Evaluation of Sequential Methods for the Determination of Butterfat Fatty Acid Composition with Emphasis on *trans*-18:1 Acids. Application to the Study of Seasonal Variations in French Butters

Robert L. Wolff[a,*], Corinne C. Bayard[a], and Robert J. Fabien[b]

[a]ISTAB, Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Talence, France, and [b]Maxwell Chemicals, Botany, Australia

ABSTRACT: The successive steps of an integrated analytical procedure aimed at the accurate determination of butterfat fatty acid composition, including *trans*-18:1 acid content and profile, have been carefully checked. This sequential procedure includes: dispersion of a portion of butter in hexane/isopropanol (2:1, vol/vol) with anhydrous Na_2SO_4, filtration of aliquots of the suspension through a microfiltration unit, subsequent preparation of fatty acid isopropyl esters (FAIPE) with H_2SO_4 as a catalyst, and analysis of total FAIPE by capillary gas–liquid chromatography (GLC). Isolation of *trans*-18:1 isomers was by silver-ion thin-layer chromatography (Ag–TLC), followed by extraction from the gel of combined saturated and *trans*-monoenoic acids with a biphasic solvent system. Analysis of these fractions by GLC allows the accurate quantitation of *trans*-18:1 acids with saturated acids (14:0, 16:0, and 18:0) as internal standards. A partial insight in the distribution of *trans*-18:1 isomers can be obtained by GLC on a CP Sil 88 capillary column (Chrompack, Middelburg, The Netherlands). All steps of the procedure are quite reproducible, part of the coefficients of variation (generally less than 3%, mainly limited to butyric and stearic acids) being associated with GLC analysis (injection, integration of peaks) and, to a lesser extent, to FAIPE preparation. FAIPE appear to be of greater practical interest than any other fatty acid esters, including fatty acid methyl esters (FAME), for the quantitation of short-chain fatty acids, because peak area percentages, calculated by the integrator coupled to the flame-ionization detector, are almost equal (theoretically and experimentally) to fatty acid weight percentages and do not require correction factors. With this set of procedures, we have followed in detail the seasonal variations of fatty acids in butterfat, with sixty commercial samples of French butter collected at five different periods of the year. Important variations occur around mid-April, when cows are shifted from forage and concentrates during winters spent in their stalls to fresh grass in pastures. At this period, there is a decrease of 4:0–14:0 acids and of 16:0 (–2 and –6%, respectively), while 18:0 and *cis*- plus *trans*-18:1 acids rise suddenly (2 and 5%, respectively). These modifications then progressively disappear until late fall or early winter. Other variations are of minor quantitative importance. Although influenced by the season, the content of 18:2n-6 acid lies in the narrow range of 1.2–1.5%. *Trans*-18:1 acids, quantitated by GLC after Ag–TLC fractionation, are at their highest level in May–June (4.3% of total fatty acids), and at their lowest level between January and the end of March (2.4%), with a mean annual value of 3.3%. The proportion of vaccenic (*trans*-11 18:1) acid, relative to total *trans*-18:1 isomers, is higher in spring than in winter, with intermediate decreasing values in summer and fall, which supports the hypothesis that the level of this isomer is linked to the feed of the cattle, and probably to the amount of grass in the feed.

JAOCS 72, 1471–1483 (1995).

KEY WORDS: Butterfat, capillary column, fat extraction, fatty acid composition, fatty acid isopropyl esters, fatty acid methyl esters, flame-ionization detector, gas–liquid chromatography, response factor, seasonal variations, silver-ion thin-layer chromatography, *trans*-octadecenoic acids, vaccenic acid.

Ruminant fats, mainly cow milk fat, may represent an important dietary source of energy for humans. In France, they account for 40–45 g/person/day (1), which is almost half the total daily per capita intake of fats and oils. Consequently, it is useful to perfect our knowledge of the fatty acid composition of cow milk fat and its possible variations. Innumerable studies have been devoted to these subjects, and it is generally recognized that the proportions of individual fatty acids may vary largely, partly because their accumulation in milk fat triglycerides is under the control of several physiological and dietary parameters (2). Superimposed to potential variations of natural origin are nonnegligible and erratic variations that are frequently linked to uncontrolled analytical procedures, and it is seldom an easy matter to evaluate which part is attributable to each kind of variation. For example, depending on the authors, the content of butyric acid in cow milk fat would vary between 0.8–6.8% (3–5).

In a previous study (6), we started to reinvestigate the seasonal variations of the fatty acid composition of milk fat, with particular attention to its *trans*-18:1 acid content and profile, by analysis of commercial butter samples. However, this

*To whom correspondence should be addressed at ISTAB, Laboratoire de Lipochimie Alimentaire, Université Bordeaux 1, Allée des Facultés, 33405, Talence Cedex, France.

Copyright © 1995 by AOCS Press

study was limited to the analysis of two sets of butters collected in spring and fall (totalling 24 samples). Seasonal variations of the fatty acid composition of milk fat have been recognized for a long time, and several attempts were made to follow the fatty acid composition of milk fat throughout the year (5,7–12). Unfortunately, and because the experiments were frequently limited to one herd or two, results were sometimes erratic, and conflicting conclusions were reached. To complete our observations on the seasonal variations of fatty acids in butterfat, we have analyzed 36 supplementary samples of butter purchased in three other periods of the year (mid-summer, early and late winter).

It was also important to distinguish between true seasonal variations and any possible interfering variations linked to analytical procedures. The main original feature of our procedures is the sequential use of isopropanol for both fat extraction (6,13,14) and the subsequent fatty acid ester preparation (6,15), in lieu of methanol. Although the use of fatty acid methyl esters (FAME) has been thoroughly evaluated (16), a few authors prefer fatty acid butyl esters (17,18). Except in our laboratory, fatty acid isopropyl esters (FAIPE) have not been employed to any extent by lipid analysts. As will be described later, FAIPE are interesting for the quantitation of short-chain fatty acids. We therefore undertook a systematic study of the influence of each step of the procedure on the final results. In the present report, we demonstrate that the methods we have designed and employed to study butterfat fatty acids are sufficiently accurate and precise to allow detection of some seasonal variations.

Because we worked with a fairly large number of butter samples from each period of the year, individual variations among animals become negligible, and all variations linked to the breed, stage of lactation, age, and calving period are minimized. Peculiarities in regional animal husbandry cannot be eliminated. To complete this study, we also examined the *trans*-18:1 acid content of butterfat throughout the year by the same analytical methodology [isolation by silver-ion thin-layer chromatography (Ag–TLC)] as used in our previous works (1,6). The use of a highly efficient capillary column, coated with a cyanopropyl polysiloxane stationary phase (1,6), for gas–liquid chromatography (GLC) of FAIPE isolated by Ag–TLC also allowed a partial insight into the distribution of individual *trans*-18:1 isomers, which shows that their profile also depends on the season.

EXPERIMENTAL PROCEDURES

Samples and standards. Thirty-six samples of fresh butter were purchased in local supermarkets (Bordeaux, France) during three periods: July–August 1994, the first week of January 1995, and the last week of March 1995. Most of the samples were major national brands, and most of these brands were the same as those purchased in October–November 1992 and May–June 1993 for a previous study (6). In the present study, we combine data previously published for spring and fall butters (6) and the original data for summer and winter butters, which total sixty samples. Individual, even-numbered saturated free fatty acids from 4:0 to 18:0 (approximately 99% pure) were obtained from Fluka (Buchs, Switzerland). These acids were used to prepare a 50-mg/mL solution (in hexane) with approximately the same composition as that shown by these acids in the saturated acid fraction of butterfat. Individual *trans*-18:1 acids with ethylenic bonds between positions 5 and 15 were a kind gift from Dr. L. Svensson (Pharmacia, Stockholm, Sweden).

Preparation of fat solutions. About 1 g of butter, weighed in a 50-mL Teflon beaker, was dispersed in 10 mL isopropanol with an Ultra-Turrax T 25 equipped with an S 25 N 10 G shaft (Janke & Kunkel GmbH & Co. KG, Staufen, Germany). Hexane (15 mL) was added, and the suspension was dispersed a second time. A sufficient amount of anhydrous Na_2SO_4 was added, and the suspension was dispersed a third time. An aliquot (2.5 mL, containing *ca.* 80 mg fat) of the suspension was withdrawn with a 5-mL, all-glass syringe and filtered into a Teflon-lined, screw-capped 100 × 18 mm Pyrex tube through a disposable microfiltration unit (Millex-GV, 0.2 μm pore size; Millipore, Molsheim, France) (14). These steps take less than 10 min. To ensure a tight seal with the Teflon liner of the cap during the esterification reaction, the lip of the tube was finely ground with emery paper.

Preparation of FAIPE and FAME. FAIPE were prepared as follows, essentially according to Wolff and Fabien (15). Isopropanol (1.8 mL) and 0.25 mL concentrated H_2SO_4 were added to the clear butterfat solutions, generally prepared in duplicate. The tubes were tightly capped and vigorously shaken, and the reaction was allowed to proceed at 100°C for 1 h. If evaporation of the solution occurred, the tube was discarded. At the end of the reaction, the tubes were cooled under tap water, and a 5% aqueous solution of NaCl (5 mL) was added. The tubes were vortexed for *ca.* 30 s and allowed to stand for 1 min or so. The upper phase was withdrawn and replaced by an equal volume of pure hexane. After vortexing and standing a second time, the upper phase was withdrawn and pooled with the first one. A third extraction was performed in the same manner. To prepare FAIPE with the weighed mixture of even-number saturated fatty acids from 4:0 to 18:0, 1.5 mL of the standard solution was mixed with 2.8 mL isopropanol and then handled as described previously. FAME were prepared with the standard mixture in a similar way. Methanol (2.8 mL) and 0.25 mL concentrated H_2SO_4 were added to 1.5 mL of the standard solution, giving a single-phase solution. FAME were then prepared exactly as the FAIPE.

