Analysis of Milkfat by HPLC

Ekkehart Frede[*,a] and **Herbert Thiele**[b]
[a]Federal Dairy Research Center, Kiel, F.R.G., and [b]Bruker-Franzen Analytik, Bremen, F.R.G.

A routine method for HPLC analysis of milkfat has been developed, which takes into account the specific phenomena of this complex and far-ranging system of triglycerides. The usual amount of injection of 1 mg has been reduced to 30 or 10 μg of fat. By this the composition of the mobile phase (acetone:acetonitrile, 35/65) and the temperature of the column (Nucleosil C18-5 μ, 15 cm + Microspher C18-3μ, 10 cm, in series, T = 30–35 C) could be adjusted to a relatively high selectivity without inducing the high-melting fat components to crystallize on the column. A further advantage resulting from this consisted in permitting the eluent to be recycled over long periods of time. The extremely low amounts of samples necessitated a highly sensitive detection ($\Delta n = 5 \times 10^{-7}$ RI units full scale deflection) which could be realized by an interferential refractometer in connection with a thermostat to keep up a stable temperature of the whole HPLC system ($\Delta T < 0{,}005$ K). By this it was possible to separate milkfat into 45 to 50 different types of triglycerides. By comparing soft and hard milkfats and fractions of milkfats, every fourth peak, starting with C 42, could be attributed to saturated triglycerides; apart from this, easily recognizable qualitative features appearing in the chromatograms permitted conclusions about the feeding regimen and energy supply of the cattle. Furthermore, due to the high stability of separating conditions achieved and due to computer software developed for this purpose it was possible to obtain and compare a large number of chromatograms under the same conditions.

In recent years reversed phase (RP) HPLC has been developed into a highly accurate method for separating natural oils and fats into triglyceride (TG) classes. In many cases it provides a valuable supplement or even alternative to gas chromatography (GLC). The main reason for this is that TG classes separated by HPLC differ in their relative molecular mass and at the same time in their number of double bonds. In GLC, as far as packed columns are concerned, the separation is carried out mainly according to the molar mass or, in other words, to the acyl carbon number (CN). For the analysis of olive oil this means, for example, that the TG class with CN = 54 containing stearic (S), oleic (O) and linoleic (L) acids is eluted by a packed GLC column as one single band, whereas in HPLC it is divided into at least five subclasses, SOS, SOO, OOO, LOO and LOL (1,2). In general, it can be found that RP-HPLC with propionitrile as mobile phase is well suited to a differentiating characterization, especially of vegetable oils.

The HPLC of animal or vegetable fats with high-melting components is a less simple task. Here, an acceptable compromise between dissolving or eluting power and separation power of the mobile phase (3,4) has to be found. In this respect milkfat causes even more difficulties. It contains considerable amounts of high-melting and, in well separating eluents, sparingly soluble TGs. Apart from this it shows the broadest TG spectrum of all natural fats, ranging from at least C24 to C54.

Gaining good resolution of this broad band of TGs in an isocratic HPLC run—the usually employed RI detector forbids any gradient elution—forms a special challenge. There are two possibilities:

(i) A gradient elution not compatible with our conditions could be replaced to some extent by temperature programming, i.e., by a continuous increase in column temperature during a chromatographic run.
(ii) By injecting extremely low amounts of sample down to 10 μg onto the column instead of the common amount of about 1 mg, the solubility conditions might be improved to a degree that the long chain saturated fatty acid TGs will not crystallize from the solution during analysis, and therefore will not affect the stationary phase and falsify the chromatogram.

In the case of one-component mobile phase, temperature programming has proved to be compatible with RI detection even at increased sensitivites (4×10^{-5} RI units full scale deflection) (2). Using propionitrile, the advantages of low and high temperatures could be combined without any compromise in one chromatogram: high selectivity for the more polar TGs in the front part of the chromatogram and complete elution and detection of the saturated long chain fatty acid TGs at the end. A number of oils and fats, such as palm oil, coconut oil, cocoa butter and milkfats, have been separated by this method more effectively than previously was possible (2).

In the case of milkfat, however, any identification of TG bands eluted in close succession remains difficult. It also has not yet been clarified whether, due to the high amount of oleic acid and saturated fatty acids, trisaturated and dioleo-mono-saturated glycerides frequently superimpose, e.g. as PPP and SOO, of LaMP (La = Lauric, M = myristic acid) and POO do. It is true, in palm oil PPP and SOO are completely resolved under temperature gradient conditions (2) but result in one common peak at a constant temperature of 30 C under otherwise equal conditions (5).

When a mixture of acetone-acetonitrile is used as a mobile phase instead of propionitrile, TG pairs, which probably are quite frequent in milkfat, are not "critical." Otherwise, the selectivity of these binary eluents is, in general, lower than that of propionitrile (3,2).

An attempt has therefore been made to improve HPLC separation of milkfat by using a mixture of acetone-acetonitrile as eluent. Two milkfat chromatograms obtained by this method and recently published by Deffense (3) seem to confirm these suppositions, even though not all of the high-melting components could have been eluted.

Because temperature programming does not work in

*To whom correspondence should be addressed.

