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Triglyceride Analysis with Glass Capillary Gas Chromatography

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ABSTRACT

The analyses of triglycerides on capillary columns is reported. Applications in which this technique can be used include: rapid identification of fats and oils, measurement of butter fat or coconut oil content in margarine or chocolate, monitoring of processes such as fractionation, transesterification or heat treatment. Although separation of all isomers within a group of triglycerides with identical carbon numbers is not usually feasible by gas chromatography (GC) alone, the resolution obtained with capillary columns gives more information than that obtained with packed column GC. The conditions used in this work are described. Persilylated columns coated with nonpolar gum phases could be used for ca. 1 yr with hundreds of injections.

INTRODUCTION

Fats and oils are largely characterized by highly developed procedures to analyze the fatty acid methyl esters (FAME), (1). These relatively volatile compounds are amenable to gas chromatography (GC) analyses and give good fingerprint chromatograms for different fats and oils. Simultaneously, this GC method has been widely used to determine the *trans* isomers of 18:1 fatty acids produced by hydrogenation or prolonged heat-treatment of fats or oils, despite the fact that the *cis* and *trans* isomers cannot be satisfactorily separated even with capillary columns (2). HPLC on $AgNO_3$-coated silica gel gives better results for these separations (3).

The analysis of triglycerides complements the chromatography of FAME if it is used for identification purposes or to measure butterfat or coconut oil content. The method is extremely simple, since sample preparation consists of merely diluting the fat with a solvent.

The technical aspect of the GC analysis requires some attention. As soon as a suitable column and an appropriate injection technique is used, the method becomes routine, even though molecular weights of some triglycerides exceed 1000 daltons.

Triglyceride analysis on packed columns has been done for nearly 20 years (4,5) and is a rapid, routine method (6). On nonpolar stationary phases, it is possible to separate the triglycerides according to their total carbon number (not including the odd-numbered ones). An attempt has been made to separate triglycerides based on their degree of unsaturation (7). However, the Silar 10C-phase used for this purpose has to be operated at its temperature limit.

The use of capillaries provides the well known advantages of increased resolution and relatively short analysis time. Little has been published on this technique: Schomburg et al. (8) shows some chromatograms; Schulte reports some applications (9); and a recent paper (10) shows a number of capillary GC runs on very short columns and gives information on the column and chromatographic conditions in detail. Although separations on 4-6-m capillaries are much better than on packed columns, we believe that considerably more information could be obtained using 15-20-m columns.

Apolar stationary phases such as OV 1 and 101, SE 30 and 52 separate the triglyceride peaks according to the total number of carbon atoms. The composition of these groups is complex; (a) there are many isomers of the same molecular weight with different combinations of various fatty acids, e.g., 12-12-12, predominate in coconut oil (11), which is well separated from 4-14-18, predominate in butter fat (12) (Fig. 1); (b) triglycerides composed of the same fatty acids may exist as stereoisomers, differing in the position of the fatty acids on the glyceride moiety; (c) a considerable proportion of the fatty acids is unsaturated, giving a large number of possible isomers differing in the number of double bonds as well as their distribution in the triglyceride molecule.

With the separation technique used presently, it is impossible to separate more than a small number of these isomeric triglycerides within one run. Nevertheless our experiments have shown that characteristic peak patterns are obtained for most fats.

At this point, it might be asked why we prefer nonpolar phases over polar ones. Nonpolar phases have good selectivity for structural and stereoisomers as they are characteristic in butter fat e.g., But the separation of unsaturated species is less satisfactory: resolution may be quite high, but peaks of different identity are mixed, not grouped according to the number of double bonds as on polar phases. There is little chance to develop simple rules to interpret or predict the appearance of unsaturated trigly-

