Composition of molecular distillates of butter oil: isolation and identification of components other than glycerides

M. J. McCARTHY, A. KUKSIS, and J. M. R. BEVERIDGE

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada

SUMMARY Molecular distillates of butter oil consisting of the four most volatile 2.5% cuts (obtained by a redistillation of the first 10% cut), the next most volatile 40% cut, and the 50% residue obtained from 777 pounds of oil were subjected to fractionation by adsorption and gas–liquid chromatography.

Only the most volatile 2.5% cut, which contained 10% unsaponifiable matter, was examined in detail. It consisted of 9% sterol, 74% triglyceride, 7% diglyceride, 4% monoglyceride, 5% free fatty acid, and a small amount of cholesterol esters and hydrocarbons. Of the unsaponifiable material, which represented 56% of the total unsaponifiable lipid present in the original oil, about 89% was sterol, 6.3% hydrocarbon, and 1.2% carbonyl material, the rest being made up of unidentified compounds of varying polarity. Ninety-five per cent of the total sterol was free cholesterol. The hydrocarbon fraction was a complex mixture of the C_{17} to C_{48} odd and even carbon number hydrocarbons of the normal, iso, and 1-cyclohexyl (?) series. Although a number of these hydrocarbons were unsaturated, only squalene could be positively identified.

The distribution of the fatty acids in the various lipid classes was markedly different from that of the original butter oil. The fatty acids of the cholesterol esters were particularly rich in the minor acids of butter fat. The small carbonyl fraction was not fully examined.

THE COMPOSITION of milk fat or butter fat has been the subject of numerous investigations, and several components of this fat have received special attention. Thus, the fatty acids (1, 2), triglycerides (3, 4), and such ingredients of the unsaponifiable matter as sterols (5) and the steam-volatile carbonyl compounds (6) have been studied in detail. Since none of the materials reported to occur in butter appeared to account fully for the hypercholesterolemic activity of the more volatile fractions of butter oil (7), it was of interest to examine the chemical composition of these distillates, and to determine the nature of the materials that become selectively concentrated in the early cuts.

Previous publications (4, 8) from this laboratory have already detailed the triglyceride composition of the molecular distillates of butter oil. It remains for the present report, which has appeared elsewhere in preliminary form (9), to account for the nonglyceride components.

MATERIALS AND METHODS

The molecular distillates of butter oil were prepared by Distillation Products Industries, Rochester, N.Y., and have been described (8). The closed vessels were heated in hot water to liquefy the oils before any samples were removed.

Reagents and Solvents

All reagents and solvents were reagent grade chemicals and were used without further purification.

The reference cholesterol sample was a high purity material supplied by American Cholesterol Products, Inc., Edison, N.J. The lanosterol was obtained from Mann Research Laboratories, New York 6, N.Y. The Δ^7-cholesterol was a gift from Dr. W. W. Wells. All these materials gave correct physical constants without further purification. The methyl esters of the saturated C_6–C_{18} straight-chain and even-numbered fatty acids, and methyl oleate, were purchased from Applied Science Laboratories, State College, Pa.

Elementary Analyses

Phosphorus was determined by the method of Harris and Popat (10) and sulfur by the sodium fusion method (11). The nitrogen was estimated essentially as described

TABLE 1 DISTRIBUTION OF UNSAPONIFIABLE MATERIAL DURING MOLECULAR DISTILLATION OF BUTTER OIL

Fraction	% Original Oil	Total Unsaponifiable Matter* % Distillate	% Original Oil	% Original Unsaponifiable	Sterols† as % Distillate Total	Free	Bound	Tocopherols‡ % Distillate	Vitamin A‡ % Distillate
				Original Distillation					
Original oil	100	0.37	0.37	100	0.25				
1st cut (D-1)	10	2.53	0.25	68.8	2.38	2.27	0.11	Tr.	Tr.
2nd cut (D-2)	40	0.2	0.08	20.9	0.05	0.04	0.01		
Residue (D-3)	50	0.08	0.04	10.3	0.01	0.01	0.00		
				Redistillation of First Cut (D-1)					
1st cut (D-1)	10	2.53	0.25	68.8	2.38	2.27	0.11	Tr.	Tr.
R-1	2.5	8.28	0.21	56.3	8.1	7.69	0.38	Tr.	Tr.
R-2	2.5	1.49	0.04	10.0	1.37	1.28	0.1		
R-3	2.5	0.25	0.01	1.6	0.07	0.09	0.0		
R-4	2.5	0.11	0.0	0.8	0.0	0.01	0.01		