Ag–TLC fractionation of FAIPE. FAIPE (typically 0.5 mL of the FAIPE solutions, containing about 8 mg FAIPE, and reduced to *ca.* 0.1 mL under a stream of N_2) were fractionated by TLC on silica-gel plates impregnated with $AgNO_3$. Commercial precoated plates (DC-Vertigplatten Kieselgel H; Merck, Darmstadt, Germany) were dipped in a 5% (wt/vol) $AgNO_3$ solution in acetonitrile for 20 min, partially air-dried, and activated at 120°C for 20 min. The $AgNO_3$ solution can be used several times with no apparent effect on the fractionation. The developing solvent was a mixture of hexane/di-

ethyl ether (90:10, vol/vol). At the end of the chromatographic runs, the plates were briefly air-dried and sprayed with a 0.2% (wt/vol) 95% ethanolic solution of 2′,7′-dichlorofluorescein. The plates were then examined under ultraviolet (UV) radiation. When *trans*-18:1 acids had to be quantitated, the bands corresponding to the saturated and *trans*-monoenoic acids were scraped off together into an aluminum foil, and the gel from the two bands was transferred into the same test tube. Methanol (1.5 mL), 2 mL hexane, and 1.5 mL of a 5% (wt/vol) aqueous solution of NaCl were added successively to the gel (6). This order is essential for the quantitative recovery of FAIPE. Thorough mixing followed each addition. After standing for *ca.* 1 min (or a brief centrifugation), the upper hexane phase was withdrawn almost quantitatively and concentrated under a light stream of N_2 in a waterbath (40°C). The residue was dissolved in a small volume of hexane (generally 200 µL) for further GLC analyses. For identification purposes only, all detected bands, and also plate zones, where no material was visible under UV (total: 10 fractions), were scraped individually and handled as described previously.

GLC analyses. The great majority of analyses of FAIPE (and FAME) was carried out on a Carlo Erba HRGC chromatograph, fitted with a flame-ionization detector (FID) and a split injector, and coupled to an LT 430 temperature programmer (Carlo Erba, Milano, Italy). The split ratio was adjusted in such a manner that the butyric acid peak exactly spanned the chart width, the attenuation of the integrator being set to 8. Separations were performed on CP Sil 88 fused-silica capillary columns (50 m × 0.25 mm i.d., 0.20 µm film; Chrompack, Middelburg, The Netherlands). For the separation and quantitation of total FAIPE, the column temperature was maintained at 65°C for 6 min, and then increased at a rate of 5°C/min up to 185°C and maintained at this point until the end of the chromatographic runs (45 min). The same conditions were used to identify peaks after Ag–TLC fractionation or to quantitate FAIPE and FAME prepared with the standard mixture. When saturated plus *trans*-monoenoic acids had to be analyzed for quantitative determinations of *trans*-18:1 acids, the columns were operated isothermally at 160°C. The inlet pressure of the carrier gas (helium) was 120 kPa in all instances. The detector and the injector were maintained at 250°C.

Unless otherwise stated, samples were injected by the traditional manual injection technique. In this technique, the ester solution is withdrawn air bubble-free up to the 2 µL mark in a 10-µL Unimetrics microsyringe (Shorewood, IL). The solution is then pulled back into the syringe barrel. The needle is rapidly and completely inserted into the hot injector, immediately followed by fast plunger pushing. The needle is maintained 5 s in the injection port and then quickly removed. The quantity of esters injected was approximately the same (within a few percents) for all samples and analyses. This was checked by comparing the total surfaces given by the integrator for the sum of all peaks. Quantitative analyses were performed with an SP 4290 integrator (Spectra Physics, San Jose, CA).

RESULTS AND DISCUSSION

Identification of peaks obtained by GLC of FAIPE. The chromatograms in Figure 1 illustrate how peaks were identified after Ag–TLC fractionation. Due to the high efficiency of this step, no overlaps between the different fatty acid families occur. We limit our description to the zone 8:0-conjugated

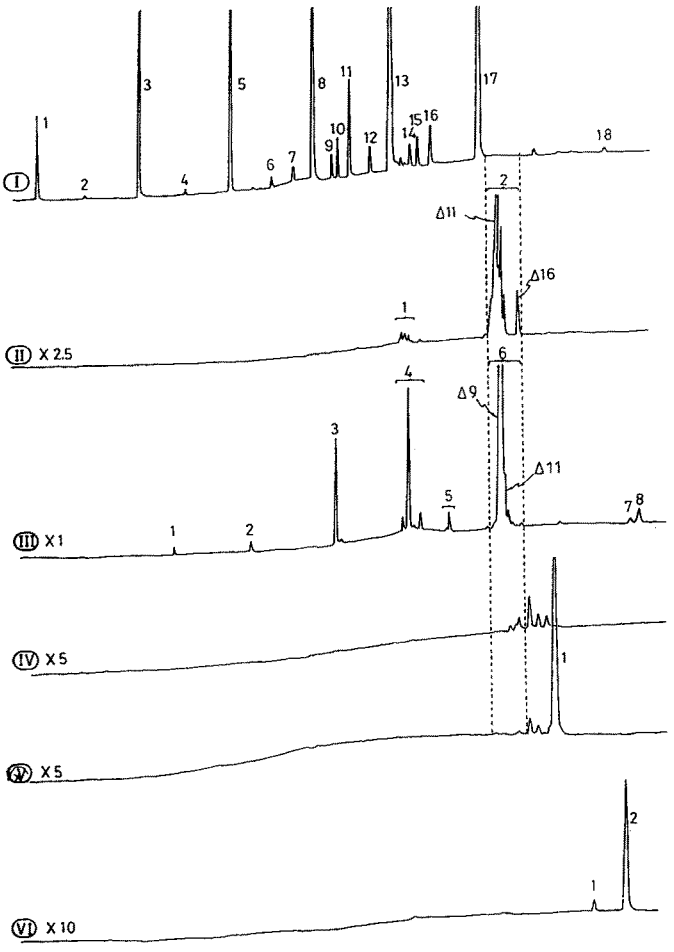

FIG. 1. Partial chromatograms of fatty acid isopropyl esters prepared from butterfat and fractionated by silver-ion thin-layer chromatography. The plates were prepared and developed as described in the text. Analyses were done on a CP Sil 88 capillary column (50 m × 0.25 mm i.d., 0.20 µm film; Chrompack, Middelburg, The Netherlands); see text for operating conditions. Identification of peaks: fraction I (saturated acids): 1, 8:0; 2, 9:0; 3, 10:0; 4, 11:0; 5, 12:0; 6, 13:0; 7, *iso*-14:0; 8, 14:0; 9, *iso*-15:0; 10, *anteiso*-15:0; 11, 15:0; 12, *iso*-16:0; 13, 16:0; 14, *iso*-17:0; 15, *anteiso*-17:0; 16, 17:0; 17, 18:0; 18, 20:0. Fraction II (*trans*-monoenoic acids): 1, 16:1 (several isomers); 2, 18:1 (several isomers). Positions of double bonds are counted from the carboxylic end. Fraction III (*cis*-monoenoic acids): 1, 10:1; 2, 12:1; 3, 14:1; 4, 16:1 (several isomers); 5, 17:1 (two isomers); 6, 18:1 (several isomers); 7, 20:1; 8, conjugated 18:2 acid(s). Positions of double bonds are counted from the carboxylic end. Fractions IV and V (octadecadienoic acids): 1, *cis*-9,*cis*-12 18:2; other peaks are unidentified. Fraction VI (octadecatrienoic acid): 1, *cis*-6,*cis*-9,*cis*-12 18:3 (?); 2, *cis*-9,*cis*-12,*cis*-15 18:3. Concentrations of the fractions relative to fraction I as indicated on chromatograms. Dotted lines delimit the elution zone of *trans*- and *cis*-18:1 acids.

18:2 acids because shorter chains (4:0–6:0 acids), due to their great volatility, are entirely lost during the procedure (Ag–TLC fractionation, solvent evaporation). For these acids, identification was by comparison with FAIPE prepared from commercial standards.

The saturated acid fraction ($R_f = 0.82$) is typical of milk fat, with straight, even-numbered chains being major components. Between these peaks, odd-numbered and branched (*iso* and *anteiso*, eluted in this order) fatty acids are also present. Under our chromatographic conditions, the last eluting saturated fatty acid is 20:0 (Fig. 1), which accounts for *ca.* 0.1% of total fatty acids.