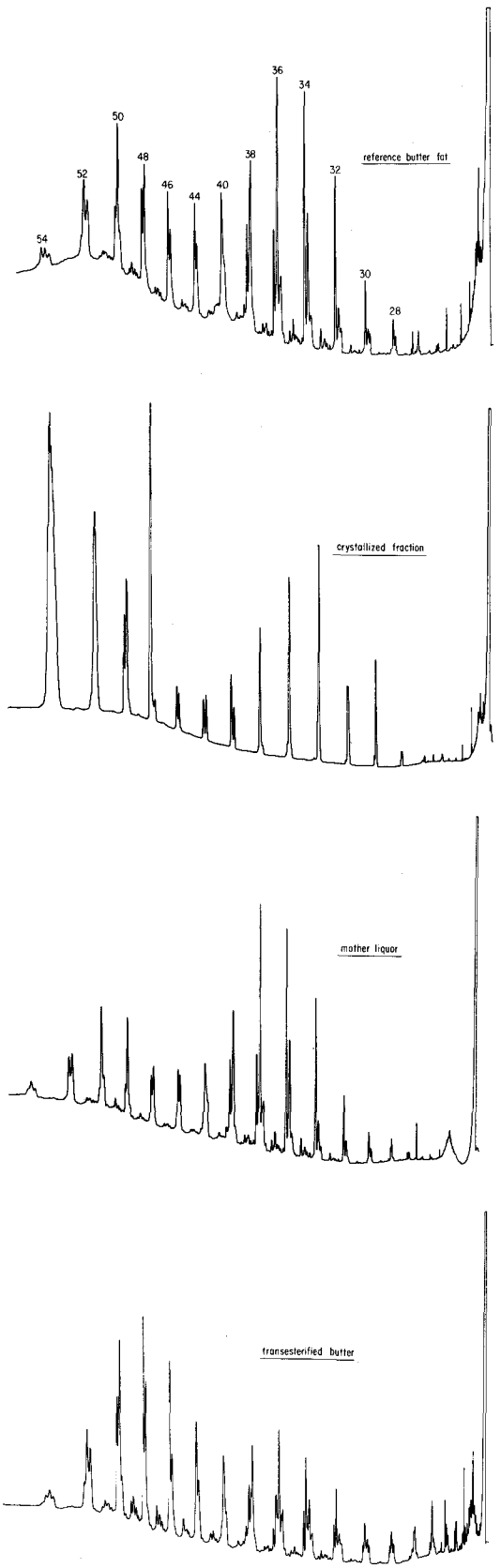

FIG. 1. Chromatograms of untreated butter, a crystallized fraction, the mother liquor of the same fractionation and the transesterified fat. Note the strong changes within each group of triglycerides whereas the molecular weight distribution did not change accordingly. Column: 14 m/0, 3 mm/0.08 μm OV-1, 0.8 at H_2 as carrier gas, temperature program 4 C/min (240-340 C). Cold on-column injection at 60 C (0.5 μl, hexane as solvent).

cerides in a chromatogram. Although differences among various plant oils and fats can be observed, they are difficult to characterize. The answer to the given question is pragmatic: we have not been able to produce polar columns with comparable lifetimes, similar efficiency and the same inertness as nonpolar columns.

PROCEDURE

Equipment: gas chromatograph: Model 4160, (Carlo Erba, Strumentazione, Italy) equipped with FID and 2 injectors, a vaporizing split/splitless and a cold on-column injector.

The fat was diluted 1:1000-1:10,000 in hexane. For butter fat or margarine it was not necessary to dry this solution even when it turned turbid from humidity. This sample (0.5-1 μl) was introduced by rapid movement of the plunger directly into the oven-thermostated column using the cold on-column injection technique. During the sampling the column was kept at 50-60 C to control the evaporation speed of the solvent. After 20-30 sec (after which most of the solvent is evaporated) the column was rapidly heated to a temperature between 240 and 300 C, immediately starting a temperature program of ca. 4 C/min to 340-370 C (temperatures depending on the kind of fat and the film thickness of the stationary phase).

Capillary columns (15 m x 0.30 mm) were used with persilanized support, coated with a film of 0.12 μm of OV 1. The inlet pressure of the carrier gas (hydrogen) was 0.7 atm. The FID fuel gases had to be adjusted to produce a minimum of sensitivity drift during the temperature program (13), i.e., 40 ml/min of hydrogen and 500 ml/min air for the instrument used.