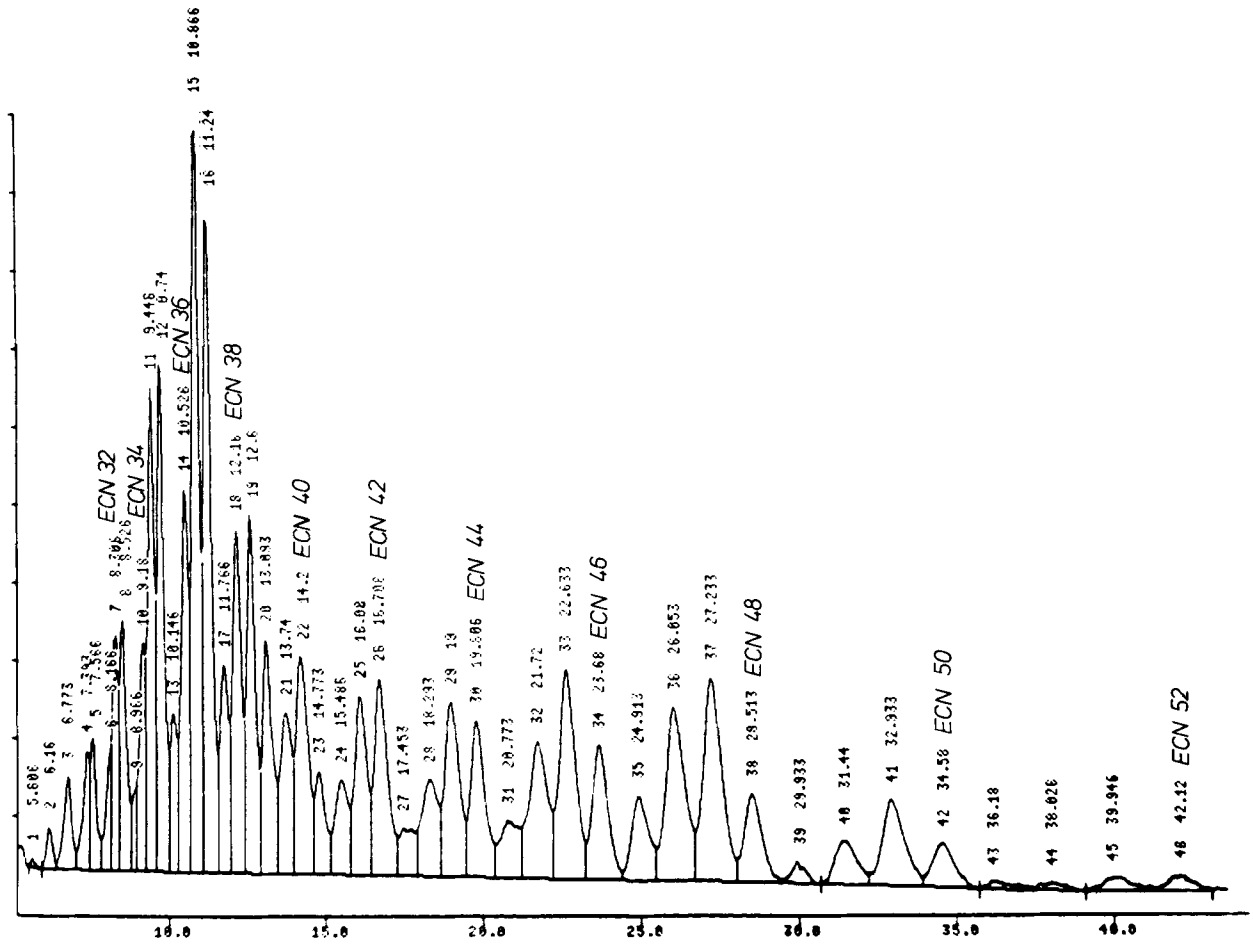

FIG. 1. Separation of milkfat triglycerides under conditions as noted in text. Temperature, 30 C; sample quantity, 30 μg.

combination with a binary eluent (2), the second of the aforementioned possibilities, injection of very low amounts of sample, was used. For this, however, the normal adjustment of sensitivity of an RI detector (32 − 4 × 10^{-5} RI units full scale deflection) does not suffice. Detector sensitivities higher than this require a precisely thermostated chromatographic system as well as a pulsation-free pump with an alomst absolutely constant flow-rate (6). Extremely low amounts of sample injections may permit a recycling of eluents. Some further advantages would be connected with this. For example, the whole HPLC system may be made insensitive to any fluctuation of atmospheric pressure. This also would be a prerequisite for an automatic HPLC analysis of milkfat.

Therefore, the task was to find an optimal solution to the technical problems mentioned and to examine in detail how many injections are feasible in a recycling operation without apparent changes in the system.

Finally, it also was an aim of this work to develop the electronic recording of chromatograms necessary for automatic operation, in connection with the facility of a variable reconstruction as well as an automatic and manual re-integration at a freely programmable personal computer. Specific demands, which the complex TG system of milkfat puts on a data station, were to be taken into special account.