Only two groups of peaks could be observed in the *trans*-octadecenoic fraction ($R_f = 0.71$), one in the C_{16} region, the other in the C_{18} region. *Trans*-16:1 acids are present in trace amounts (3% of the *trans*-18:1 acids, or about 0.07% of total fatty acids) and could not be individually identified. On the other hand, *trans*-18:1 acids are quite abundant (2.3% of total fatty acids in the sample we used for this experiment). The major isomer is vaccenic (*trans*-11) 18:1 acid. The last-eluting *trans*-18:1 isomer has its ethylenic bond in position 16 (1,6,19), and due to its relative abundance, it can be used in chromatograms of total FAIPE as a landmark to delimit the *cis*- plus *trans*-18:1 acid group (Fig. 1). It appears that this isomer has been confused with 19:0 acid in some instances (16). This acid is known to be present in butterfat and may correspond to the small peak eluting between 18:0 and 20:0 acids in the saturated fraction (peaks 17 and 18, respectively, in fraction I of Fig. 1). This peak represents *ca.* 0.06% of total fatty acids and elutes largely after the *trans*-16 18:1 isomer, in admixture with isomeric 18:2 acids when total FAIPE are analyzed. Temperature programming is not the best means to determine the distribution profile of individual *trans*-18:1 isomers. For this purpose, the *trans*-18:1 acid fraction is analyzed isothermally at a relatively low temperature (1,6).

Cis-monoenoic acids ($R_f = 0.58$) range from 10:1 to 20:1 acids, and this fraction also includes some late-eluting conjugated 18:2 acid(s). 15:1 Acid was not detected. For 14:1, 16:1, 17:1, and 18:1 acids, the main isomer always belongs to the Δ9 series (20). The 10:1 acid probably also has its ethylenic bond in the terminal 9-position (21). The 16:1 acids show two supplementary isomers, probably the Δ7 and Δ11 (20) (before and after the Δ9 isomer, respectively). The 17:1 acids also include the minor Δ8 isomer (20), which elutes before, and is partly resolved from, the main Δ9 isomer. *Cis*-17:1 acids are sometimes reported as being mixed with a branched 18:0 acid, but this last acid does not exceed *ca.* 0.06% of total fatty acids. From semilog plots of chainlengths vs. retention times (results not shown), the branched 18:0 acid should be the *iso*-18:0 isomer. The *cis*-18:1 acid region includes the Δ11 isomer, which appears as a shoulder on the descending edge of the main Δ9 peak, and several other minute components follow it.

The dienoic acid fractions ($R_f = 0.22$ and 0.16) appear complex, and apart from the main all-*cis* 18:2n-6 acid, show several minor isomeric forms that elute before it. However, these isomers do not seem to interfere to a great extent with the *trans*- plus *cis*-18:1 acid group during GLC (Fig. 1). The identity of the all-*cis* 18:2n-6 acid (and that of the all-*cis* 18:3n-3 acid) was confirmed by co-injection of a mixture of FAIPE prepared from butterfat and soybean oil. However, it would seem that the 18:2n-6 acid peak is not pure and that it may contain a trace amount of an isomeric 18:2 acid that is not resolved from the main all-*cis* peak under our routine chromatographic conditions (small shoulder at the base of the leading edge of the 18:2n-6 acid peak; Fig. 1). Other specific conditions allow this separation (results not shown). The isomeric 18:2 acids are believed to be *cis* or *trans* methylene and nonmethylene-interrupted dienes. Finally, the 18:3n-3 acid, all-*cis* form ($R_f = 0.05$), could be isolated as an almost pure compound, with perhaps a trace of 18:3n-6 acid (Fig. 1).

These identifications allow the quantitation of about forty different fatty acids in routine analyses in the range 0.03–33%. However, slight variations in column efficiency may occur from one batch to another. Consequently, some overlaps are sometimes unavoidable. For example, the *cis*-14:1 and *anteiso*-15:0 acids are not always separated, and they can be quantitated only by indirect means (6). This also holds true for the *cis*-9 16:1 and *iso*-17:0 acids. Older studies in which packed columns were used did not distinguish between these close-eluting fatty acids, and they were reported as either branched or monounsaturated acids.

Conversion factors of FAME and FAIPE analyzed by GLC with an FID. Craske and Bannon (22) have defined a practical correction factor (F_p), determined empirically, that can be used in the conversion of peak area percentages to FAME weight percentages. This factor can be expressed as:

$$F_p = F_t \times F_e \qquad [1]$$

where F_t is the theoretical response factor for FAME in the FID as determined by Ackman and Sipos (23), and F_e is an error factor introduced to compensate for different experimental errors in esterification (selective extraction, losses due to volatility of some components), as well as errors linked to the GLC analysis itself (inadequate injection technique, nonlinearity of detector response). Craske and Bannon (22) argued that the optimization of analytical conditions (esterification, GLC analysis) makes $F_e = 1$, therefore reducing Equation 1 to $F_p = F_t$. The only necessary correction is then the theoretical response factor. With the F_t factors being known for all esters, the weight percentage of component X becomes:

$$\%\, X = 100 \times \frac{F_{tx} \times S_x}{\sum (F_{ti} \times S_i)} \qquad [2]$$

where S stands for peak area. For FAME analysis, the values calculated correspond to the weight percentage of a given ester relative to the total weight of FAME.

Ackman and Sipos (23) calculated F_t as the ratio of the ester molecular weight to the weight of active carbon atoms present in the molecule. For FAME, the carbonyl carbon is the only one not giving any response in the FID, all other car-

bon atoms giving a response equal to 1. The expression for F_t becomes:

$$F_t = \frac{\text{ester molecular weight}}{\text{weight of active carbon atoms}} = \frac{14n + 46 - 2m}{12n} \quad [3]$$

where n is the number of carbon atoms in the ester and m is the number of ethylenic bonds. Some experimental data have led to the belief that Equation 3 is only valid for esters of fatty acids that contain more than 9 carbon atoms (24). However, Bannon *et al.* (25–27) have demonstrated the high accuracy obtained by using F_t as calculated in Equation 3 when applied to FAME containing 4–22 carbon atoms and 0–6 ethylenic bonds. Under optimal analytical conditions, i.e., when $F_e = 1$ and $F_p = F_t$, the theoretical response factors allow accurate determination of weight percentages of FAME from peak areas established by the integrator coupled with the FID.

If one wishes to calculate the weight percentages of fatty acids instead of the weight percentages of fatty acid esters, a correction term needs to be applied to the response factors. The resulting factor, termed theoretical conversion factor (F'_t) because it is used to convert area percentages of FAME peaks into weight percentages of fatty acids, is linked to the theoretical response factor F_t by:

$$F'_t = F_t \times \frac{\text{fatty acid molecular weight}}{\text{FAME molecular weight}} = \frac{14n + 32 - 2m}{12n} \quad [4]$$

These new factors allow individual fatty acid (not ester) weight percentage determination relative to total fatty acid (not ester) weight according to:

$$\% X = 100 \times \frac{F'_{tx} \times S_x}{\sum (F'_{ti} \times S_i)} \quad [5]$$

The mode of calculation applied to FAME, where methanol is used for esterification, can be extended to higher alcohols [sometimes used by a few authors (28–30)], and general equations for the theoretical response factor for esters and the theoretical conversion factor for fatty acids can be obtained:

$$F_t = \frac{14(n + n') + 32 - 2m}{12(n + n' - 1)} \quad [6]$$

$$F'_t = \frac{14n + 32 - 2m}{12(n + n' - 1)} \quad [7]$$

where n' is the number of carbon atoms in the alcohol. Table 1 gives the calculated F'_t values for esters of saturated acids that contain 4–18 carbon atoms and alcohol chains that contain 1–5 carbon atoms. These values are relative to the esters of palmitic acid for which F'_t were taken as equal to 1 (for reasons explained later). It is clear from Table 1 that alcohols with three carbon atoms lead to conversion factors that are the closest to 1 over the whole range 4:0–18:0 acids (1.03–1.00, respectively). Other alcohols give rise to esters that need larger conversion factors. Alternately stated, for alcohols with three carbon atoms, one can approximate $F'_t = 1$ independently of the fatty acid chainlength, and Equation 5 becomes:

$$\% X = 100 \times \frac{S_x}{S_i} \quad [8]$$

which indicates that fatty acid weight percentages are equal to peak area percentages.

TABLE 1
Relative Conversion Factors Used to Transform Peak Area Percentages (given by the integrator coupled to the flame-ionization detector) into Fatty Acid Weight Percentages for Saturated Fatty Acids from 4:0 to 18:0 Esterified with Alcohols of Different Chainlengths (C_1–C_5)[a]

	Alcohol				
Acid	C_1	C_2	C_3	C_4	C_5
4:0	1.375	1.169	1.031	0.933	0.859
6:0	1.208	1.100	1.020	0.957	0.906
8:0	1.125	1.063	1.013	0.972	0.938
10:0	1.075	1.038	1.008	0.982	0.960
12:0	1.042	1.022	1.004	0.990	0.977
14:0	1.018	1.009	1.002	0.995	0.990
16:0	1.000	1.000	1.000	1.000	1.000
18:0	0.986	0.993	0.998	1.004	1.009

[a]Values are relative to palmitic acid esters taken as equal to 1.000.

To verify these theoretical considerations, we have prepared a weighed mixture of straight, even-numbered saturated fatty acids (4:0–18:0 acids) resembling that present in the saturated acid fraction of butterfat. These acids were esterified either to FAME or to FAIPE in the presence of concentrated H_2SO_4 as a catalyst. Each esterification experiment was repeated five times. FAME and FAIPE prepared from the standard solution were analyzed exactly under the same conditions as FAIPE prepared from butterfat. The results of these analyses are plotted in Figure 2, in which we compare the theoretical and experimental conversion factors. For both FAME and FAIPE, experimental data closely match theoretical values. However, it is also clear that FAME need nonnegligible, not to say large, conversion factors, mainly for the shortest chains, whereas FAIPE show experimental conversion factors close to 1, as predicted by the theory. Discrepancies introduced by approximating F'_t to 1 for FAIPE of all chainlengths are of the order of experimental errors (see below). Thus, the choice of FAIPE instead of FAME practically eliminates the necessity of applying conversion factors. Results given by the integrator directly correspond to fatty acid weight percentages, provided the integrator is able to suitably determine the beginning and the end of a peak.