RESULTS AND DISCUSSION

Application in Food Analysis

Measurement of butter fat and coconut oil content. According to the literature, the quantitative composition of some special fat mixtures, such as peanut/coconut oil have been determined on the basis of triglycerides (14). However, the method still is not used for several applications where it might be very useful: butter fat content in margarines or chocolate products have usually been determined using methylbutyrate as a marker; methyllaurate was used for coconut oil and palm kernel fat. The butyric acid content in triglycerides of milk of different origin and different seasons varies considerably, which requires the use of large tolerance limits. Our faster and more precise method quantitates typical triglyceride peaks: the fat is dissolved in hexane; a known amount of an internal standard, e.g., the alkane C_{40} (eluting between the triglyceride groups 34 and 36), is added (usually 3%/100% butter); and quantitation is carried out on the basis of peak heights calibrated for a number of peaks of pure butter fat. Within certain limits butter fat and coconut oil can be quantitated even when they are mixed. Figure 2 shows that a minimum of 10% coconut oil in butter or 20% butter in coconut oil is required for a reasonable determination. If they occur separately, much smaller fractions of the content can be quantitated easily.

Fractionated fats. Fractionation of a fat can be more sensitively monitored on the triglyceride basis than by the classical analysis of FAME, since it is a direct analysis of the species affected by such processes (e.g., in a crystallized fraction, where tripalmitin is strongly enriched). The FAME analysis of the methylpalmitate reflects this incorrectly, because other triglycerides, also containing palmitic acid, are not enriched at the same time. Figure 3 gives an example of a fractionated margarine; Figure 1 shows

a crystallized butter, which is used in the baking industry. In both cases, the distribution of the molecular weights of the triglycerides (as observed by packed column GC) is changed. For the butter fat an increase in the proportion of larger triglycerides is observed for the crystallized fraction. However, the stronger differences are only evident by improved resolution of triglycerides with the same number of carbon atoms: some peaks increase, others decrease as can be seen at the 44 and 46 peaks of the fractionated margarine fat in Figure 3. The molecular weight distribution would not reflect these changes at all. The chromatograms show that the late peaks of each group beyond triglyceride 40 are enriched in the crystallized fraction, reflecting the high tendency of the saturated molecules to solidify. Further identification of different peaks would