EXPERIMENTAL

HPLC system. The HPLC system consisted of LC 41 chromatograph (Bruker, Bremen, Federal Republic of Germany), containing a trihead pump with electronic control and minimum flow pulsation; 1 nucleosil-C18-column, 5 μ, 150 × 4 mm (Macherey & Nagel, Düren, F.R.G.) and 1 microspher-C18-column, 3 μ, 100 × 4.6 mm (Chrompack, Mülheim, F.R.G.) in series; injection block 7125 (Rheodyne, Cotati, California); interferential refractometer 931 (Optilab, Höganäs, Sweden) with 12 μl cell and waste valve system; thermostated eluent reservoir (capacity volume 2 1), Thermostir (Optilab) with magnetic stirrer; a computer system containing a QX10 data system, including a dual floppy disk system, FX80 printer and monitor (all Epson, Düsseldorf, F.R.G.), for collection, storage and manipulation of chromatograms by a specially designed interface and the chromatography software (Bruker).

Thermostatting system. It contained an ultra thermostat 7911 Bt (Heto, Bir-Kenrod, Denmark) with a controlling accuracy ΔT = 0.01 K for regulating the temperature of a self-constructed aluminum block containing the columns and the injection block, of a double-walled PVC housing around the aluminum block, of the flow-cell as well as the optical bench of the refractometer and the eluent reservoir; a self-constructed heat exchanger made of copper, supplied with water for

cooling the motor of the pump, instead of the air ventilation provided by the manufacturer; a kryothermostat F 40 (Julabo) for regulating the temperature inside the LC 41-box isolated by styropor lining by a self made copper coil system, and for regulating the three pumpheads by plastic hose-loops.

Solvents and samples. Eluant: Acetone and acetonitrile LiChrosolv (Merck, Darmstadt, F.R.G.) were mixed 65:35 (v/v) and ultrasonically degassed under vacuum.

Samples: All milkfats (from German and foreign dairies as well as from a small herd of the Federal Dairy Research Center) and fractions of milkfats (produced by crystallization under cooling from the melt or from solutions in acetone) were dissolved in the eluent (3 mg/ml or 1 mg/ml) and were then filtered by a Fluoropore-filter (0.5 μ) (Millipore Corp., Neu-Isenburg, F.R.G.).

Performance. Flow rate of the mobile phase was 0.8 ml/min. The temperature of the system was set at 30 or 35 C. Via a small opening in the double-walled PVC housing 10 μl of the preheated solution were entered manually into the sample loop by means of an equally preheated syringe (Hamilton, Boneduz, Switzerland), and the injection valve was operated from the outside by means of a self-constructed hydraulic device.

The chromatograms were stored on diskettes to be re-integrated later by specially designed software.

RESULTS AND DISCUSSION

Qualitative considerations. Figure 1 shows an HPLC-chromatogram as one typical example of 60 chromatograms taken over a two-month period under the above conditions. During this time the mobile phase was not renewed but was continuously recycled. At the end of the series of experiments the mobile phase had been contaminated by 60 × 30 μg = 1.8 mg of fat, which corresponds to a concentration of about 1 mg/l. This concentration led neither to an observable deterioration of separation nor to a delay of retention times. This proves that the uncommonly low sample injection with an amount of only 30 μg of fat onto the column not only lessens or perhaps completely eliminates the risk of column deterioration, but also permits use of the advantages of a recycling operation. Apart from saving preparation time and solvents, one main advantage is that separating conditions can be kept stable during a