* Gravimetric estimation.
† Estimated by Liebermann-Burchard reaction.
‡ Spectrophotometric estimation.

by Lang (12). Melting points were taken in sealed capillaries in a Hershberg circulating oil bath melting point apparatus.

The determinations of the free and total "fast" and "slow" acting sterols were based on digitonide precipitations and were performed as described by Cook (13). Glycerol was determined by the method of Hanahan and Olley (14) and methyl ketones by the methods described by Patton and Tharp (6).

Spectrophotometric Analyses

Ultraviolet and visible spectra were recorded on a Beckman DK-2 ratio recording spectrophotometer in ethanol solution. Infrared spectra were determined on a Perkin-Elmer Infracord spectrophotometer equipped with sodium chloride optics, using carbon disulfide as the solvent. Optical rotations were taken on 2% chloroform solutions in a Hilger Model 412 polarimeter. The Liebermann-Burchard (LB) chromogens were measured in a Beckman Model B spectrophotometer at 620 mμ after 90 sec (for fast acting sterols) and after 30 min (for slow acting sterols).

Methods of Lipid Segregation

The acidic and neutral lipids were separated by extracting an ethereal solution of the distillate with 0.05 N K_2CO_3. The unsaponifiable matter was prepared as suggested by the Association of Official Agricultural Chemists (15).

Chromatographic Methods

The techniques of adsorption chromatography of sterols and other lipids were similar to those previously described (16). The fatty acids (8), sterols (17), and hydrocarbons (18) were determined by gas–liquid chromatography (GLC).

RESULTS AND DISCUSSION

Elementary Analyses

From an academic point of view and for various practical reasons indicated below it was of interest to examine the molecular distillates of this complex natural product for their content of nitrogen, phosphorus and sulfur.

Butter contains about 1% protein, most of which is removed by heat coagulation and centrifugation during the preparation of butter oil. Any protein not removed in this manner could be expected either to remain in the distillation residue or to be decomposed and possibly distilled into the more volatile distillates in the form of small nitrogen-containing fragments. The low values obtained for nitrogen (100–200 ppm) on all the distillates and the residue indicate that the starting material (butter oil) was essentially free of nitrogenous matter.

The phospholipid concentration of butter oil has been reported to be 0.011% (19), which would correspond to a phosphorus concentration of 4.4 ppm. This material, unless decomposed or otherwise lost during the preparation of the oil or molecular distillation, would be expected to accumulate in the distillation residue. The phosphorus values found were extremely low (15–27 ppm) for all distillates and the residue, and are near the practical lower limit of determination with the common analytical methods.

All distillates gave negative tests when checked with either lead acetate or sodium nitroprusside reagents, indicating that sulfur was not present in concentrations greater than 10 ppm. Sulfhydryl compounds, such as goitrin (5-vinyl-2-thio-oxazalidone), which show strong antithyroidal and hypercholesterolemic activity, have been detected in the milk from cows fed *Brassica oleracea mollerii* or *Cruciferus* weeds (20). In view of the above

sulfur analyses it would appear that these "brassica factors" or compounds of this class are not responsible for the hypercholesterolemic effect of the butter oil distillates (7).