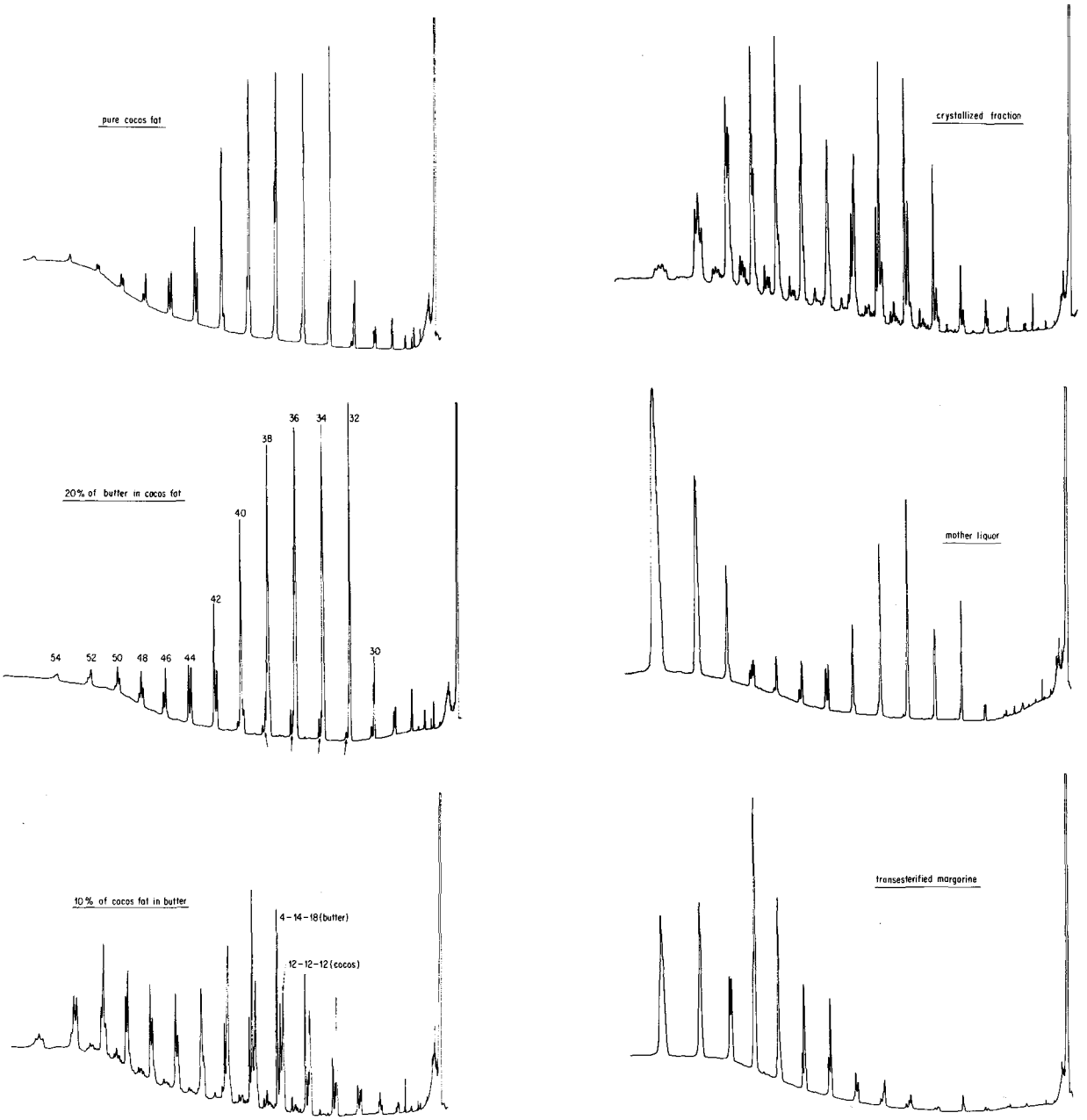

FIG. 2. Determination of butter fat/coconut oil mixtures by their triglycerides; limitations toward extreme compositions. Coconut oil (10%) can be easily determined in butter, but 20% of butter in coconut oil causes problems. A convenient quantitation can be achieved using an internal standard. For column and conditions, see Fig. 1.

FIG. 3. Chromatogram of a commercial margarine, containing hydrogenated soybean oil and coconut oil. This margarine was fractionated and transesterified. Later peaks of each triglyceride group (probably representing the more saturated molecules) are concentrated into the crystalline fraction. Column and conditions as in Fig. 1.

probably facilitate the monitoring of fractionation processes.

Transesterification. Transesterification can be monitored by packed column GC, as shown by, e.g., Eckert (6). However, increased resolution as obtained with capillary columns gives more detailed information of the changes during such reactions. The butter and the margarine shown in Figures 1 and 3 may serve as examples. While the molecular weight distribution is strongly changed in the margarine, corresponding shifts are moderate only for the butter fat. Moreover a closer look at the fine structure within each group reveals dramatic changes (see groups 32-40 of the butter fat in Fig. 1). Again, much identification would be necessary to fully exploit the available information.

Heat-treated fats. Polyunsaturated fatty acids tend to polymerize when fats or oils are submitted to strong heat treatment. This process can be quantitated by the reduction of the fatty acid content using FAME analyses. The triglyceride analysis yields a complementary result as it reflects changes in the whole triglyceride molecule. Apart from the absolute reduction of triglyceride concentration there is usually a distinct change in their composition. Figure 4 shows the triglyceride chromatograms of a frying oil based on beef tallow, before and after heating. The late peaks of each group are increased because these largely saturated triglycerides show little tendency to polymerize.

Remarks on the Technique

Columns for triglycerides. To be suitable for triglyceride analysis, capillary columns should fulfill 2 requirements: (a) they have to be thermostable up to at least 330 C. (350 C is preferable). This includes a support surface with sufficient inertness to avoid catalytic degradation of the stationary phase (causing high column bleeding and short column lives). Furthermore, the deactivation of the support has to be sufficiently thermostable to avoid tailing of the triglyceride peaks after extended use of the column.