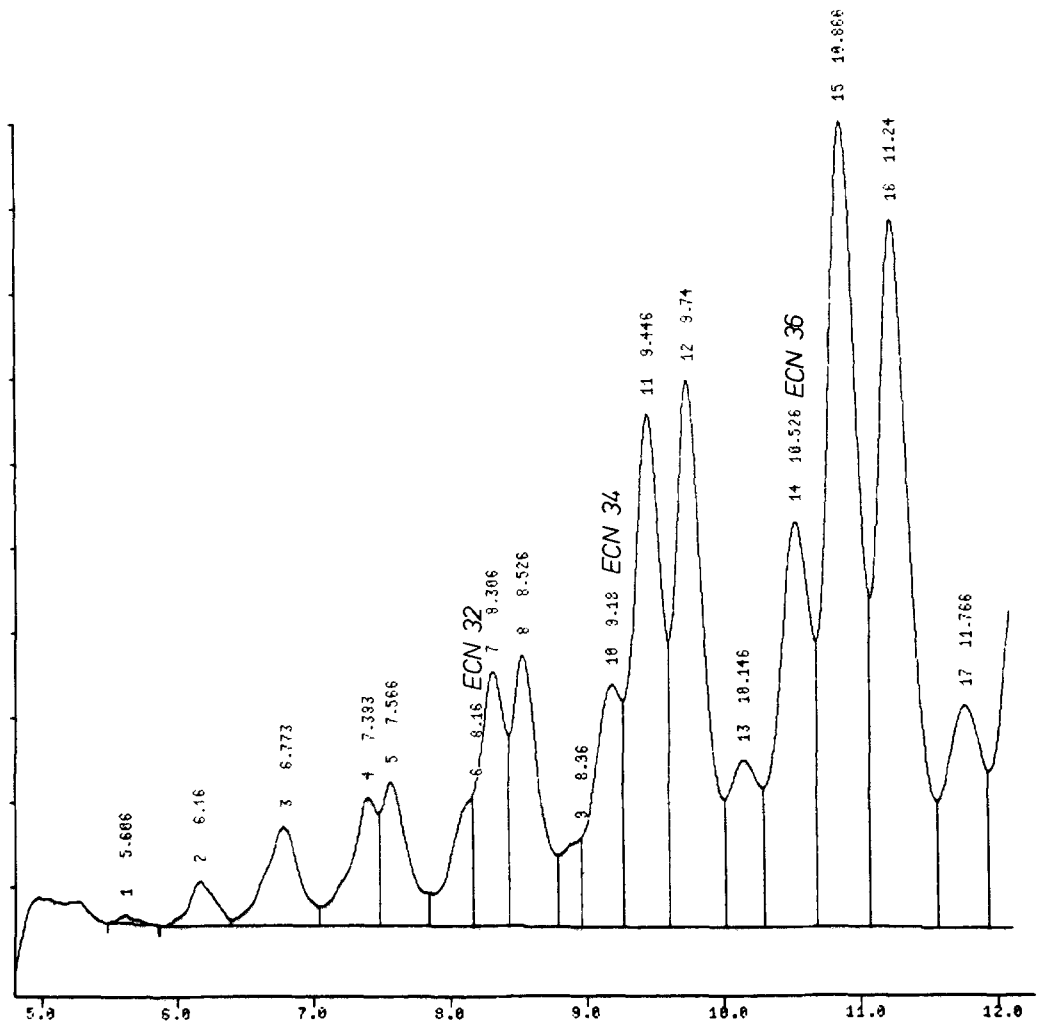

FIG. 2. The front part of the chromatogram of Fig. 1 on an enlarged time scale.

long series of chromatographic runs. The noise ratio was sufficiently low to obtain even small peaks with a height of $\Delta n = 5 \times 10^{-8}$ RI units and within a time frame of min. Probably due to differences in room temperature, however, long-term baseline drifts changing in both directions could not be avoided, even though considerable efforts had been undertaken to thermostat the system (see below). When the chromatogram was supposed to be displayed on the monitor during a run, the detector had to be re-adjusted to zero once every 6 hr. In all other cases this was not necessary due to the far-reaching dynamic range of the detector and data system (± 1000 mV at a maximum peak height of 300 mV). Therefore, a 24-hr automonitored run controlled by an autosampler would be possible without any problems. Each time before storing the data files on diskette, the data system produces an automatic translation of the chromatograms on zero level so that afterwards they can be recalled directly for reconstruction and reintegration and be displayed on the monitor.

Forty-six different peaks can be distinguished in the chromatograms (Fig. 1). If the column temperature is raised by 5 K, the retention time of the last peak will be shortened by 7.5 min (see below) and the traces of TG classes eluted even later appear. On the other hand, the resolution especially within the front part of the chromatogram will diminish considerably, so that under our conditions a column temperature of 30 C will represent a fair compromise for the acquisition of milkfat chromatograms. The number of peaks obtained by this method is lower than that obtained when propionitrile is applied (2). At the same time only the last peaks are separated almost down to the baseline. Most peaks, however, seem to have a uniform pattern in their retention times and intensities, so that association with certain TG classes might well be facilitated. Especially the peaks 27 to 46 can be classified visually as five quartets. Adding saturated TGs such as trimyristin, tripalmitin, dipalmistostearin and distearopalmitin to the milkfat sample resulted in an exact correspondence of their retention time to that of the last peak of each quartet. A comparison with further standards (trilaurin, tricaprin, tricaprylin and coconut oil) has shown that this periodicity, i.e. every fourth peak corresponding to an even ECN, continues until peak 4. Here the group structure of four changes. The peak of ECN 36, for instance, occupies the second position in the quartet and not the last. When considering the first peaks of the chromatogram on an extended time scale (Fig. 2), it might be concluded that at a higher resolution they would be separated into four peaks as were the group of peaks 11-14.

If the logarithms of the capacity factors of all peaks are graphically drawn against the even ECNs of the related quartets as shown in Figure 3, four parallel straight lines are obtained. They are all spaced equidistantly. This means that the whole milkfat chromatogram is of uniform structure and each TG class is distinct from its adjacent classes by half a unit of ECN.

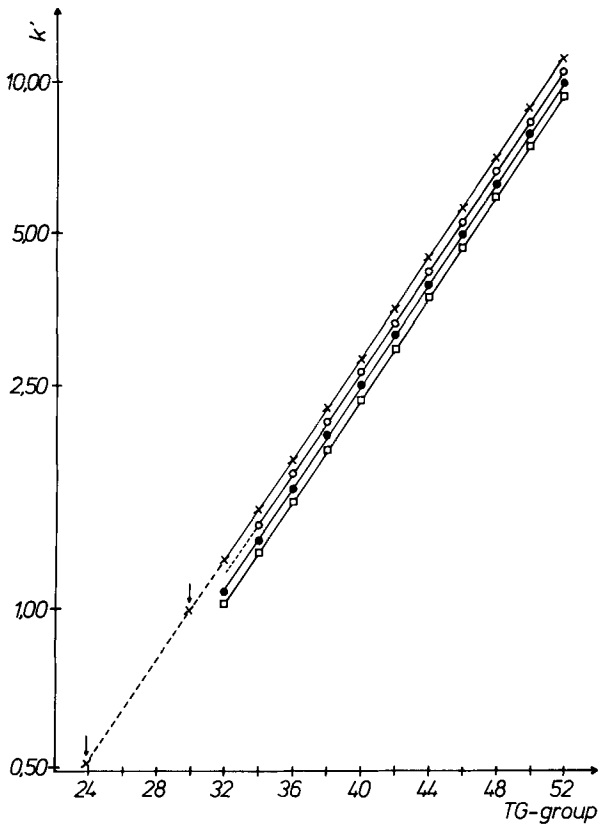

FIG. 3. Capacity factor k' of milkfat triglycerides and, respectively, standards (see text) drawn on a logarithmic scale versus the even equivalent carbon number (ECN) of the related quartets. The points designated by arrows refer to standards only (tricapyrlin and tricaprin).