Distribution of Unsaponifiable Matter

The distribution of the total unsaponifiable lipid during the original molecular distillation and redistillation was determined gravimetrically following saponification. The averages of duplicate determinations are given in Table 1. Since a sample of the original oil was not available, its unsaponifiable lipid content was estimated by multiplying the determined percentage of unsaponifiable matter in each distillate by the respective weight percentage of each distillate and adding the products. The estimated value of 0.37% is somewhat lower than the 0.65% reported in the literature (5). In the original distillation 68.8% of the total unsaponifiable matter distilled into the most volatile 10% fraction (D-1), which was subsequently redistilled into 4 fractions of equal weight: R-1, R-2, R-3, and R-4 in order of decreasing volatility. In the redistillation 82% of the unsaponifiable lipids of fraction D-1 was distilled into distillate R-1.

Table 1 gives also the distribution of free and bound LB reactive material. About 95% of the total sterol was free, and there was no significant concentration of the bound sterol in any of the fractions. Some 89% of the unsaponifiable matter of distillate R-1 was sterol, which indicated that the sterol content of the unsaponifiable matter of the original oil had been about 61%. The latter value is in reasonable agreement with the 65% estimate for cholesterol reported by Morice (5). About 2% of the total distilled LB positive material was "fast" acting, and essentially all of it was found in the free form in the most volatile distillate. Gas chromatography, however, gave no indication of the presence of any Δ^7-sterols in this fraction.

A spectrophotometric examination of 2% oil solutions in chloroform showed optical densities in the range of 0.110 to 0.210 at 295 and 325 mμ and indicated that the concentrations of vitamin A and tocopherol were negligible for all distillates.

Distribution of Acidic Lipids

During the initial characterization of the various distillates it was noted that the R-1 distillate contained large amounts of acidic material. Removal of these acids by extracting with a mild alkali provided an easy access to them and considerably simplified the subsequent adsorption chromatographic fractionation of the neutral lipid. All the free acidity was associated with free fatty acids.

Table 2 gives the distribution of this material among the distillates and indicates the individual fatty acid concentration found in the most volatile fraction. When the estimated total amount was expressed as acid degree, the value (0.63) obtained for the original oil was well within the range (0.1 to 0.9) described for the free fatty acid content of freshly churned butterfat (21). These acids, therefore, may be assumed to have originated in the milk fat, and are not a result of triglyceride degradation during the molecular distillation.

A comparison of the composition of the free fatty acids with that of the total acids reveals that the free acid fraction contains a greater proportion of the longer chain acids. Thus, the C_{18} acids, which in normal butter fat comprise about 40% of the total, make up 59% of the free fatty acid portion.

Adsorption Chromatography of Unsaponifiable Matter

In order to isolate and identify all the components of the unsaponifiable lipids, the material obtained from the most volatile distillate (R-1) was subjected to repeated adsorption chromatographic fractionation on silicic acid. The approximate composition of the fractions is shown in Table 3.

The major component of the unsaponifiable matter was sterol, accounting for about 89% of the total material. By rechromatography on silicic acid and crystallization from methanol over 90% of this fraction was obtained in crystalline state [mp 149.5–151°; $(\alpha)_D$ −39°]. The infrared spectrum and the GLC behavior of this material were identical with that observed for an authentic sample of cholesterol. The LB positive material from the 5% ether in petroleum eluate on crystallization from methanol yielded 5–8 mg of yellow-brown crystals.

TABLE 2 FREE FATTY ACID CONTENT OF BUTTER OIL DISTILLATES

Distillate	Acid Degree*	% Acidic Material as Palmitic Acid	Fatty Acid Composition† Weight %	
R-1	20.6	5.28	Capric	1.4
			Lauric	3.4
			Myristic	10.0
			Myristoleic	2.2
			Palmitic	15.8
			Palmitoleic	8.3
			Stearic	10.8
			Oleic	48.1
R-2	0.47	0.12		
R-3	0.39	0.10		
R-4	0.16	0.04		
D-2	0.23	0.06		
D-3	None	None		

* Acid degree is equal to the number of milliliters of 1 N alkali required to titrate 100 g of fat to the phenolphthalein end point.
† Analyses made by GLC as indicated in the text.

