterfat TGs by RPLC yielded a complete separation of butyrates, caproates and longer chain length TGs within the same carbon number (see above). As a result, GC was no longer useful for separation of these three groups of parent TG species.

*Triacylglycerol compositions of RPLC fractions*

TG compositons of each RPLC fraction and total butterfat according to the total *CN* distribution are given in Tables III and IV, respectively. Molar percentages

TABLE IV

TG COMPOSITION OF TOTAL BUTTERFAT TGs ANALYSED BY CAPILLARY GC

| Carbon number | Experimental[a] | Calculated[b] | Carbon number | Experimental[a] | Calculated[b] |
|---|---|---|---|---|---|
| 24 | 0.03 ± 0.004 | 0.08 | 40 | 10.80 ± 0.21 | 11.46 |
| 26 | 0.17 ± 0.04 | 0.33 | 41 | 1.06 ± 0.02 | 0.93 |
| 27 | 0.03 ± 0.01 | 0.02 | 42 | 6.39 ± 0.11 | 6.85 |
| 28 | 0.70 ± 0.04 | 0.81 | 43 | 1.10 ± 0.02 | 0.57 |
| 29 | 0.03 ± 0.01 | 0.07 | 44 | 5.31 ± 0.07 | 4.96 |
| 30 | 1.07 ± 0.03 | 1.42 | 45 | 1.19 ± 0.02 | 0.85 |
| 31 | 0.03 ± 0.01 | 0.14 | 46 | 5.44 ± 0.06 | 5.21 |
| 32 | 2.17 ± 0.06 | 2.57 | 47 | 1.62 ± 0.06 | 1.19 |
| 33 | 0.37 ± 0.02 | 0.52 | 48 | 6.21 ± 0.02 | 5.73 |
| 34 | 4.91 ± 0.11 | 5.34 | 49 | 2.15 ± 0.12 | 1.33 |
| 35 | 1.09 ± 0.05 | 1.29 | 50 | 7.80 ± 0.08 | 7.73 |
| 36 | 9.98 ± 0.02 | 10.04 | 51 | 2.08 ± 0.14 | 1.08 |
| 37 | 1.90 ± 0.02 | 2.03 | 52 | 7.69 ± 0.15 | 8.01 |
| 38 | 12.61 ± 0.26 | 13.45 | 53 | 0.74 ± 0.19 | 0.20 |
| 39 | 1.67 ± 0.02 | 1.38 | 54 | 3.66 ± 0.21 | 4.41 |

[a] Means (mol%) ± S.E.M. from five GC analyses.
[b] Calculated from the relative proportions (Table II) and TG compositions (Table III) of the 46 RPLC fractions of butterfat.

were calculated from chromatographic peak areas without applying any correction factors according to chain length or/and degree of unsaturation. Some correction factors were determined from standard TGs in the $C_{24}$–$C_{54}$ range (results not shown), but remained low enough to be included in the range of measurement errors. Blanks were not subtracted as for fatty acid compositions because blanks performed and analysed under the same GC conditions contained no traces of TGs in the range $C_{24}$–$C_{54}$. No RPLC fraction appeared to be made up of only one TG species as several GC peaks were found for each fraction, even if 31 fractions exhibited one major even-carbon number peak (50–96%). TGs with the same CN were found to be present in a higher number of RPLC fractions (e.g., TG $C_{40}$ in the 23 RPLC fractions 4–26) than would have been otherwise expected. This results from extensive interesterification of the short- and longer-chain fatty acids in butterfat.

The odd-carbon number peaks were in low proportions, except in some minor RPLC fractions (fractions 10, 14, 32, 40 and 45) where their proportions exceeded those of the even-carbon number peaks. The odd-carbon number TGs accounted for 15.1% of the butterfat TGs when determined from the profile of the total butterfat (Fig. 4 and Table IV) or 11.6% when calculated from the distribution of the odd-carbon number peaks in each RPLC fraction (Table III) and from the proportions of the RPLC fractions in the total butterfat (Table II). They would make up 13.5% if this percentage was calculated from the proportion of the odd-carbon number fatty acids in the total fatty acids (ca. 4.5%), admitting that the odd-carbon number TGs occur with a stoichiometry of only one odd-carbon number fatty acid per TG molecule [1].