Repeatability and reproducibility of FAIPE preparation and analysis. To check the precision of each step of our analytical procedures, we have used one single sample of butter, purchased in January (the same as that used for identification of FAIPE; see above). The whole procedure (fat extraction,

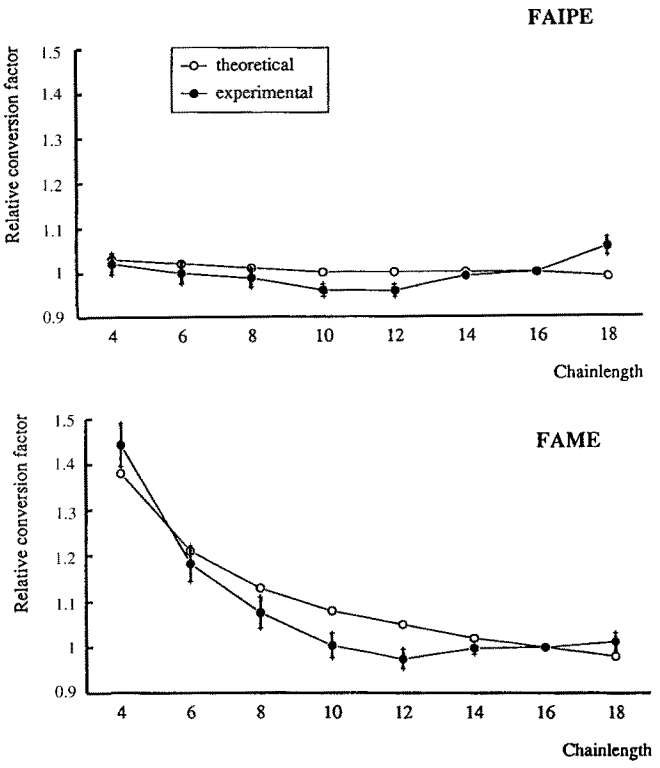

FIG. 2. Comparison of the theoretical and experimental relative conversion factors (used to transform peak area percentages calculated by the integrator linked to the chromatograph into fatty acid weight percentages) for fatty acid methyl esters (FAME) and fatty acid isopropyl esters (FAIPE). Values are relative to palmitic acid esters taken as equal to 1.000. Experimental data points represent the means of five experiments, and vertical bars correspond to the standard deviations.

FAIPE preparation, GLC analysis) was applied five times by five different operators to the same sample of butter. We then calculated the coefficients of variation (standard deviation divided by the mean times 100), which are a measure of the precision of the method, for each even-numbered acid from 4:0 to 18:0 acids and for 18:1 acids. These results, shown in Figure 3 (experiment A), indicate that the coefficients of variation are a function of the number of carbon atoms in the fatty acid molecules. They decrease from *ca.* 6% for butyric acid to 1% for palmitic acid and then slightly increase for stearic and 18:1 acids. Except for butyric acid, all coefficients of variation are less than 4%. Furthermore, the same operator applied the whole procedure five times to the sample of butter. The results (Fig. 3, experiment B) show that the coefficients of variation are similar to, although slightly lower, than those obtained when five different operators performed the analyses. Consequently, the repeatability is practically identical to the reproducibility, which indicates that the operator has little influence on the results. This is easily explained by the fact that the preparation of the fat solution (dispersion and filtration) is so simple that practically no experimental errors can be introduced during this step. This contrasts with the complicated methods proposed by other authors (28). Furthermore, we prepared five FAIPE solutions with the same fat extract and analyzed them by GLC. Here too, the coefficients of variation are practically not modified as compared to the two preceding experiments (Fig. 3, experiment C), stearic acid being a major exception (8%). The behavior of 18:0 acid in this experiment and some others is puzzling, and we attribute its relatively large coefficient of variation to chance only. Finally, we analyzed the same preparation of FAIPE (a mixture of the five preceding different solutions of FAIPE) by GLC five times. In this case, the coefficients of variation are slightly reduced, mainly for butyric acid (Fig. 3, experiment D), with all of them being less than 3%. This indicates that part of the variation comes from the GLC analysis itself, and another part from the step of FAIPE preparation. It might be possible to avoid the extraction step of FAIPE, and the reaction solutions themselves might be directly used for injection. However, we did not dare to inject sulfuric acid, even diluted, onto the column (which is rather expensive), although others do so with apparently no evident degradation of the column or any other severe drawbacks. This shortcut would be an interesting simplification of the method.

We repeated experiment D ten days later, after the FAIPE solution had been left on the bench at ambient temperature. Except for 18:0 acid, which shows a rather random behavior, the results were similar to those of the preceding experiment (Fig. 3, experiment E). We also tested another mode of injection. In this technique (solvent flush) the needle is first filled with hexane. The plunger is withdrawn to the 1 μL mark, the tip of the needle is immersed in the FAIPE solution, and the plunger is pulled back to the 3.5 μL mark. This gives results similar to the conventional injection technique used for all of our other analyses (Fig. 3, experiment F). This means that the injection mode does not affect the coefficients of variation. No differences were observed when the temperature of the injection port was increased by 10%, up to 275°C (Fig. 3, experiment G). Changing the complete chromatographic system for another one (Carlo Erba 4160 model), equipped with a similar capillary column and operated under comparable conditions (same total surface and retention times of peaks), did not modify the coefficients of variation that remained less than 3% (Fig. 3, experiment H). However, under these conditions, we noted a significant difference in the apparent content of butyric acid, which was 10% higher than with the habitual chromatographic system (results not shown).

In all experiments, the lowest coefficient of variation corresponds to 16:0 acid. Parodi (31) also noted this peculiarity for fatty acid butyl esters analyzed on a packed column. This is the reason why we chose 16:0 acid and not 18:0 acid as the reference compound to calculate theoretical and experimental relative conversion factors. Variations in GLC analysis evidently include variations linked to the injection parameters, but also to the integrator itself. One major problem with the integrator is how it determines what constitutes the beginning and end of a peak, particularly in zones of sloping baseline (during temperature programming, for example) or with skewed peaks. Obviously, these problems can be alleviated by use of personal computer-based systems that allow correc-