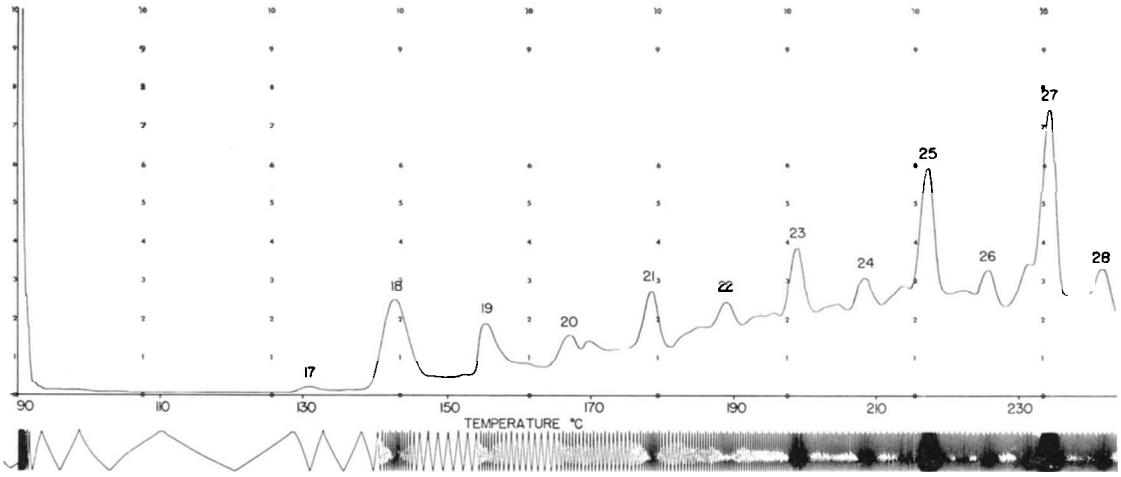

FIG. 1. **Gas–liquid chromatogram obtained** with the butter oil hydrocarbons. Chromatography conditions and peak identification are described elsewhere (18). (Fig. 1 continued on opposite page)

With the LB reagent this purified material gave an intense greenish-yellow, slightly fluorescent color. The infrared spectrum and GLC of this material, however, failed to confirm its identity with lanosterol. These results confirm previous studies (5) on the total unsaponifiable matter of butter fat, where the major component (65%) was also found to be cholesterol. It should be noted that the unsaponifiable matter as prepared under the conditions suggested by the Association of the Official Agricultural Chemists (15) contained some 20% of free fatty acid.

The failure to concentrate significant amounts of ketonic material into the molecular distillates of butter oil supports the belief that fresh milk fat is essentially free from such materials. The traces of methyl ketones found in the saponification products and certain steam distillates apparently owe their presence to the heat treatment (6).

The material eluted with petroleum ether alone was shown by GLC (18) to consist of at least 42 different hydrocarbons. Figure 1 illustrates a qualitative gas chromatographic run obtained with these materials and indicates the identity of the major components by their carbon numbers. Represented apparently were all the C_{17} to C_{48} odd and even carbon number hydrocarbons of the normal, iso, and 1-cyclo-hexyl (?) series. Furthermore, a number of the peaks showed shoulders indicative of the presence of unsaturated components. On the basis of gas chromatographic behavior alone, however, only squalene could be identified with any degree of certainty. A comparison of the GLC patterns obtained with the hydrocarbon fractions from the

TABLE 3 COMPOSITION OF FRACTIONS OBTAINED BY SILICIC ACID CHROMATOGRAPHY OF THE UNSAPONIFIABLE MATTER FROM R-1*

Fractions in Order of Elution	Eluting solvent	Volume of Eluting Solvent	Compounds	Fraction Weight	% Original Material	Remarks
		liters		*mg*		
I	Petroleum ether	2.9	Hydrocarbons†	118	4.3	LB negative; greasy, light yellow solid; mp 28–32°
II	1% ethyl ether in petroleum ether	1.6	Ketones‡	27	1.1	Carr-Price negative; yellow oil, LB negative
III	5% ethyl ether in petroleum ether	2.5	Fatty acids	470	20.0	White, waxy solid; mp 29–31, 23–27, 38.5–42.5°; LB negative; plus heavy yellow oil, LB negative
IV	10% ethyl ether in petroleum ether	2.9	Sterols	1,637	69.5	Crystalline solid; m.p. 133–157°, $(\alpha)_D$ − 39°; LB positive
V	25% ethyl ether in petroleum ether	2.0	Unknown	34	1.4	Yellow oil; LB negative
VI	Absolute ethyl ether	1.4	Unknown	47	2.0	Orange oil; LB negative
VII	Methanol	2.0	Unknown	76	3.1	Dark brown, amorphous solid; LB negative