As expected, the mean CN values calculated from the data given in Table III are

consistent with those calculated from the fatty acid compositions and shown in Table II. However, differences exceeding one unit were observed for eighteen RPLC fractions. The fact that the odd-carbon number fatty acids were not taken into account in Table II could account for the differences, especially in some minor RPLC fractions (fractions 10, 32, 40 and 45) rich in odd-carbon number fatty acids. However, no explanation could be given when differences reached major (>3%) RPLC fractions (fractions 13, 16, 20 and 21) where the odd-carbon number TGs were in low proportions. The same differences were observed for the *PN* because in Table III *PN* values were calculated using the *DB* values given in Table I. When the TG composition of the total butterfat on the basis of *CN* was calculated from the TG compositions of the RPLC fractions (Table III) and the proportions of each RPLC fraction in the butterfat (Table II), the results were in good agreement with the experimental data (Table IV). These TG compositions are close to that determined by Amer *et al.* [10] under similar conditions.

*Identification and elution order of triacylglycerol species in the RPLC fractions*

The identity of a molecular species of TG is defined here by that of its component fatty acids, but the positioning of fatty acids is not determined. The fourteen different fatty acids listed in Table I could theoretically yield 560 different TG species in total ($PN = 12$–$60$) and 541 species in the studied RPLC fractions 1–47 ($PN = 22$–$52$). The identities of molecular species of TG present in an RPLC fraction often could not be assigned only by the examination of the chemical compositions of the fraction, as given in Tables II and III, because of their evident complexity. An RPLC fraction can gather together up to 26 different TG species distributed among nine classes (fraction 5). The identities of TG species, therefore, were also based on the general rules of elution order in RPLC, consistent with chromatographic data, such as relative retention times of standard TGs, and comparison with results obtained by other workers for mixtures of natural or synthetic TGs. Table V gives an example of the determination of the identities of TG species. There is not much point in calculating the equivalent carbon numbers (*ECN*) for each molecular species of TG, based on the retention times of the homologous series of standard simple TGs, because they would not be of any help in the determination of identities in this work, in contrast to the case with less complex fats [27].

First, we observed that TGs containing caprylic or longer chain saturated and monounsaturated fatty acids were resolved within a *PN* into four RPLC peaks corresponding to TG classes 111, 011, 001 and 000 in order of increasing retention times, as generally described [38]. Within a class, no apparent resolution of parent TGs was observed on the basis of the chain length of acyl moieties in the range $C_8$–$C_{20}$, even if a certain segregation within an RPLC peak was probable. Indeed, Nurmela and Satama [34] using two LiChrospher 100 CH-18 (5 $\mu$m) columns connected in series and a non-linear acetonitrile–acetone gradient elution programme showed that 10 10 10 and 8 8 14, and also 10 10 14 and 8 8 18, could be partially resolved with longer retention times for caprylates. When TGs contained a caproic or butyric residue in combination with two longer chain fatty acids, the retention time was lengthened with a time equivalent to one or two more RPLC peaks as follows: $X$:1 $X$:1 $X$:1, $X$ $X$:1 $X$:1, $X$ $X$ $X$:1 $+$ 6 $X$:1 $X$:1, $X$ $X$ $X$ $+$ 6 $X$ $X$:1 $+$ 4 $X$:1 $X$:1, $X$:1 $X$:1 $X$:1 ($PN + 2$) $+$ 6 $X$ $X$ $+$ 4 $X$ $X$:1, $X$ $X$:1 $X$:1 ($PN + 2$) $+$ 4 $X$ $X$. This sequence was complete only in