(b) At temperatures above 300 C triglycerides are fairly labile compounds. While FAME at 150 C are only saponified on surfaces which completely absorb weak acids (as the test compound 2,6-dimethylphenol [15]), triglycerides decompose at 330 C on columns which elute weak acids almost perfectly. Satisfactory results were not obtained from those columns with basic bare glass surfaces beneath the stationary phase nor from columns prepared according to the "barium carbonate" method (16). In agreement with Schulte (9), "barium carbonate" columns had relatively short lifetimes and some of them even partially degraded triglycerides. However, persilanized columns (17,18) gave good results, even if programmed up to 360 C. These columns lasted for many runs without a notable change in the test chromatogram (checking adsorption, separation efficiency and retention according to Grob et al. [15]). Because of the low catalytic activity, a low bleed rate of such columns is achieved and degradation of triglycerides did not cause problems. As Grob describes (19), there was some discrimination of the biggest triglycerides, with losses ranging from 3 to 15%. This was assumed to result from degradation of these compounds at high temperature. However, this only brought the response down near 1 if normalized on the lower boiling triglycerides as tri-10 (instead of being ca. 9% higher for the tri-18 compared to tri-10).

Regarding selectivity for triglycerides, there was little difference between the gum phases such as OV 1, SE 30, 52 or 54. The unsubstituted methylpolysiloxanes seemed to be slightly preferable because of a somewhat lower elution temperature at comparable conditions (ca. 6 C lower for OV 1 compared to SE 52). Furthermore, unsaturated compounds are more clearly eluting before the saturated analog on the apolar phases. Film thickness varied between 0.08 and 0.12 μm as a compromise between a sufficient capacity (overriding baseline drift and ghost peaks) and low retention. The low retention reduces the thermal stress on the column and degradation of triglycerides.

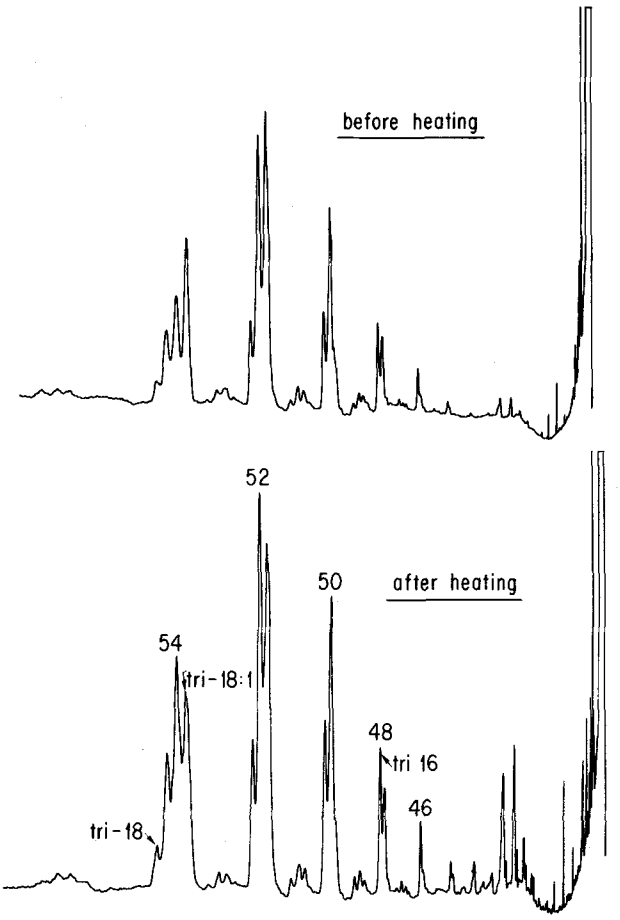

FIG. 4. Frying oils on the basis of beef tallow before and after heat-treatment. Positions of tri-16, tri-18 and tri-18:1 are marked. Besides a reduction of the total of original triglycerides, there is a relative change in peak size within each group of peaks, increasing the proportion of the saturated (later) peaks of a group at the cost of the unsaturated species (earlier peaks).