* 2.35 g of lipid was applied to 140 g of silicic acid. The total recovery was 2.41 g (102%).
† This material was shown by GLC to contain at least 42 different hydrocarbon components (18).
‡ Intense absorption in the 5.70–5.85μ region in the infrared due to the presence of a carbonyl function.

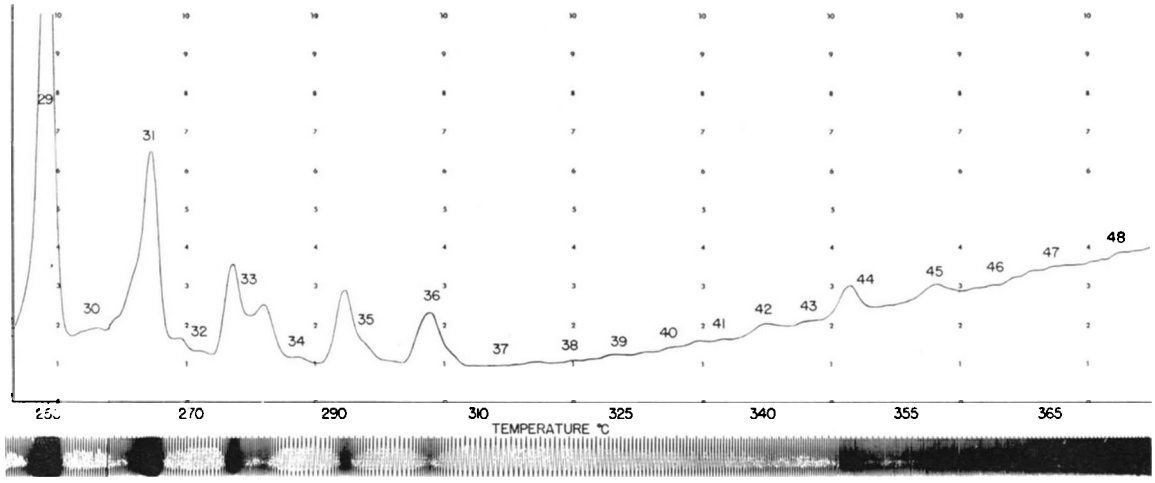

Fig. 1 (Concluded)

unsaponifiable material and the neutral lipid showed that considerable amounts of squalene had been destroyed during the saponification, as had been other unsaturated materials. Squalene made up 42% of the hydrocarbon mixture of the neutral lipid, but only 16.3% of the hydrocarbon mixture of the unsaponifiable matter.

The more polar components of the unsaponifiable matter that were eluted after the sterols were not examined beyond the extent indicated in Table 3.

Adsorption Chromatography of Neutral Lipid

The small amounts of the esterified sterol indicated by the preliminary analyses and the absence of detectable amounts of other alcohols in the unsaponifiable matter precluded the presence of significant quantities of nonglyceride esters in this distillate. The main purpose of adsorption chromatography was, therefore, the separation of triglycerides from any mono- and diglycerides that might be present. The solvents used and their volumes, together with the approximate composition of the major fractions, are recorded in Table 4. The major portion (73.8%) of the neutral lipid of this distillate (R-1) consisted of triglycerides, and there was little segregation of this material on the silicic acid. These triglycerides have since been extensively analyzed by GLC in both the glyceride and the fatty acid methyl ester form. The results have been presented elsewhere