TABLE V

DISTRIBUTION OF MOLECULAR SPECIES OF TRIACYLGLYCEROLS IN RPLC FRACTION 12[a]

| $CN$[b] | Class[c] | $PN$[d] | Molecular species[e] | Content (mol%) | |
|---|---|---|---|---|---|
| | | | | In the RPLC fraction | In the total butterfat TGs |
| 34 | 000 | 34 | 6 12 16 | 11 | 0.456 |
| | | | 6 14 14 | 4 | 0.166 |
| | | | 6 10 18 | 4 | 0.166 |
| 36 | 001 | 34 | 4 14 18:1 | 43 | 1.783 |
| | | | 4 16 16:1 | 10 | 0.415 |
| | | | 4 18 14:1 | 2 | 0.083 |
| 38 | 002 | 34 | 4 16 18:2 | 10 | 0.415 |
| 42 | 111 | 36 | 14:1 14:1 14:1 | 0.1 | 0.004 |
| 44 | 013 | 36 | 8 18:1 18:3 + 10 16:1 18:3 | | |
| | | | + 12 14:1 18:3 | 0.2 | 0.008 |

[a] 4.15% in total butterfat TGs (see Table II).
[b] $CN$ = carbon number of the TG species in a TG class.
[c] Each digit represents the number of double bonds ($DB$) of each of the three acyl chains.
[d] $PN$ = partition number ($PN = CN - 2DB$) of the TG species in a TG class.
[e] The position of the three acyl chains on the glycerol skeleton is not determined.

the series of RPLC fractions 12–17 where $PN = 36$. The same sequence, except for some butyrates, was found by Weber et al. [20,21], who analysed butterfat TGs on a Shandon ODS Hypersil (5 $\mu$m) column (250 × 4 mm I.D.). In contrast to our findings, they assigned the TG 4 12 14 to RPLC fraction 5, which preceded fraction 6 containing 4 10 16 as expected, although both TGs have same carbon number. In the same way, they found an earlier elution time for 4 16 14:1 than for 4 12 18:1, and for 4 14 14 than for 4 12 16 + 4 10 18. Baron et al. [22] reported that not only some butyrates (4 $X$ $X$) were separated according to the chain length of the other two acyl moieties but TGs $X$ $X$ $X$ and $X$ $X$ $X$:1 with $X$ in the range $C_8$–$C_{18}$ were also resolved. For example, 18 14 8 + 12 16 12 + 14 12 14, 16 8 16 and 18 12 10 belonged to three different RPLC peaks in increasing elution order. In their work [22], ascribing the molecular species of TGs to a given RPLC fraction did not seem to follow clear elution rules. According to our results obtained with standard mixed TGs (Fig. 3) it is hardly likely that such complete separations can occur under the RPLC conditions used by other workers [20,21,22], even if the RPLC columns used appeared to give slightly better separations.

The order of resolution of the TGs containing linoleic acid was consistent with that determined by Semporé and Bézard [27], who analysed peanut oil TGs using the same RPLC column. Their chromatogram indicated that 16 18:1 18:1, 16 18 18:2 and 16 16 18:1 were separated and eluted in order of increasing retention time, but 16 18 18:2 and 16 16 18:1 were only partially separated and eluted clearly later than 16 18:1 18:1. In this work, TGs of classes 001 and 002 within a $PN$ were found in the same RPLC fraction, probably because class 002 was always present in a much smaller proportion than class 001 as a result of the relative proportions of 18:1 (22.2%) and

18:2 (2.1%) in butterfat. TG classes within a *PN* were separated as follows: 222 + 122 + 112 + 111, 022 + 012 + 011, 002 + 001 and 000 in increasing elution order. This elution order was still apparently valid when within such a series of TGs one of the three saturated acyl residues was a short-chain fatty acid. In contrast, Weber *et al.* [20,21] found that 16:0 16:0 18:2 was associated with 14:0 18:1 18:1 in an RPLC fraction and that both these TGs were retained for a longer time than 16:0 18:1 18:2 but a shorter time than 14:0 16:0 18:1. According to Barron *et al.* [22], long-chain TGs within a *PN* were eluted in increasing order as follows: 012, 011, 002 and 001.

