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Determination of Fatty Acids in Butter Fat Using Temperature-Programmed Gas Chromatography of the Butyl Esters

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ABSTRACT

Substituting n-*butanol for the methanol specified in the* AOAC Official methods of analysis (*sections 28.056–28.059*) *yields the butyl esters of the fatty acids with complete recovery of the volatile short-chain acids. Fatty acid butyrates can be determined using a continuous multistage temperature-programmed gas chromatographic method, with a 10% apolar 10C column. The results of the analysis in 1982, of fifty butters were compared with those of thirty butters analyzed in 1974. There were no practical significant differences in the fatty acid compositions of the 1974 and 1982 butters. A urea fractionation technique was used to concentrate minor components for identification and estimation.*

INTRODUCTION

Short-chain volatile fatty acid methyl esters are often lost during analysis (Parodi, 1967; van Wungaarden, 1967; Shehata *et al.*, 1970; Iverson & Sheppard, 1977). Recoveries are generally better when butyl esters, rather than methyl esters, are used (Gander *et al.*, 1962; Sampugna *et al.*, 1966; Parodi, 1970; Iverson & Sheppard, 1977). In a collaborative study of a gas chromatographic (GC) method for the determination of fatty acid composition, low recoveries and relatively poor within- and between-laboratory precision were reported for the methyl esters of butyric acid (Firestone & Horwitz, 1979).

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Parodi (1972) has discussed the variation in the fatty acid composition of milk fat. However, few data have been reported on the fatty acids present in trace amounts in milk fat. To determine these trace fatty acids, the fat/oil must be fractionated so that fatty acids exhibiting similar GC retention times are in different fractions. A fractionation procedure has the advantage of yielding amounts of the minor acids; in particular, the long-chain acids, large enough for identification. The fractionation techniques are usually based on column chromatographic separations (Herb *et al.*, 1951, 1962; Keeney, 1956) or urea complexing (Schlenk & Holman, 1950; Schlenk, 1954; Iverson *et al.*, 1965; Iverson & Weik, 1967; Egge *et al.*, 1972; Strocchi & Bonago, 1975; Aurousseau & Bauchart, 1980; Massart-Leen *et al.*, 1981). Strocchi & Holman (1971) used thin-layer silver nitrate fractionation and GC–mass spectrometry (MS) to determine positional and *cis–trans* isomers in milk fat; they quantitated 87 trace components. Melcher & Renner (1976) reported 53 fatty acids in milk fat. Herb *et al.* (1962) and Iverson *et al.* (1965) presented data on the fatty acids in milk fat obtained using fractionation techniques. Ackman & Hooper (1973) reported considerable variation in the relative amounts of isoprenoid fatty acids. Patton & Jensen (1975) reviewed the composition of milk fat.

Recently, there has been renewed interest in determining the order in which fatty acid–urea adduct formation proceeds (Aurousseau & Bauchart, 1980). This fractional crystallization method separates fatty acids and will yield 100- to 500-fold concentration of branched chain acids (Egge *et al.*, 1972). More importantly, when used in a stepwise procedure, coupled with overloading the gas chromatograph and hydrogenation of the fractions, fatty acids can be identified. Some investigators have indicated that, for unequivocal identification, combined GC–MS must be used (Ryhage, 1966; Strocchi & Holman, 1971; Egge *et al.*, 1972).

Some investigators have suggested that, if the fatty acid composition of butterfat is known, the addition of non-milk fat to butterfat should be detectable (Roos, 1963). To determine the adulteration of milk fat with other fats, a fatty acid should be chosen that is present in milk fat in substantial amounts and can be measured accurately. Jebson & Curtis (1981) found that myristic acid could be used to detect the addition of soybean oil or sunflower oil to milk fat. The error of measuring the amounts of adulterant increases considerably when the composition of the added oil is unknown and even more if the identity of the added oil is

unknown. An experienced analyst using butyrate esters might determine butyric acid more reliably and thus reduce the inherent errors and increase the reliability of using this fatty acid as an index of adulteration. Butter is also unique in having a relatively large amount of pentadecanoic acid.