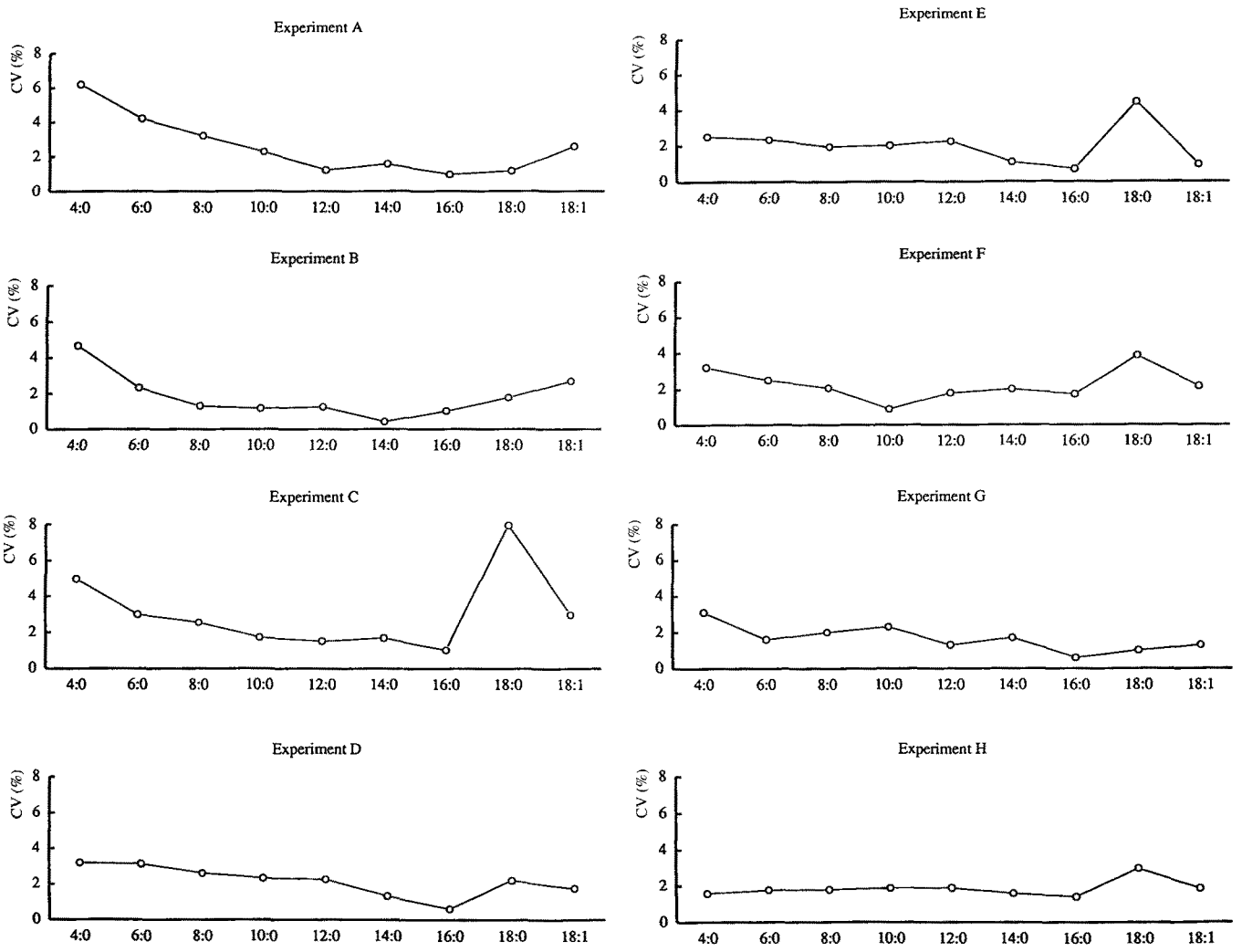

FIG. 3. Coefficients of variation (CV; ratio of standard deviation to the mean times 100) of fatty acid weight percentages as a function of chain-length. All experiments were realized with the same sample of winter butterfat, and except otherwise stated, the same chromatograph and the same injection technique were used (see the Experimental Procedures section). Experiment A, whole procedure applied five times to the sample of butter by five different operators. Experiment B, whole procedure applied five times to the sample of butter by one single operator. Experiment C, five different preparations of fatty acid isopropyl esters (FAIPE) with the same butterfat extract. Experiment D, five injections of the same sample of FAIPE (mixture of preparations used in experiment C). Experiment E, five injections of the preceding solution of FAIPE kept ten days on the bench at ambient temperature. Experiment F, same as experiment E, except that a different injection technique (solvent flush) was used. Experiment G, same as experiment E, except that the injector temperature was increased by 10% (275°C). Experiment H, same as experiment E, except that injections were made on another chromatographic system.

tion of integration errors post-run, but this was not possible with our system. On the other hand, we have no explanation for the occasional erratic behavior of 18:0 acid.

Between-sample variations of individual fatty acids. A curious fact is that what we observed with a single sample of butter is also observable with several samples of butter from spring and fall (6) and several kinds of goat and ewe cheese (1). For all samples, the coefficients of variation for the butyric acid content are similar and close to 5%—fall butters, 5.6%; spring butters, 3.4%; ewe cheeses, 5.4%; goat cheeses, 4.6%. Similar values were obtained in the present study—summer butters, 5.6%; winter butters, 5.1 and 5.7%. When a single sample of butter is analyzed several times by repeating the whole procedure, or part of it, the coefficient of variation for butyric acid is comprised between 3 and 6% (Fig. 2). This means that the content of butyric acid in butters for a given season, or in ewe and goat cheeses, does not vary much from one sample to another. The observed between-sample variations are within the experimental errors. Provided the same procedure is applied to all samples, the butyric acid content may thus be considered a constant. Alternately stated, large variations noted in the literature for the butyric acid content of butterfat (or more generally of milk fat) from commercial origin should probably be linked to variations among different procedures rather than to true physiological or environmental differences.

In our study, and for a given season, the regional origin of butters is certainly an important factor that can affect the fatty acid composition. Despite the fact that one single brand of butter is manufactured with a considerable quantity of pooled, homogenized, and standardized milks, it is assumed that the collection of milk is generally made in regions with well-marked geographic boundaries. All regions do not have the same pasture potential, and hence, differences surely exist in feeding practices between regions. This also applies to major national brands, such as those analyzed in the present study, because they are manufactured in numerous dairies distributed all over the country. Regional variations are, in fact, differences in feeding practices and are probably related to the amount or quality of grass in the feed.

If one considers the coefficients of variation for individual fatty acids, two periods are distinguishable—winter and other seasons (Fig. 4). In winter, the between-sample coefficients of variation are close to the coefficients of variation inherent in the experimental methodology (Fig. 4), except for 18:0 acid, the behavior of which could not be experimentally controlled. Alternately stated, the between-sample variations are practically within the experimental errors. This means that regional variations are reduced in winter, probably because feeding practices are more uniform in the country during this season. The situation is quite different for other seasons (Fig. 4). The between-sample coefficients of variation for some fatty acids (4:0, 6:0, and 14:0) are close to the coefficients of variation inherent in the experimental methodology, whereas they are larger for other fatty acids (8:0, 10:0, 12:0, 16:0, 18:0, and 18:1). This does not mean that 4:0, 6:0, and 14:0 do not vary, but that their variations are within the experimental errors, and thus, that they cannot be detected. Nevertheless, we can deduce that not all fatty acids are affected to the same extent from one sample of butter to another in a given season. However, the variability appears to be almost constant for a given fatty acid, independently of the season, from spring through autumn (Fig. 4).

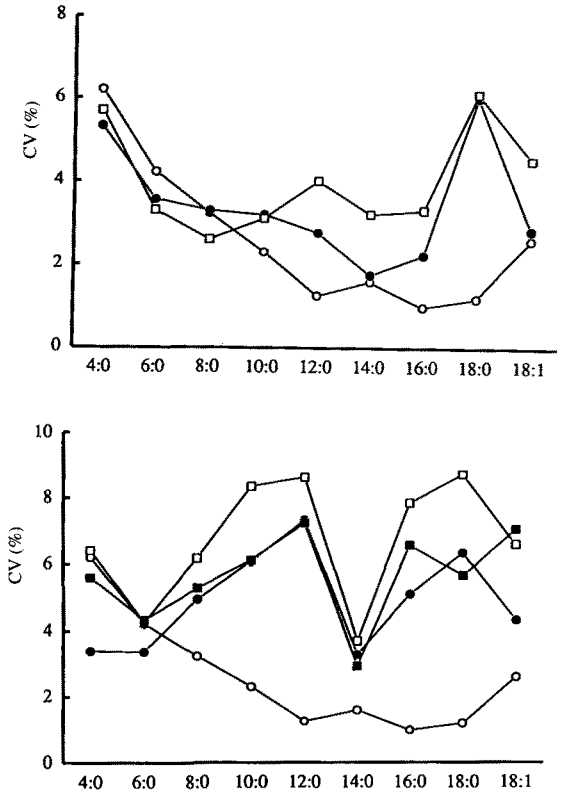

FIG. 4. Comparison of the between-sample coefficients of variation (CV) of the main fatty acids in winter butters on the one hand, and in other seasons on the other hand, with the CV inherent in the methodology (experiment A in Fig. 3). Top panel: ○, Methodology; ●, January; □, March. Bottom panel: ○, Methodology; ●, May–June; □, July–August; ■, October–November.

Repeatability of the determination of trans-*18:1 acid content and profile.* A preceding study showed that the recovery of fatty acids from the gel after Ag–TLC fractionation is not selective (6). To demonstrate this, a weighed mixture of 16:0, 18:0, and *trans*-9 18:1 acids was analyzed before and after separation. The fractionation and elution from the gel did not modify the fatty acid composition of the standard mixture (6).

In the present study, we have fractionated the same solution of FAIPE prepared with a sample of winter butter by Ag–TLC five times. The results of this experiment (Table 2) indicate that the procedure is highly repeatable: with 14:0, 16:0, and 18:0 as internal standards, the coefficient of variation for the content of *trans*-18:1 acids is less than 4% (2.33 ± 0.09%). Five injections of the same *trans*-18:1 acid fraction (plus saturated acids) led to similar results (Table 2), indicating that the fractionation plus elution steps did not add much to the coefficient of variation linked to the GLC analysis alone.

Because our capillary column allows a fairly good (although incomplete) resolution of several individual *trans*-18:1 isomers, we also established the relative distribution of these acids. Table 2 summarizes these results. For the main Δ11 (plus Δ10) isomer, the coefficient of variation is less than 2%, independently of the experiment (five distinct fractionations, or five injections of the same fraction). Here too, it can be concluded that the fractionation plus elution steps do not considerably increase the coefficients of variation linked to GLC analyses. Although difficult to prove, it would appear that the main source of variations does not necessarily come from the chromatograph itself (injector, column, detector), but rather from the integration unit. The limiting step in the precision of the whole procedure would thus be the precision with which the integrator measures the area of peaks, that is the precision in the recognition of what is the beginning and end of a peak, particularly when they are partly fused. Any contamination of the *trans*-monoenoic acid fraction by *cis*-18:1 acids, not visible after Ag–TLC fractionation, is immediately detected by an abnormally high proportion of the *trans*-12 18:1 isomer, which elutes along with *cis*-9 18:1 acid.

Seasonal variations of butterfat fatty acid composition. Table 3 gives the complete and detailed fatty acid composition of butterfats analyzed throughout the year. However, seasonal variations are more perceptible in the plots of Figure 5.

TABLE 2
***Trans*-18:1 Acid Content and Distribution Determined on a Single Sample of Winter Butter After Fractionation by Silver-Ion Thin-Layer Chromatography (Ag–TLC) of Fatty Acid Isopropyl Esters (FAIPE)[a]**

	Total *trans*-18:1 acids[b]	Individual *trans*-18:1 isomers[c]					
		Δ6–Δ9	Δ10 + Δ11	Δ12	Δ13 + Δ14	Δ15	Δ16
Fractionations[d]	2.33 ± 0.13	9.03 ± 1.32	47.74 ± 0.75	9.78 ± 0.41	17.90 ± 0.69	6.75 ± 0.17	8.79 ± 0.98
Injections[e]	2.29 ± 0.16	8.64 ± 0.40	47.77 ± 0.32	9.59 ± 0.22	17.95 ± 0.05	6.97 ± 0.13	9.10 ± 0.14

[a]Effects of multiple fractionations and injections. [b]As weight percent of total fatty acids. [c]As weight percent of total *trans*-18:1 isomers. [d]Five different fractionations by Ag–TLC of a single FAIPE preparation. [e]Five successive injections of one FAIPE fraction (a mixture of the five preceding fractions).