Injection technique. The results of our studies comparing precision and accuracy of different injections for triglycerides (split, splitless and cold on-column) have been published elsewhere (19). They can be summarized as follows: split injections produced results with strong discrimination and very high standard deviations. Splitless injections gave results reflecting a discrimination resulting from insufficient elution out of the syringe needle. Since the different triglycerides eluted in similar proportions, discrimination seemed to be tolerable for many applications as long as a triglyceride was chosen as an internal standard. However, standard deviations were still on the order of 10%. Cold on-column injection had little discrimination and standard deviations of normalized results on an internal standard were 1-3%. Optimal conditions for cold on-column injections are described in (20). According to Monseigny et al. (10), the moving needle (or solid) injection is a viable alternative with little discrimination, and standard deviation is reported to be between 2 and 4%.

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The Determination of Cocoa Butter Equivalents in Chocolate

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ABSTRACT

A method of determining cocoa butter equivalents in chocolate and cocoa butter is described. The method relies on a new approach for interpreting data obtained by triglyceride gas liquid chromatography (GLC). This technique provides information on the composition of a fat according to the carbon number of the triglycerides (C_n). Examination of the data for a wide range of cocoa butters shows that a straight line relationship between the C_{50} and C_{54} contents exists. This relationship has been used as the basis for a quantitative method determining the amount and type of cocoa butter equivalent added to chocolate. The application of the method to both plain and milk chocolate is described. The method is also used to determine the amount of milk fat in chocolate.

INTRODUCTION

Throughout the world there is an increasing interest in the use of fats to partially replace cocoa butter in chocolate. The reasons, partly economic, are that certain fats can also provide significant product improvement (1). In the United Kingdom, Denmark and Ireland the addition of 5% non-cocoa butter fats, apart from milk fat, has been permitted for many years.

The type of fats that have been used in chocolate are very similar in chemical composition to cocoa butter. These fats mainly contain symmetrical triglycerides and for this reason it has been impossible to determine the amount of symmetrical-based fats in mixtures with cocoa butter.

Although there has been no method of monitoring the level to which added fats are used, the manufacturers have mutually agreed to abide by this 5% restriction. However it is desirable that some analytical method be made available which would quantitatively determine the amount of added fat in chocolate. The objective of the work described in this paper was to develop a method which would detect and quantify symmetrical triglyceride cocoa butter equivalents (CBE) when used at the 5% level in chocolate. Ideally, the method should also be quantitative at even lower levels of addition. The method should be suitable for both milk and plain chocolate, as well as rapid and easy enough for routine use.

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The method is required to detect the high quality symmetrical-type CBE which have physical and chemical properties similar to those of cocoa butter. These CBE include Coberine, Illexao, illipe, shea fractions and palm fractions. Ideally, the method should also detect hardened fats but other methods are presently available for their characterization.

The determination of added fats has been the subject of much research and the position was reviewed in 1959 (2). At that time, modern chromatographic methods were being applied to oils and fats for the first time and this earlier work is of little interest today. Chromatographic methods for the general characterization of mixtures of oils and fats have been reviewed by Mani (3) and more recently for confectionery fats by Fincke (4).

Early attempts to detect added fats depended on the determination of the fatty acid composition of cocoa butter and, in particular, the content of lauric acid (5). Bonar (6) was able to detect 5% Coberine in cocoa butter using a preliminary low temperature crystallization as developed by Purr and Hettich (7,8). Methods dependent on lauric acid are unsuitable when milk fat is present. Iverson and Harrill (9,10) isolated the minor acids in cocoa butter, shea and illipe by urea fractionation. Although there was a suggestion that the method could be used to detect oils in cocoa butter, most operators would find the method difficult. The use of fatty acid analysis as a means of detecting added fats is therefore severely limited because some of them have a fatty acid composition similar to cocoa butter. Large additions of, e.g., palm fraction must be added before this method will detect it with certainty.

Sterol analysis is often used for identifying specific oils and fats. Fincke (11) has reported sterol analyses for several samples of illipe, Calvetta, Coberine and cocoa butter. Bracco et al. (12) have used sterol analyses to help characterize illipe/cocoa butter mixtures. Sterol analyses do have value in specific instances, e.g., detection of shea nut oil and its fractions or rapeseed oil. They are limited for detecting replacer fats because of their inability to detect palm fractions, major components of many products.

Sterol analysis is further limited because a preliminary step to isolate sterols is necessary and this is tedious. Quantitation is also virtually impossible because of the wide