The goals of the present study were: (1) to demonstrate that a single programmed-temperature GC method can be used to measure the major fatty acids having chain lengths of 4 through 18 carbons; (2) to generate a pool of milk fat fatty acid composition data; (3) to determine whether major shifts in fatty acid composition of milk occurred over several years and (4) to identify trace fatty acids present in milk fat.

MATERIALS AND METHODS

The butters used in this study were purchased from local grocery stores in Maryland near the District of Columbia from June, 1973 to June, 1974 for the first study and from June, 1981 to December, 1981 for the second study. The first and second studies are referred to as the 1974 and 1982 studies, respectively.

The following solvent extraction technique was used to extract butterfat from butter. Weigh 6·25 g of butter into a flask and add 10 ml of distilled water and 50 ml of petroleum ether. Warm the mixture on a steam bath for 3 min and transfer the contents of the flask to a 125-ml separatory funnel containing 10 ml of water. Rinse the flask with two 10-ml portions of petroleum ether and add these to the separatory funnel. Swirl the contents of the separatory funnel, let the layers separate and draw off the aqueous layer. Repeat the washing of the organic phase with a second 20-ml portion of water, let the layers separate and discard the aqueous layer. Add approximately 5 g of anhydrous sodium sulfate to the separatory funnel, shake vigorously and filter the extract through a powder funnel, containing a loose-fitting glass wool plug and approximately 5 g of anhydrous sodium sulfate, into a 125-ml Erlenmeyer flask. After the petroleum ether extract has passed through the powder funnel, rinse the separatory funnel with two 10-ml portions of petroleum ether and pass the washes through the same powder funnel. Evaporate the combined petroleum ether extracts on a steam bath to approximately 15 ml, transfer to a 25-ml volumetric flask, dilute to volume with petroleum ether and store in a refrigerator for subsequent analysis.

Preparation of butyl esters

The butyl esters of the fatty acids were prepared according to the *Official methods of analysis* (AOAC, 1984), except that butanol was substituted for methanol as described by Iverson & Sheppard (1977). The esters were separated from the reaction products by using a separatory funnel rather than the Babcock flask-centrifuge that was used in the 1982 study. The procedure was as follows. Transfer 1 ml of petroleum ether containing 200 mg of extracted butterfat into a 125-ml Erlenmeyer flask fitted with a 13-cm air condenser. Add 6 ml of 0·5N NaOH in butanol through the condenser. Add boiling chips and heat the mixture on a steam bath to a gentle reflux for 4 min. Then cool the Erlenmeyer flask for approximately 2 min and add 10 ml of 12·5% boron trifluoride–butanol reagent (w/v) followed by 2 ml of petroleum ether. Heat the mixture on a steam bath to a gentle reflux for 3 min, cool to near room temperature and transfer to a 125-ml separatory funnel containing 75 ml of distilled water. Add 15 ml of petroleum ether and shake vigorously for 30 s. Let the layers separate. Discard the aqueous butanol phase and wash the petroleum ether phase with four 75-ml portions of distilled water, which are discarded. Transfer the petroleum ether phase containing the butyl esters to a 50-ml Erlenmeyer flask through a powder funnel containing a glass wool plug and approximately 2 g of anhydrous sodium sulfate. Evaporate the extract on a steam bath under a stream of nitrogen to approximately 3 ml, transfer to a 5-ml volumetric flask, dilute to volume with petroleum ether and store at 20°C until analyzed.

Gas chromatography

In both the 1974 and 1982 studies 10% Silar 10C coated on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA 16801) was used with a 3·6 m × 3·2 mm stainless steel column. A dual column gas chromatograph with dual hydrogen flame detectors, automatic integration unit and computer printout capability was used in the 1974 study. In 1982, a hydrogen flame detector with automatic integration and chart printout was used. The stainless steel column bore was silane-treated, as was the glass wool used to plug the column. The GC conditions given here should be considered as broad guidelines to the method finally utilized. In this study, performance was considered satisfactory when the distance between each of the saturated fatty acid