Butyric acid apparently varies with the season, being slightly higher in late winter than in spring. On the other hand, the 6:0 and 8:0 acids do not display any seasonal trends of statistical significance (Fig. 5). The small differences that can be observed between successive points are within the experimental variations inherent in our procedures. For these acids, our observations are in fairly good qualitative agreement with those of Huyghebaert and Hendrickx (12), who analyzed 235 samples of commercial Belgian butter. This study is interesting for comparison, because it is assumed that cattle feeding practices are probably the same, on the average, in Belgium and France. However, our absolute values for 4:0, 6:0, and 8:0 are each *ca.* 0.3–0.5% higher than in the preceding study. On the other hand, our observations disagree with those of Gray (5), who reported an almost continuous decrease of 4:0 acid from 6.8% in July to 3% in May, and also a 50% decrease of 6:0 and 8:0 acids during the same period. In contradiction to the two preceding studies (5,12), we only noted minute variations in the content of 10:0 and 12:0 acids (Fig. 5). A more pronounced decrease of 14:0 acid can be observed in April, but

TABLE 3
Fatty Acid Composition (weight percent) of French Butters Collected at Different Periods of the Year

Fatty acid[a]	January[b]	March	May–June	July–August	October–November
4:0	3.95 ± 0.21	4.40 ± 0.25	3.83 ± 0.13	4.21 ± 0.27	4.29 ± 0.24
5:0	0.03 ± 0.005	0.05 ± 0.01	0.03 ± 0.01	0.02 ± 0.005	0.04 ± 0.01
6:0	2.53 ± 0.09	2.67 ± 0.09	2.38 ± 0.08	2.52 ± 0.11	2.55 ± 0.11
7:0	0.03 ± 0.005	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.005	0.03 ± 0.01
8:0	1.52 ± 0.05	1.56 ± 0.04	1.42 ± 0.07	1.46 ± 0.09	1.52 ± 0.08
9:0	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
10:0	3.45 ± 0.11	3.46 ± 0.11	3.14 ± 0.19	3.13 ± 0.26	3.28 ± 0.20
10:1	0.34 ± 0.02	0.34 ± 0.01	0.30 ± 0.01	0.34 ± 0.02	0.35 ± 0.02
11:0	0.07 ± 0.005	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
12:0	3.99 ± 0.11	3.95 ± 0.16	3.57 ± 0.26	3.50 ± 0.30	3.75 ± 0.27
13:0	0.11 ± 0.005	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.02	0.10 ± 0.01
iso-14:0	0.12 ± 0.02	0.12 ± 0.03	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.02
14:0	12.08 ± 0.21	11.93 ± 0.38	11.03 ± 0.36	11.16 ± 0.41	11.32 ± 0.33
iso-15:0	0.23 ± 0.04	0.31 ± 0.07	0.34 ± 0.04	0.36 ± 0.05	0.27 ± 0.02
anteiso–iso-15:0	0.37 ± 0.11	0.49 ± 0.09	0.58 ± 0.04	0.59 ± 0.08	0.67 ± 0.10
14:1	1.12 ± 0.05	0.95 ± 0.07	0.91 ± 0.07	1.05 ± 0.14	0.88 ± 0.07
15:0	1.16 ± 0.09	1.20 ± 0.10	1.15 ± 0.05	1.19 ± 0.09	1.13 ± 0.08
iso-16:0	0.28 ± 0.05	0.27 ± 0.04	0.27 ± 0.02	0.29 ± 0.07	0.24 ± 0.03
16:0	33.34 ± 0.74	32.52 ± 1.06	27.05 ± 1.37	28.27 ± 2.20	29.27 ± 1.91
16:1	0.27 ± 0.04	0.20 ± 0.06	0.35 ± 0.04	0.37 ± 0.07	0.27 ± 0.06
iso-17:0	0.30 ± 0.05	0.29 ± 0.14	0.42 ± 0.02	0.43 ± 0.08	0.34 ± 0.02
16:1Δ9	1.54 ± 0.14	1.57 ± 0.10	1.43 ± 0.08	1.46 ± 0.12	1.67 ± 0.16
anteiso–iso-17:0	0.52 ± 0.06	0.56 ± 0.05	0.51 ± 0.02	0.53 ± 0.06	0.45 ± 0.03
17:0	0.51 ± 0.04	0.54 ± 0.02	0.70 ± 0.04	0.57 ± 0.05	0.65 ± 0.03
17:1	0.24 ± 0.02	0.25 ± 0.02	0.38 ± 0.03	0.33 ± 0.04	0.35 ± 0.04
18:0	9.01 ± 0.54	8.95 ± 0.55	10.96 ± 0.69	10.54 ± 0.91	9.61 ± 0.54
18:1[c]	19.16 ± 0.54	19.67 ± 0.89	24.00 ± 1.03	22.93 ± 1.50	22.62 ± 1.58
18:2 isomers	0.41 ± 0.12	0.41 ± 0.07	0.93 ± 0.08	0.48 ± 0.10	0.68 ± 0.12
18:2n-6	1.37 ± 0.14	1.49 ± 0.27	1.17 ± 0.15	1.26 ± 0.15	1.35 ± 0.15
20:0	0.12 ± 0.01	0.10 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.13 ± 0.01
18:3n-3	0.25 ± 0.05	0.25 ± 0.05	0.60 ± 0.07	0.53 ± 0.10	0.46 ± 0.09
20:1	0.12 ± 0.01	0.12 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.12 ± 0.01
18:2 conjugated	0.38 ± 0.06	0.46 ± 0.05	0.74 ± 0.16	0.65 ± 0.17	0.48 ± 0.10
Others	1.04 ± 0.17	0.78 ± 0.33	1.21 ± 0.31	1.24 ± 0.38	0.91 ± 0.36

[a]Identification as described in the text. [b]Mean ± SD for twelve samples of butter at each period. Values for May–June and October–November are from Reference 6. [c]*Cis* and *trans* isomers.

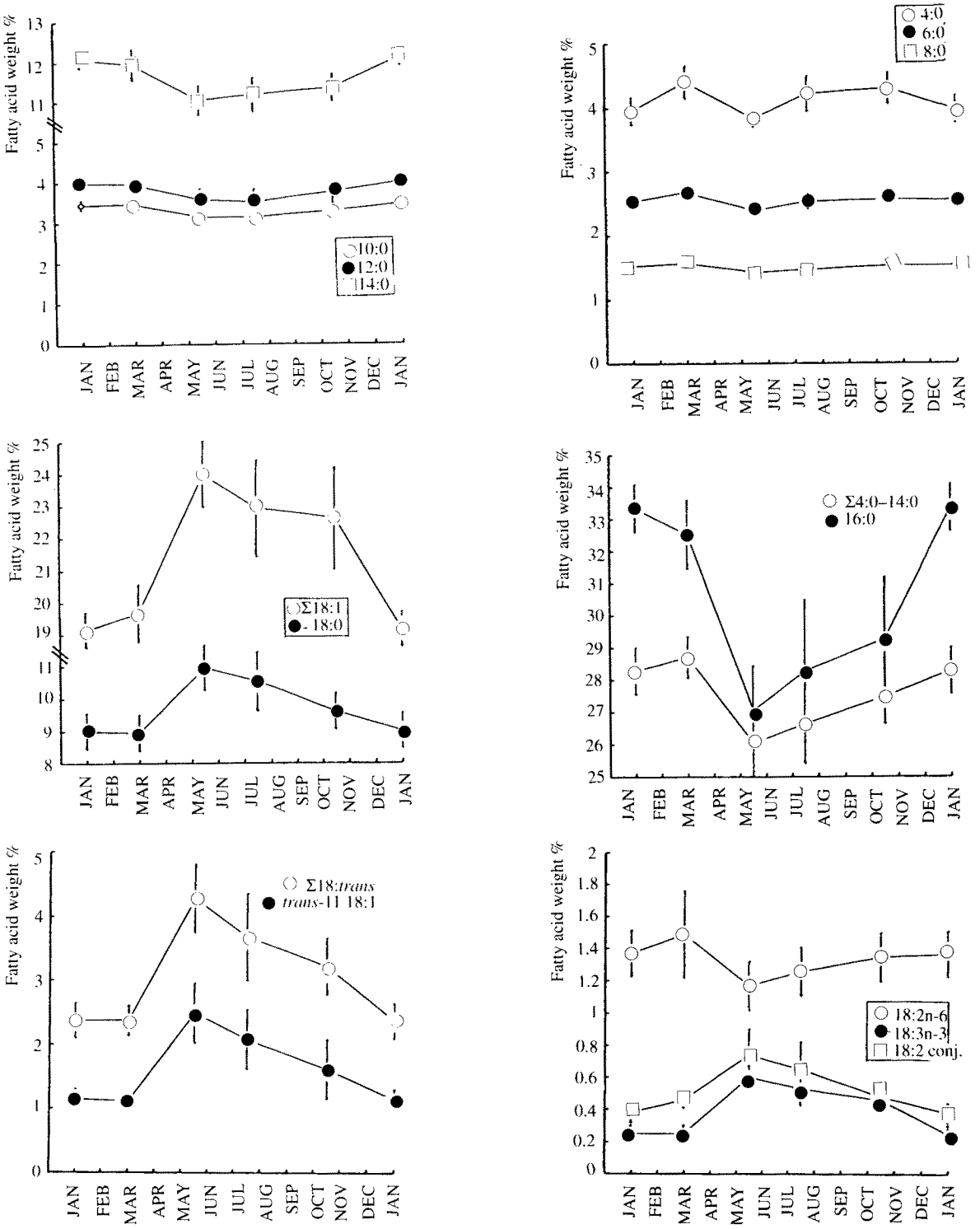

FIG. 5. Evolution of some fatty acids (as weight percentages of total fatty acids) in the fat from commercial French butters purchased at different periods of the year. Each point is the mean of analyses of 12 different brands. Vertical bars correspond to standard deviations. Conj., conjugated.