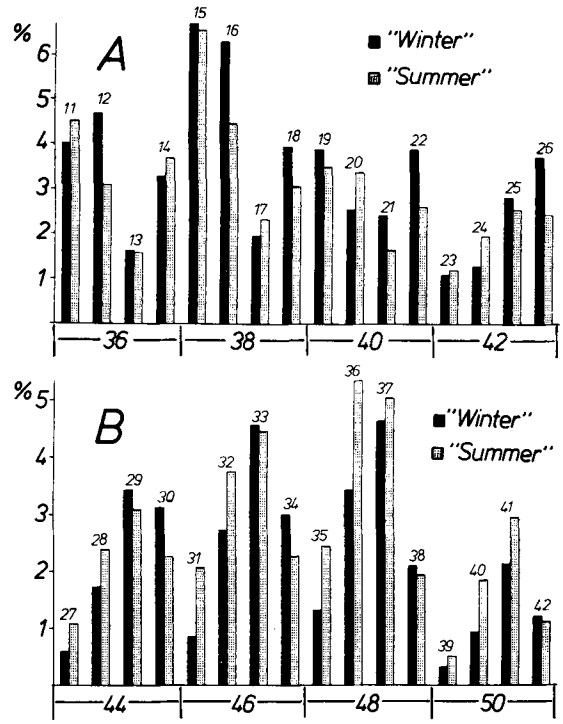

FIG. 4. Peak intensities of a winter and a summer milkfat (A) ECN 34.5-ECN 42, (B) ECN 42.5-ECN 50. The quartets have been designated by the ECNs of their last peak. The individual peak pairs are numbered as in Fig. 1.

When compared to cocoa butter (2) and olive oil (1,2), the mono-, di- and trisaturated TGs OOO, POO, POP, SOO, SOP and SOS were found to possess the same ECNs as milkfat TG peaks 35, 36, 37, 40, 41 and 45. From this it follows that oleic acid with 15.5 units contributes to the ECN of the triglyceride in which it is contained; this forms a contrast to 15 units when the mobile phase consists of propionitrile (5). Because of their high content of oleic acid and saturated fatty acids this is important to milkfats, because saturated and dioleo-monosaturated TGs now will no longer superimpose. E.g., the aforementioned pair SOO and PPP, which is difficult to separate by propionitrile, is far apart in the chromatogram of Figure 1 and includes a clearly separated third type of triglyceride (peak 39) as well.

As has been mentioned already the chromatogram of Figures 1 and 2 is typical for all milkfats despite their quite different compositions. Especially the division of the chromatogram into quartets always remains the same. Only the relative peak intensities show differences. This will be discussed in the next paragraph in more detail.

Semiquantitative analysis. Most peaks in the chromatogram of Figure 1 are not baseline-separated. In consequence it is not yet possible to give reliable area percentages of most individual peaks. If the evaluation is confined to a comparison of differently composed milkfats, or differently modified milkfats (e.g. gained by fractionation), an integration by drawing perpendiculars, as shown in Figure 1, will appear to be quite useful. All peak groups between ECN 34 and 52 are computed by the integrator as quartets. Of the peak group ECN 32 only three peaks are resolved, and of the groups ECN 30, 28 and 26 only one. The peak group ECN 24 is quite often so faint as to be lost in the injection peak. Peak group ECN 54 behaves correspondingly, though it exists as well, as will be shown. Figure 4 shows the area percentages of peaks 11 to 42 of a winter fat and a summer fat in the form of histograms. Several values amount to less than 1%. At an injection volume of 30 μg this signifies that 100 ng of fat can still be detected. Peaks with even ECNs 44, 46, 48 and 50 are larger for winter fat than for summer fat. All other peaks within this range (Fig. 4B) behave oppositely, with only two exceptions (29 and 33). In winter, milkfat contains more myristic and palmitic acid and less stearic and oleic acid and at the same time has higher melting temperatures than in summer. This gives another indication that most of the TGs, which form the last peaks of the quartets ECN 44 to ECN 50, are saturated (e.g. MPM, MPP, PPP and SPP); i.e. that their equivalent acyl-C-numbers are also their true acyl-C-numbers, whereas all other TG classes generally contain double bonds. In Figure 4A this tendency partly continues toward the lower ECNs, but at group 36 it comes to a complete stop. The reason for this might be that here two anatropous tendencies overlap, i.e. more oleic acid, but less short chain fatty acids, especially less butyric acid in summer.