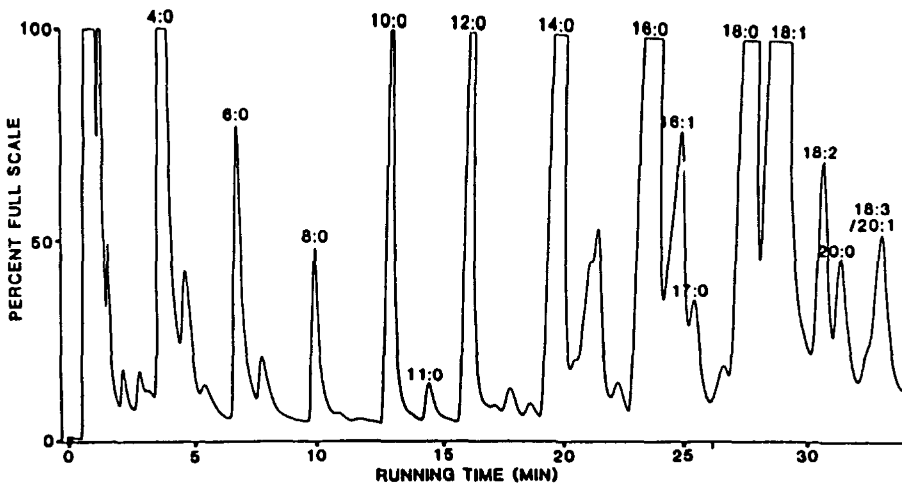

Fig. 1. Temperature-programmed GC determination of fatty acid butyrate derivatives from butterfat (see text for operating conditions).

butyrate peaks was about the same (see Fig. 1). The following operating conditions were used with the 1982 GC unit: detector and injector, 275°C column, initial temperature, 105°C held for 2 min, then programmed at 5°/min to 130°C, at 4°C/min to 150°C, at 3°/min to 227°C, and 227°C, held to end of analysis. The procedure used is similar to that reported by Iverson & Sheppard (1975, 1977).

Accurate quantitation was assured by preparing a quantitative reference mixture with triglycerides (Nu Chek Prep, Inc., PO Box 172, Elysian, MN 56028) converted to fatty acid butyl esters. The butyl ester mixture approximating the composition of milk fat was used to determine the GC response of individual fatty acids. The use of fatty acid butyrate response factors (area corrections) would not appreciably change the quantitative values. There are inherent difficulties in quantitation when the instrumental responses for individual fatty acid esters vary, thus reducing the quantitative value of the analysis (Herb & Martin, 1970; Iverson & Sheppard, 1977; MacGee & Allen, 1977).

Fatty acid fractionation

The fatty acids were fractionated by urea adduction as described by Iverson *et al.* (1965) and Iverson & Weik (1967) and similar to the procedure of Egge *et al.* (1972). Dissolve the methyl esters in a methanol–

urea mixture by heating in a steam bath and cool to form inclusion compounds with urea crystals. Remove the inclusion products by filtration and repeat the procedure to form seven or eight fractions as described by Iverson & Weik (1967). The procedure was used four times on composites of six to ten butters in this study.

The identification procedure for the fatty acids, especially the ones present in trace amounts, is not necessarily as straightforward as for the major components. In GC of the methyl esters there are critical pairs such as 16:2, 17:1; 18:3, 20:1; 20:4, 22:1; 20:5 and 24:1. Also, large amounts of 16:1 will prevent the detection of iso- and anti-C:17 components. The distribution coefficients (Strocchi & Bonago, 1975) and the partition coefficients (Aurousseau & Bauchart, 1980; Iverson & Weik, 1967) show that the critical pairs would be concentrated in separate fractions. Identities were confirmed by comparison with available authentic standards coupled with a detailed knowledge of GC retention times. Finally, the structure assignments were further confirmed by comparison of the non-hydrogenated and hydrogenated fatty acid patterns of the urea-adducted fractions in the same procedure as Ghosh & Ghosh (1976) used.

RESULTS AND DISCUSSION

The calibration/response data obtained using the fatty acid–butyrate mixture showed a surprisingly uniform instrumental (area) response, regardless of the fatty acid chain length (Table 1), indicating that the temperature program was approaching nearly ideal conditions. The response factor data obtained are so close to 1·0 that, using these factors to correct area response would not add appreciably to the quantitative value of the raw data obtained.