it does not exceed 1% (Fig. 5). Our data do not support recent results obtained for French butters by Bornaz *et al.* (32), who observed variations of 14:0 acid in the range of 11 to 20% (summer and winter, respectively). To our knowledge, nobody else found such high values for 14:0 acid in milk fat, except when cows are fed experimental diets supplemented with myristic acid (2). If one considers the sum of 4:0 to 14:0 acids, the small individual variations add to each other, and a

definite variation can be observed around mid-April (Fig. 5). Its level decreases by *ca.* 2% and then progressively returns to the winter level in an almost linear manner.

Palmitic acid is apparently the saturated acid that is the most sensitive to seasonal variations. Its level is quite constant during winter at about 33% of total fatty acids (Fig. 5). In April, there is a sharp decrease, and the level of 16:0 acid drops to 27% in May–June. Then, the content of 16:0 acid progressively increases until late fall–early winter. A decrease of similar amplitude was reported by Huyghebaert and Hendrickx (12) during the same period, but their absolute values for palmitic acid were 5% less than ours (28 and 22% in winter and spring, respectively). The origin of this discrepancy is unclear. Concomitant with the decrease of palmitic acid is an inverse evolution of 18:0 acid, although of lesser amplitude (about 2%; Fig. 5). The main variation that compensates for the depression of 4:0 to 16:0 acids at the beginning of spring is an increase in the levels of *cis*- plus *trans*-18:1 acids (Fig. 5), which rise from a low of 19% in winter to a high of 24% in spring. Here, too, our data closely agree, on a qualitative basis, with those of Huyghebaert and Hendrickx (12), although there are discrepancies in the absolute levels of 18:1 acids (low, *ca.* 27%; high, 31%). Almost half of the increase (2%) is attributable to *trans*-18:1 acids (see next section).

Polyunsaturated fatty acids from the essential n-6 and n-3 series in milk fat are practically limited to two main species, 18:2n-6 and 18:3n-3 acids. From our study, it would appear that the level of 18:2n-6 acid is almost constant throughout the year, with a mean annual range of 1.2–1.5% of total fatty acids (Table 3, Fig. 5). The precision of our methods is on the verge of allowing detection of subtle seasonal variations, i.e., a small decrease of 18:2n-6 acid in spring. Our values are slightly lower than generally reported, and this is certainly due to the use of a capillary column that readily separates 18:2n-6 acid from most of its geometrical and positional isomers (Fig. 1). Although Huyghebaert and Hendrickx (12) reported a higher value for 18:2n-6 acid (2.4%), they did not note any significant seasonal variations. On the other hand, these investigators observed that the 18:3n-3 acid level in butterfat was quite sensitive to the seasons, maximum values (*ca.* 2.9%) being reached between May and October, with a steady decrease between October and April, down to 1.4–1.6% (12). Our own values (Table 3, Fig. 5) are considerably lower, the maximum of 18:3n-3 acid being 0.6%. This difference can by no means be attributed to some damaging effect of our procedures, which have been shown not to alter polyunsaturated fatty acids (15). Despite the relative scarcity of 18:3n-3 acid, some seasonal trends could be unambiguously observed. α-Linolenic acid is at its lowest level in winter (0.2%) and suddenly increases in April to reach a maximum of 0.6% in May–June. Our values should be considered as accurate, because no other fatty acids interfere with 18:3n-3 acid under our chromatographic conditions (Fig. 1). Parallel to the evolution of 18:3n-3 acid, the conjugated 18:2 acid, an intermediary metabolite in the biohydrogenation of dietary polyunsaturated acids, increases in April, reaches a maximum in spring and summer and then decreases until winter (Fig. 5). Our GLC data are in excellent qualitative and quantitative agreement with those of Boatman *et al.* (9), who analyzed the polyunsaturated acids in milk fat by biochemical (lipoxidase) and chemical (alkali isomerization) means. In this study, in which the milk from cows reared in the United States was analyzed, the annual ranges for 18:2n-6 and 18:3n-3 acids were 1.2–1.7 and 0.3–0.6%, respectively. Both acids were shown to vary with the season. There was a decrease of 18:2n-6 acid and an increase of 18:3n-3 acid, concomitant with the availability of pasture in spring, an observation confirmed by our GLC study.

Practically all trends noted in the present study agree well with those detailed in the report by Gallacier *et al.* (33) on seasonal variations of butter from three dairies in the west of France. However, the amplitudes of variations are of lesser importance in our study than in that of Gallacier *et al.* (33), researched in 1982. This may perhaps reflect some evolution in feeding practices during these last twelve years. On the other hand, it should be stressed that the wider choice of samples in our study may also be responsible for the more uniform fatty acid distribution in butters observed throughout the year.

Most of our observations can be logically explained by the change in the dietary habits of cows with seasons. In France, if the climatic conditions are favorable, most cows traditionally go back to pastures around mid-April. Some regional variations do occur. Fresh grass lipids contain a high proportion of 18:3n-3 acid (mainly esterified to glycolipids and phospholipids) (34), an excellent substrate for biohydrogenation by rumen microorganisms (35). The result of this hydrogenation mechanism is the formation of stearic acid, which is desaturated by the animal to oleic acid, and the formation of intermediate *trans*-18:1 acids (mainly vaccenic acid). Consequently, the level of most C_{18} fatty acids (18:0, *cis*- plus *trans*-18:1, 18:3n-3, and conjugated 18:2 acids) increases in milk fat. However, 18:2n-6 acid apparently does not follow this trend. On the other hand, 4:0 to 14:0 acids, and part of 16:0 acid, which are of endogenous origin and synthesized in the mammary gland, are depressed. Following this first period in the spring, as the grass progressively diminishes in pastures, cows are fed a mixed diet with grass, concentrates, and roughage in various proportions. As the amount of grass in the diet diminishes, the C_{18} acids progressively decrease in milk fat until winter, when there is practically no more grass. Alternately, the decrease of C_{18} acids in summer and fall may follow the content and nature of lipids in grass at different degrees of maturity (34). If one considers the coefficients of variation for individual major fatty acids (Fig. 4), it is clear that these variations are lower in winter than in any other season. This supports the view that the proportion of grass (absent from feed in the winter and consequently invariable, variable in other seasons, and depending on geographic and climatic conditions and on husbandry) is the major parameter that influences the fatty acid composition of milk fat in commercial products such as butter.

Trans-*18:1 isomer content and profile.* Seasonal variations in the *trans*-18:1 acids content of milk fat have been recog-

nized for more than three decades, and the first report of GLC analyses of these isomers is apparently attributable to Patton *et al.* (7), in 1960. In a preceding study (6), we discussed the limits of the methods for *trans*-fatty acid quantitation in milk fat—infrared absorption at *ca.* 970 cm^{-1} and GLC alone or GLC coupled with Ag–TLC. As a general rule, GLC/Ag–TLC methods give the most accurate results in the determination of *trans*-18:1 acids. However, it does not seem that this procedure has been applied frequently to follow seasonal variations in milk fat. Gray (5) used Ag–TLC fractionation, but the *trans* content was determined by densitometry after charring. Qualitatively, at least, it is known that the level of *trans*-18:1 acids in milk fat vary with the season, being higher in summer than in winter (6,7), but absolute values conflict (6). On the other hand, nothing is known about possible variations in the distribution profile of individual isomers with the season.

In the present study, we observed that the *trans*-18:1 acid level is at its minimum value in winter (2.4%) and at its maximum value in May–June (4.3%) (Table 4, Fig. 5). The *trans*-18:1 acid evolution follows that of total 18:1 acids. From experimental data and interpolated values from the graph in Figure 5, we calculated a mean annual value of 3.3% *trans*-18:1 acids. A similar value recently has been reported for German butters (36). This is *ca.* 15% less than our previous estimate (6), based only on butters from spring and fall (3.8%). However, it must be emphasized that the value of 3.3% can only be used for those countries where animal husbandry is practiced, such as in western European countries. For countries where cows are reared on pastures throughout the year, slightly higher values would probably fit best. Minimum and maximum values observed for individual butter samples were 1.7% (winter) and 5.1% (spring). Incidentally, the mean *trans*-18:1 acid content in cow milk fat is intermediate between those found in beef meat lipids (*ca.* 2%) and tallow (*ca.* 5%) (1). Apparently, this does not depend on the period of the year (unpublished results). The tissue would thus have some influence on the esterification rate of *trans*-18:1 acids to lipids.