Within the initial part of the chromatogram another criterion for distinguishing between winter and summer fat becomes evident (Fig. 5). The differences in intensity of the adjacent peaks I(4)-I(5), I(7)-I(8), I(11) I(12), and

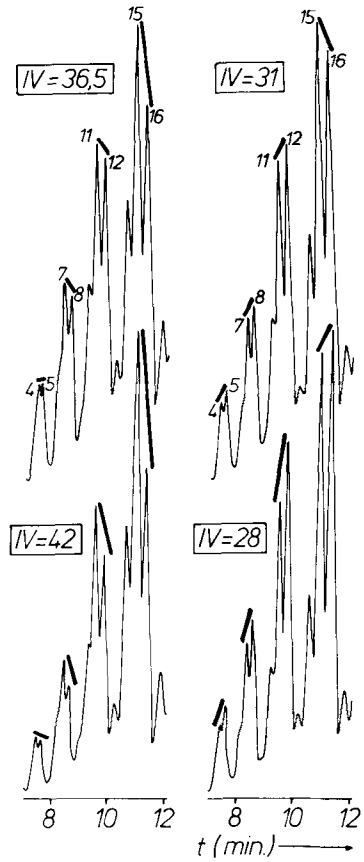

FIG. 5. Characteristic peak patterns of differently composed milkfats. IV = iodine value. The peak numbers are the same as in Figs. 1 and 2.

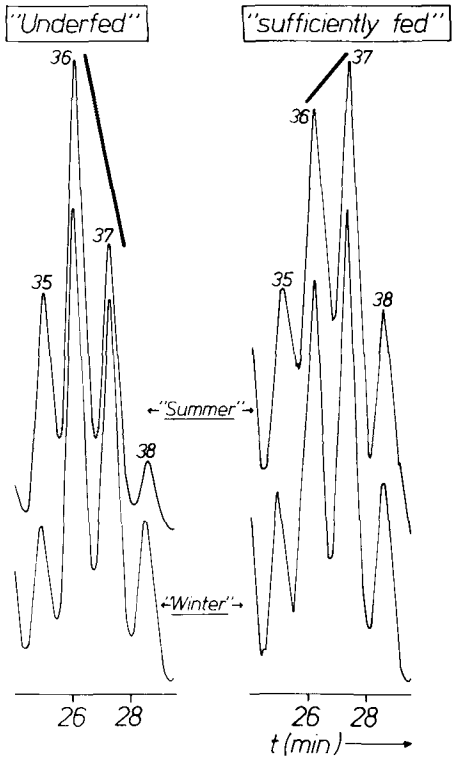

FIG. 6. Characteristic peak patterns of milkfats from normally fed and underfed cows. The peak numbers are the same as in Fig. 1.

I(15)-I(16) are greater for summer fat than for winter fat. The differences I(7)-I(8) and I(11)-I(12) are always positive for summer fat and negative for winter fat. If, in addition, the difference I(15)-I(16) is negative also, the fat in question will be a very hard winter fat with an iodine number below 28.5.

A further characteristic criterion for differently composed milkfats is shown in Figure 6. Normally the intensity of peak 36 is lower than that of peak 37 (Fig. 1). This holds true for winter fat as well as for summer fat. If the milkfat, however, originates from cows which are underfed with respect to their energy supply, peak 36 will dominate within its quartet (Fig. 6). This energy deficit may occur at the so-called 'top of lactation,' the lactation stage when the milk yield is at a maximum. This stage is characterized by the fact that especially oleic acid and palmitic acid pass from the depot fat of the cow via the liver into the mammary gland. Triglycerides which are composed of two chains of oleic acid and one chain of palmitic acid (e.g. POO) are then found in milkfat in higher concentrations (7). Peak 36 corresponds to ECN 47. This also is the ECN of the TG type POO ($16 + 2 \times 15.5$). From these physiological characteristics it can therefore be concluded that peak 36 is formed mainly by TGs of the type POO. Thus an insufficient energy supply may very quickly and simply be established by means of HPLC.

HPLC separations of fractionated milkfats. The HPLC method not only enables the analyst to distinguish between differently composed natural milkfats, but also between fractionated milkfats. Cooling melted milkfat down to certain temperatures leads to an enrichment or deconcentration of the different TGs within the solid and liquid fraction formed, depending on their different melting points. Figure 7 shows the chromatograms of some solid fractions, the so-called stearin fractions. The solubility of these fractions in the mobile phase is lower than that of unfractionated milkfat. Therefore, all the following separations were no longer carried out at 30 C, but at 35 C. In all quartets shown in Figure 7, down to ECN 42, the intensities of their respective last peaks increase with increasing fractionation temperature in such a way as to be noticeably more marked than those of all other peaks. This can be taken as another indication that they are saturated TGs. Apart from that a further quartet is observed among the higher-melting fractions: a group whose last peak corresponds to ECN 54 and thus obviously represents tristearin. Apart from a few recent separations on glass capillary columns