TABLE 4 Composition of Fractions Obtained on Silicic Acid Chromatography of the Neutral Lipids from R-1*

Fractions in Order of Elution	Eluting Solvent	Volume of Eluting Solvent	Compounds	Fraction Weight	% Original Material	Remarks
		liters		*mg*		
I	Petroleum ether	10	Hydrocarbons†	234	1.2	LB negative; greasy, light yellow solid; mp 29–34°
II	1% ethyl ether in petroleum ether	9.8	Sterol esters Ketones	317	1.6	Sterol esters LB positive; oil, ketones LB negative
III	5% ethyl ether in petroleum ether	13.9	Triglycerides‡	14,764	73.8	Oil, Sap. No. 268; molar FA/glycerol, 3.1
IV	10% ethyl ether in petroleum ether	5.1	Sterols	2,350	11.7	LB positive solid; mp 135–150°, $(\alpha)_D$ −39°
V	25% ethyl ether in petroleum ether	4.5	Diglycerides	1,371	6.9	Yellow oil; LB negative; Sap. No. 223; molar FA/glycerol, 2.1
VI	Absolute ethyl ether	4.5	Monoglycerides	784	3.9	Yellow oil to white solid; LB negative; molar FA/glycerol, 1.02
VII	10% methyl alcohol in ethyl ether	4.0	Unknown	83	0.4	Dark amorphous solid; LB negative
VIII	Methanol	2.0	Unknown	104	0.5	Dark amorphous solid; LB negative

* 20.0 g of lipid was applied to 573 g of silicic acid. The total recovery was 20.0 g (100%).

†This material was shown (18) to be a mixture of at least 42 components by gas–liquid chromatography.

‡ The triglyceride fraction from R-1 has been extensively analyzed and its fatty acid and triglyceride composition described elsewhere (4, 8).

(4, 8) together with similar studies on the triglyceride fractions from other butter oil distillates and the triglycerides of whole butter fat. For the present purposes it is sufficient to note that the triglyceride fraction from R-1 contained a high proportion of the shorter chain fatty acid triglycerides, some of which had been enriched up to 25 times during the distillation.

The amount of lipid eluted with 10% ether in petroleum (Table 4) is high because of contamination with the more polar triglyceride fractions. Rechromatography of this material gave a sterol proportion comparable to that estimated following saponification. No sterols other than cholesterol could be detected by GLC in either the crude or the refined sterol preparations.

In addition to triglycerides, the R-1 distillate contained about 7% di- and 4% monoglycerides. This was demonstrated by chemical and gas–liquid chromatographic examination of the neutral lipid components recovered from the silicic acid column with 25% ether in petroleum and absolute ether, respectively. The pooled eluates of the respective fractions showed characteristic saponification numbers and correct glycerol/fatty acid ratios. The fatty acid compositions of the three glyceride fractions are given in Table 5. The acid compositions of a reference butter fat sample and the sterol ester fraction from R-1 are included for comparison. In all the glyceride fractions the concentration of the fatty acids of medium chain length (C_{10}, C_{12}, and C_{14}) is increased while that of the C_{18} acids is markedly decreased when compared to the acid composition of the original butter fat. Thus, in case of the monoglycerides, the C_{18} acids account for approximately 22% of the total, which is only about half the concentration in the whole fat. The origin of these mono- and diglycerides is uncertain. Jensen and Morgan (22) were able to detect 0.3 to 1.8 mmoles of monoglyceride per 100 g of fat in milk after storage in a refrigerator, but were unable to find any monoglyceride in normal fresh pasteurized milk. They attributed the formation of monoglycerides during storage to milk lipase. If all of the monoglyceride had distilled into the most volatile fraction, the original butter oil would have contained 0.1% monoglyceride, which would correspond to about 0.3 mmole per 100 g of butter oil, assuming monopalmitin to be the median monoglyceride.