Concerning the TG species containing linolenic acid, the elution order was more difficult to establish because of the very small proportions of most of these TG species and a lack of comparative chromatographic data from other workers. TGs containing odd-carbon number fatty acids were not studied in this work.

*Determination of triacylglycerol species in butterfat*

The proportions of the TG species in each RPLC fraction were obtained as follows. The proportions of fatty acids and TGs in an RPLC fraction (Tables II and III) were first recalculated to eliminate the residual contaminations from the preceding and succeeding major fractions when these contaminations were obvious and easy to remove. For instance, GC peak $C_{38}$ (2.9%) in fraction 22 (see Table III) probably resulted from tailing of fraction 21 because there was no known TG $C_{38}$ in fraction 22 (see Table III) and the proportion of TGs $C_{38}$ in fraction 21 was high (67.7%). Further, the fatty acid compositions of both fractions were consistent with this contamination because a small amount of butyric acid (2.7%) was detected in fraction 22, which was expected to contain no butyrate, whereas GC peak $C_{38}$ in fraction 21 was identified as being made up of 4 16 18.

The proportions of the TG species in an RPLC fraction were then calculated as described by Bézard *et al.* [23]. The proportions had to be consistent first with those of the GC peaks of TGs (Table III), which were more accurate than the corresponding fatty acid composition because of even-carbon number fatty acids provided by odd-carbon number TGs, especially when these TGs were present in high proportions in RPLC fractions. As the odd-carbon number TG species were not identified in RPLC fractions even-carbon number fatty acids supplied in this way could not be subtracted. Further, subtracting a blank from each fatty acid distribution introduced some errors, especially for minor unsaturated fatty acids.

In a second step, the distribution of the parent TG species in an RPLC fraction was such that the fatty acid composition recalculated from the TGs species distribution was as close to the experimental fatty acid composition as possible. Sometimes, the capillary GC resolution of the TGs of an RPLC fraction clearly yielded more than one peak within a *CN*. The chromatographic data (not given in Table III) were then used for subtraction of contaminations or the determination of different TG classes within a *CN*. The total proportion of all the TG species within a *CN* in an RPLC fraction was sometimes lower than the experimental percentage of the corresponding GC peak of TGs (Table III). The difference resulted from the subtraction of contaminations from another RPLC fraction or an unidentified TG, as explained for fraction 12 (see Table V). In the GC peak $C_{38}$ (18.9%) of fraction 12, 4 16 18:2 could not exceed 10% because linoleic acid accounted for 3.4% in this fraction. The remaining 9% could be made up of a contamination from fraction 11 (TGs with $CN = 38$) and/or an unknown TG. In

the same way, no TG $C_{40}$ was given in Table V although a GC peak of corresponding TGs (5.9%) was detected experimentally (Table III) because no known molecular species of TG $C_{40}$ was identified in fraction 12.

Calculation of the proportions of TG species in an RPLC fraction was relatively easy in the fractions where the number of species did not exceed 5 (*e.g.*, fractions 16, 20, 31, 32 and 37–47) and remained smaller than the number of available data (fatty acid and TG percentages). However, many RPLC fractions contained a large number of TG species, and the proportions of all the species could not then be determined accurately, especially when a class made up of several (from two to six) parent TGs was in a low proportion, as is the case for fraction 12 (Table V). As a result, 181 minor TG species were identified but not individually quantified, and they accounted for only 4.1%. In contrast, 223 individual TG species distributed among 45 RPLC fractions were recognized, and accounted for a large proportion (79.3%) in the butterfat sample studied. The detailed results will be published elsewhere [39].

In conclusion, the methodology choosen in this work, namely fractionation by RPLC and GC analysis of fatty acids and TGs in each RPLC fraction, appears to provide sufficient data to determine a large number of TG species in a complex fat.

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