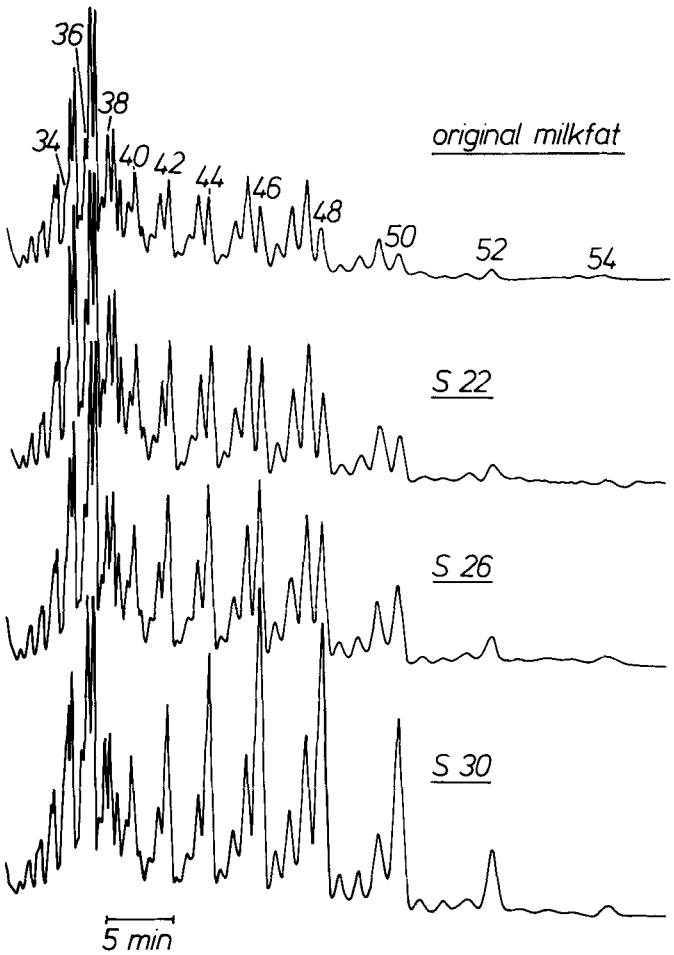

FIG. 7. Chromatograms of stearin fractions (ST) of a milkfat (T = temperature of fractionation). The peak numbers are the ECNs of the respective triglycerides. Column temperature, 35 C; sample quantity, 30 μg.

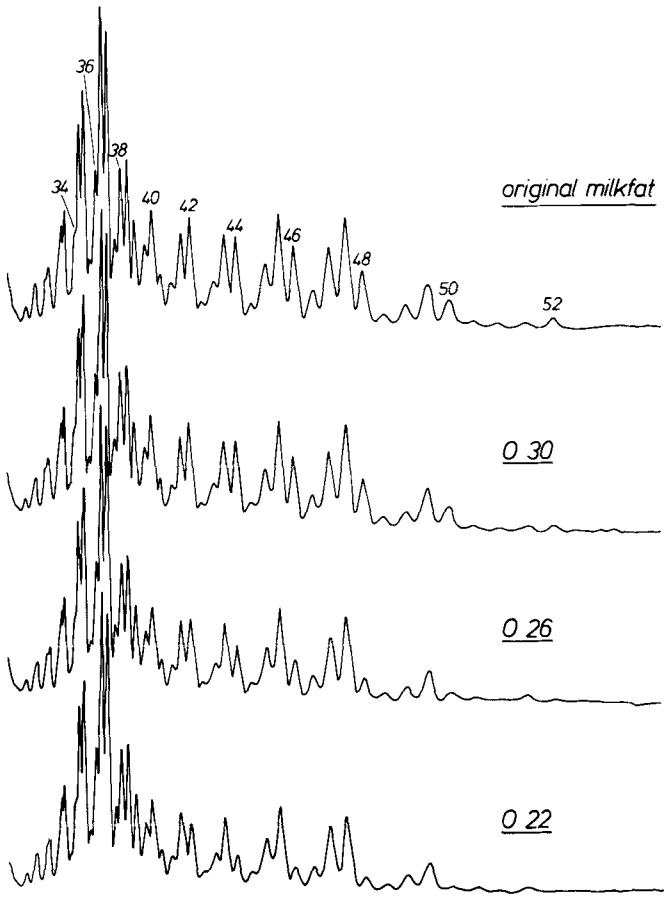

FIG. 8. Chromatograms of olein fractions (OT) of the milkfat of Fig. 7 (T = temperature of fractionation). The peak numbers are the ECNs of the respective triglycerides. Column temperature, 35 C; sample quantity, 30 μg.

(8-10), tristearin could be identified only by GLC of saturated TG fractions of milkfat previously isolated on an $AgNO_3$-impregnated thin layer plate (11). Due to its high melting point it has so far not been possible to identify tristearin in milkfat by means of HPLC. Due to partly incorrect indices of peaks, Deffense's (3) chromatogram of milkfat gives the impression of including C 54; in reality, however, the last peak of this chromatogram corresponds to C52 (retention time 110 min).

Also in the corresponding liquid fractions (Fig. 8), the so-called olein fractions, the content of long chain saturated fatty acid TGs increases with crystallization temperature, but to a lesser degree.