Table 4 summarizes the distribution profiles of *trans*-18:1 acids in butterfat throughout the year. It is clear that the level of vaccenic acid (plus *trans*-10 18:1 acid) relative to total *trans*-18:1 acids varies with the seasons, being higher in spring (*ca.* 58%) than in other seasons. The lowest level is reached in winter (*ca.* 48%). No such variations were observed in a previous study by Parodi (37), who determined the distribution pattern of *trans*-18:1 acids in spring–summer and winter milks by ozonolysis. Nevertheless, this confirms our previous hypothesis made with goat and ewe milk fats (1). The relative level of vaccenic acid is related to the feed and, more precisely, to the amount of grass in the feed. Goats do not eat very much grass, and vaccenic acid is low in their milk, even less than in the fat from winter cow milk. On the other hand, ewes eat grass throughout the year, and vaccenic acid is high in their milk, as in spring cow milk. It would also appear that the level of vaccenic acid, relative to total *trans*-18:1 isomers, in the fat from cow, goat, and ewe milks (between 45 and 58%, depending on the species and/or the feed) is lower than in beef meat lipids or tallow (68 and 64%, respectively) (1). Thus, some selectivity must exist in the esterification of individual *trans*-18:1 isomers to lipids, depending on the tissue.

Gallacier *et al.* (33) tried to follow the *trans*-16 18:1 acid content by direct GLC on a packed column. Under these conditions, it is doubtful that the peak identified as *trans*-16 18:1 acid was pure. It certainly co-eluted with 18:2 isomers and C_{19} acids. Nevertheless, this peak slightly evolved with the season, being lower in winter than in other seasons. From data in Table 4, it can be calculated that the *trans*-16 18:1 acid is *ca.* 0.2% of total fatty acids in winter, and *ca.* 0.3% in other seasons. Much more than vaccenic acid, the *trans*-16 18:1 isomer appears to be the characteristic *trans* isomer of milkfat when compared to hydrogenated oils. The mean annual proportion of *trans*-16 18:1 acid relative to total *trans*-18:1 isomers is 8.1% in butterfat, but only 0.6% in hydrogenated oils (value determined with eleven French margarines and shortenings; unpublished results).

Several authors have tried to find constant ratios between different fatty acids in butterfat to ascertain its genuineness and to detect adulterations by other fats and oils. It is assumed that such adulterations may come mainly from the addition of hard fats, such as tropical fats, beef tallow, or partially hydrogenated oils, which are cheaper than butter. Due to the seasonal variations in the content of different fatty acids in butter (4:0-14:0, 16:0, 18:0, 18:1, 18:2n-6, 18:3n-3, conjugated 18:2 acids), this appears extremely difficult, except perhaps

TABLE 4
Content and Distribution Profile of *trans*-18:1 Isomers in French Butters as a Function of the Period of Collection[a]

Collection period	Total *trans*-18:1 acids[b]	Individual *trans*-18:1 isomers[c]					
		Δ6–Δ9	Δ10 + Δ11	Δ12	Δ13 + Δ14	Δ15	Δ16
January	2.37 ± 0.27	10.1 ± 1.2	48.4 ± 3.0	8.9 ± 0.7	17.0 ± 0.9	6.6 ± 0.6	9.0 ± 1.1
March	2.37 ± 0.23	6.7 ± 1.3	47.8 ± 1.9	10.2 ± 0.7	19.4 ± 1.1	7.4 ± 0.5	8.4 ± 0.7
May–June[d]	4.28 ± 0.47	7.2 ± 0.8	58.2 ± 3.8	6.2 ± 0.7	15.4 ± 1.7	5.7 ± 0.6	7.3 ± 0.9
July–August	3.68 ± 0.68	8.0 ± 1.2	56.5 ± 3.8	7.3 ± 2.4	14.9 ± 1.4	5.7 ± 0.6	7.6 ± 1.1
October–November	3.22 ± 0.44	9.6 ± 1.3	50.4 ± 3.9	7.2 ± 0.7	17.2 ± 1.6	6.6 ± 1.6	9.0 ± 1.2

[a]Twelve different samples were analyzed at each period. [b]As weight percent of total fatty acids. [c]As weight percent of total *trans*-18:1 isomers. [d]Data for samples purchased in May–June and October–November are from Reference 6.

for 6:0 to 12:0 acids, which are less sensitive to such variations. For example, we calculated the ratio 12:0/10:0, which equals to 1.13 ± 0.03 (n = 60; minimum, 1.08; maximum, 1.19) with no significant seasonal variations. From the data of Gallacier *et al.* (33) for French butters, a value of 1.17 ± 0.03 can be calculated, and Iverson and Sheppard (4) reported a value of 1.16 ± 0.07 for U.S. butters and cheeses. In both studies, more than 100 samples were analyzed. So, adulteration with 12:0 acid-containing fats should be detectable. On the other hand, the use of absolute values could be more helpful, particularly that of 18:2n-6 acid, which varies in the narrow range 1.2-1.5% of total fatty acids. *Trans*-18:1 isomers in butterfat have a characteristic, although slightly variable profile, and this should therefore be useful in detecting adulteration with partially hydrogenated oils. In these artificially hardened fats, the relative proportions of the Δ9 and Δ10 *trans* isomers are higher than in butterfat. On the other hand, incorporation of beef tallow in butter will not modify the *trans*-18:1 pattern of butterfat because their distributions are too similar (1).

REFERENCES

1. Wolff, R.L., *J. Am. Oil Chem. Soc. 72*:259 (1995).
2. Christie, W.W., *Prog. Lipid Res. 17*:245 (1979).
3. Bitman, J., and D.L. Wood, *J. Dairy Sci. 73*:1208 (1990).
4. Iverson, J.L., and J. Sheppard, *Ibid. 72*:1707 (1989).
5. Gray, I.K., *J. Dairy Res. 40*:207 (1973).
6. Wolff, R.L., *J. Am. Oil Chem. Soc. 71*:277 (1994).
7. Patton, S., R.D. McCarthy, L. Evans, and T.R. Lynn, *J. Dairy Sci. 43*:1187 (1960).
8. Jensen, R.G., G.W. Gander, and J. Sampugna, *Ibid. 45*:329 (1962).
9. Boatman, C., D.K. Hotchkiss, and E.G. Hammond, *Ibid. 48*:34 (1965).
10. Hutton, K., R.C. Seeley, and D.G. Armstrong, *J. Dairy Res. 36*:103 (1969).
11. Senft, B., and F. Kobasa, *Michwissenschaft 25*:510 (1970).
12. Huyghebaert, A., and H. Hendrickx, *Ibid. 26*:613 (1971).
13. Wolff, R.L., and A.F.M. Castera-Rossignol, *Rev. Fr. Corps Gras 34*:123 (1987).
14. Wolff, R.L., *Sci. Alim. 13*:559 (1993).
15. Wolff, R.L., and R.J. Fabien, *Lait 69*:33 (1989).
16. Badings, H.T., and C. De Jong, *J. Chromatogr. 279*:493 (1983).
17. Sampugna, J., R.E. Pitas, and R.G. Jensen, *J. Dairy Sci. 49*:1462 (1966).
18. Iverson, J.L., and A.J. Sheppard, *Food Chem. 21*:223 (1986).
19. Lund, P., and F. Jensen, *Milchwissenschaft 38*:193 (1983).
20. Hay, J.D., and W.R. Morrison, *Biochim. Biophys. Acta 202*:237 (1970).
21. Morrison, W.R., *Topics in Lipid Chemistry,* Vol. 1, edited by F.D. Gunstone, Logos Press Limited, 1970, p. 52.
22. Craske, J.D., and C.D. Bannon, *J. Am. Oil Chem. Soc. 54*:1413 (1987).
23. Ackman, R.G., and J.C. Sipos, *Ibid. 41*:377 (1964).
24. Ackman, R.G., and J.C. Sipos, *J. Chromatogr. 16*:298 (1964).
25. Bannon, C.D., N.T. Hai, N.L. Harper, and K.L. O'Rourke, *Ibid. 247*:67 (1982).
26. Bannon, C.D., J.D. Craske, and A.E. Hilliker, *J. Am. Oil Chem. Soc. 62*:1501 (1984).
27. Bannon, C.D., J.D. Craske, and A.E. Hilliker, *Ibid. 63*:105 (1986).
28. Iverson, J.L., and A.J. Sheppard, *J. Ass. Off. Anal. Chem. 60*:284 (1977).
29. Traitler, H., U. Richli, A.M. Kappeler, and C. Monnard, *6th International Symposium on Capillary Chromatography,* Riva del Garda, 1985, p. 568.
30. Seher, A., and M. Arens, *Fat. Sci. Technol. 89*:307 (1987).
31. Parodi, P.W., *Aust. J. Dairy Technol. 25*:200 (1970).
32. Bornaz, S., G. Novak, and M. Parmentier, *J. Am. Oil Chem. Soc. 69*:1131 (1992).
33. Gallacier, J.-P., J.-P. Barbier, and S. Kuzdzal-Savoie, *Tech. Lait. 993*:13 (1984).
34. Harfoot, C.G., *Prog. Lipid Res. 17*:21 (1978).
35. Kuzdzal-Savoie, S., J. Raymond, W. Kuzdzal, and J. Petit, *Ann. Biol. Anim. Bioch. Biophys. 6*:351 (1966).
36. Precht, D., *Z. Ernährungswiss. 34*:27 (1995).
37. Parodi, P.W., *J. Dairy Sci. 59*:1870 (1976).

[Received March 31, 1995; accepted August 31, 1995]