TABLE 5 Composition of the Fatty Acids Obtained from the Glyceride and Sterol Ester Fractions of R-1*

Fatty Acid†	Weight %				
	MG	DG	TG	SE	Original Butter Oil‡
Butyric	2.9	5.3	3.2		2.0
Caproic	1.9	3.5	4.7	0.5	2.2
Caprylic	2.2	4.8	5.3	0.4	1.4
Capric	5.5	9.0	11.1	0.5	3.3
Decanoic	0.6	0.7	0.7	0.5	0.3
Lauric	6.2	8.5	10.7	0.5	3.5
Myristic	19.0	22.8	23.4	1.4	12.9
Myristoleic	1.4	1.5	0.9	1.3	2.0
Pentadecanoic	1.5	0.9	0.8	0.8	1.1
14-Me Pentadecanoic				2.1	
Palmitic	35.0	32.2	29.6	18.3	32.5
Palmitoleic	2.1	1.3	1.0	7.4	1.7
15-Me Palmitic				2.9	
16-Me Heptadecanoic				4.8	
Stearic	11.1	2.9	2.5	9.9	11.6
Oleic	9.2	6.3	6.1	36.5	25.0
Linoleic	1.4	0.3	Trace	7.2	0.5
Arachidic				3.3	
Linolenic				1.4	

* Analyses by GLC; experimental conditions as described in the text.

† Fatty acids identified on the basis of retention times and by cochromatography with selected standards.

‡ Obtained by calculation.

The diglycerides also might have been a product of milk lipase activity. The possibility that they might have originated from the decomposition of phospholipids appears unlikely because of the low average molecular weight, 505, which corresponds to that for dimyristin, 512. Phospholipids generally contain fatty acids of higher molecular weight.

Since only a small fraction of the 317 mg of lipid eluted with 1% ether in petroleum ether (Table 4) gave a positive LB test, a larger scale isolation was performed to identify the sterol esters. One hundred grams of the neutral lipid (R-1) was adsorbed on 1500 g of silicic acid and the hydrocarbons and sterol esters were removed with 18 liters of 1% ether in petroleum. This mixture (2.9 g) was then rechromatographed on 140 g of silicic acid using the usual solvent sequence. The material giving a positive LB test (fractions 26–58) was pooled and again rechromatographed, yielding approximately 105 mg of sterol ester. The fractions eluted prior to and following the sterol esters were comprised, respectively, of hydrocarbons (600 mg) and triglycerides (2.1 g).

The sterol ester fraction (105 mg) was saponified and the unsaponifiable matter (69 mg) and the fatty acids (37 mg) were recovered separately. The 3β-hydroxy sterols were then isolated from the unsaponifiable material by digitonin precipitation. Cleavage of the digitonides, followed by sterol recrystallization and GLC, indicated that essentially all the digitonin-precipitable sterol was cholesterol. The nonsterol components, which accounted for approximately 60% of the unsaponifiable matter of the sterol ester fraction, were shown to consist primarily of carbonyl material (6). Since none of the contaminating material could be demonstrated to be alcoholic, all the fatty acids associated with this fraction were assumed to have been esterified with cholesterol. This assumption was supported by the nearly correct cholesterol/fatty acid ratio obtained after allowing for contamination with the ketonic material.

When compared to the fatty acid composition of butter fat (Table 5), the fatty acids of the sterol esters showed two major differences. The content of the short-chain fatty acids was relatively low, and the proportion of the so-called minor fatty acids of butter fat was high. The total content of branched-chain fatty acids in butterfat has been estimated to be of the order of 2% of the total (23). Since the unsaponifiable matter of butter fat amounts to only 0.65% of the total weight and consists mainly of cholesterol (95%) originally in the free form, it would seem that these branched-chain acids cannot appear in the neutral fat exclusively as the wax or sterol esters (23), but must contribute to the triglycerides also, as already suggested on the basis of the GLC of the triglycerides (4, 8). The amount of cholesterol ester found in this most volatile distillate is about one-half of that (0.1–0.12%) estimated for whole butter fat by Nieman et al. (24).

The information provided by the present and the previous (4, 8) analytical studies on the chemical make-up of these distillates is currently being assessed statistically in relation to their hypercholesterolemic activity in man (7).

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