A semiquantitative evaluation of the area percentages reveals that in the stearin fractions the percentages of all TGs with even ECNs from ECN 40 upward are increased, whereas all other TGs with even ECNs up to ECN 38 are decreased in comparison with the fat from which they were derived. The opposite is valid for the corresponding olein fractions. It is possible to shift the turning point of this change—ECN 40 for all fractions of the series considered here—to higher ECNs according to a rising melting point of the stearin fraction, especially in the case of a rather limited melting range. The simplest way to obtain such fractions is to apply solvents during fractionation. Due to low viscosity combined with the higher interfacial tension of the liquid phases, purer crystals are formed, being less coated by liquid fat. Figure 9 shows the chromatograms of four milkfat fractions with different melting points which have been crystallized from acetone solution. The extremely low solubility of these fractions in the mobile phase was taken into account by further reducing the amount of injected fat to 10 μg. Almost all TGs below ECN 42, and between the long-chain saturated components, are lacking, as they have gone into the liquid fraction. Furthermore it can be seen that with increasing melting points of fractions, the peak intensities of the long-chain components increase, and those of the shorter-chained decrease. These chromatograms permit only the conclusion that the peaks with even ECNs, at least from 42 upward, represent saturated TGs such as tristearin, distearopalmitin (if some insignificant amounts of C20 and C22 fatty acids are left aside) and other mixed-acid TGs containing mainly stearic, palmitic, myristic and lauric acid. This finding was confirmed by gas chromatographic analysis of the fatty acids. Apart from the quoted fatty acids the GLC analysis showed amounts of C18:1 and C18:2 from 3.5-6% and other fatty acids (long-, short-, branched-chain and odd-numbered ones) from 5.5-7.3%.

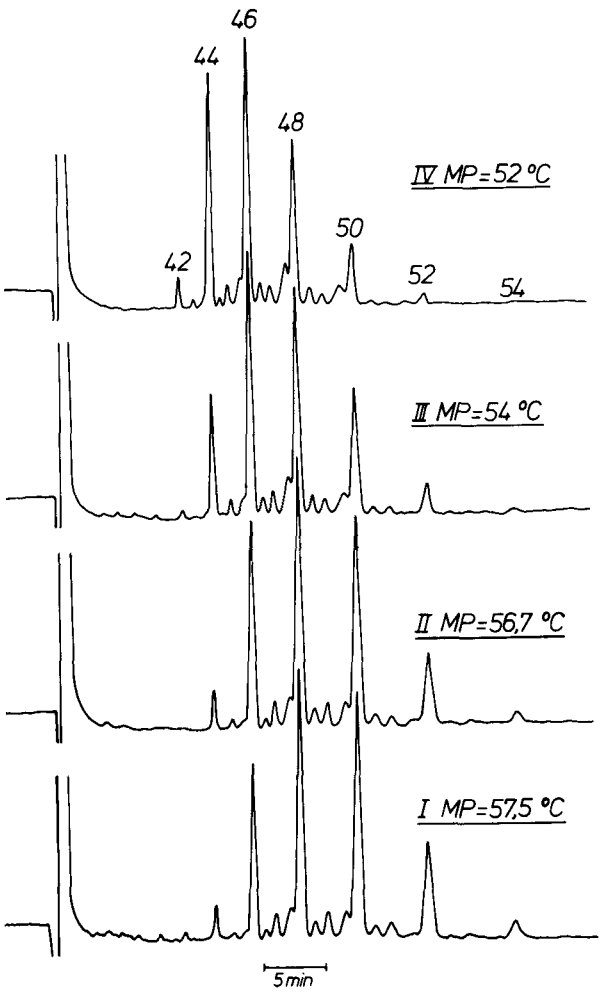

FIG. 9. Chromatograms of stearin fractions crystallized from acetone solutions. The peak numbers are the ECNs and simultaneously the CNs of the respective triglycerides (see text). Column temperature, 35 C; sample quantity, 10 μg.

Applications and outlook. The results discussed in this paper demonstrate clearly the possibilities of HPLC analysis as a supplement or even alternative to other separation techniques in analytical applications. In the context of this work, application is not limited to analysis of natural and fractionated milkfats but includes other milkfat modifications, obtained by interesterification, hydrogenation or admixture of other fats. The technological relevance is based on the fact that the individual TG types, which are distinguished by the length of their chains and their degree of saturation, are related to certain physical properties of the milkfat (melting temperatures, percentage of solid fat, or firmness). The relationship between these physical and constitutional properties merits further study to enable production of milkfat products with certain functional properties influenced by a controlled change of their TG composition.

Apart from the peaks with even ECNs, which could be attributed to classes of saturated TGs from ECN 42 upward, conclusive association of most other peaks with odd- or half-numbered ECNs to certain TG classes is not yet possible. It would be of special interest to elucidate the way in which the *cis*- and *trans*-isomeric fatty acids contained in milkfat are distributed among the individual TG types. The possibility of directly separating *cis*- and *trans*-isomeric fatty acid TGs by HPLC had been established by El Hamdy and Perkins (12). Unfortunately, however, the efficiency of any present HPLC system will not suffice to differentiate between monounsaturated *cis*- and *trans*-isomeric fatty acid TGs in milkfat. Analysis of the fatty acid methyl esters of the individual peak fractions by GLC will, therefore, be one of the next steps in continuation

of our work. Because of the small sample size (0.3 μg per peak or less) this will be possible only by a time-controlled accumulation of the peak fractions in repeated, exactly reproducible chromatographic runs, as realized in this paper.

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