The major fatty acids (as butyl esters) of the thirty butters obtained in 1974 and of the fifty butters obtained in 1982 are presented in Table 2. Student's t-test indicated no significant differences between years for 4:0, 8:0, 10:0, 12:0 and 18:0 butyl esters, while there were significant differences for 6:0, 14:0, 18:1, 18:2 and 18:3 butyl esters. The differences may be statistically correct, but are probably of no practical consequence. GC equipment and techniques improved considerably between 1974 and 1982. In general, the 1982 variability of GC values for these acids is small enough for methodological improvements to account for a major

TABLE 1
Weight Response Factor for Fatty Acid Butyl Esters Determined from a Triglyceride Mixture Approximating the Composition of Milk Fat

Fatty acid	*Area* (%)	*Actual wt.* (%)	*Response factor*
4:0	4·74	4·89	1·032
6:0	3·28	3·32	1·012
8:0	4·29	4·09	0·953
10:0	4·34	4·21	0·970
12:0	4·68	4·54	0·970
14:0	11·16	10·96	0·982
16:0	26·50	26·46	0·998
18:0	9·80	9·84	1·004
18:1	25·68	25·90	1·008
18:2	3·68	3·82	1·038
18:3	1·84	1·95	1·060

portion of the significant differences observed. Our results agree with those of Toppino *et al.* (1982) in Italy, who analyzed 235 samples of domestic and imported butter, and those of Homer (1983) in Finland, who reported the relative amounts of fatty acids with greater than twenty carbons.

The butyl butyrate weight values may be expressed in terms of moles of butyric acid by using the conversion factor 2·26. The calculated mole per cent butyric acid mean values and standard error of the mean are $10{\cdot}55 \pm 0{\cdot}284$ for 1974 and $10{\cdot}94 \pm 0{\cdot}161$ for 1982. These values compare favorably with the mean value of 10·41 mol % determined by Keeney (1956) on 500 samples.

It is difficult to compare fatty acid composition data unless the values are converted to a uniform basis. Posati *et al.* (1975) emphasized the many factors involved in comparing the fatty acid composition of milk fat determined by various workers; namely, to interpret the data properly it is necessary to know whether they are given on a fatty acid basis or a methyl ester basis, whether response factors have been used and whether the data have been normalized. The data of Hendricks & Huyghebaert (1970), Parodi (1970), Gray (1973), Posati *et al.* (1975) and Rauramaa (1976) were normalized to 90% for the major fatty acids (i.e. major fatty acids comprise 90% of total fatty acids) by the present authors (Table 3). The data for the major fatty acids obtained in the present study are in agreement with those already published.

TABLE 2

Major Fatty Acids of Butters Obtained in 1974 and 1982 Determined as Butyl Esters

Fatty acid	*Thirty Butters 1974*[a]		*Fifty Butters 1982*[b]	
	Mean wt. %	*Std. error of mean*	*Mean wt. %*	*Std. error of mean*
4:0	4·67	±0·126	4·84	±0·071
6:0	2·58	±0·050	2·20	±0·030
8:0	1·36	±0·031	1·30	±0·016
10:0	3·07	±0·057	2·88	±0·033
12:0	3·46	±0·057	3·33	±0·034
14:0	11·58	±0·092	10·76	±0·078
16:0	28·75	±0·276	26·20	±0·265
18:0	10·73	±0·116	10·76	±0·102
18:1	25·46	±0·365	24·10	±0·206
18:2	1·84	±0·049	2·37	±0·038
18:3	0·26	±0·012	1·13	±0·037

[a] Duplicate analysis of each butter.
[b] Triplicate analysis of each butter.

TABLE 3

Comparison of Fatty Acid Composition (wt.%) of Milk Fat as Methyl Esters Normalized to 90% for the Major Fatty Acid Components

Fatty acid	*Present study*[a]	*Posati* et al. *(1975)*[b]	*Parodi (1970)*[c]	*Gray (1973)*[d]	*Rauramaa (1976)*[e]	*Hendricks & Huyghebaert (1970)*[f]
4:0	3·84	3·56	3·27	4·10	2·97	3·56
6:0	2·06	2·02	1·95	2·31	1·76	1·94
8:0	1·20	1·15	1·30	1·26	1·30	1·11
10:0	2·76	2·59	2·49	2·79	2·65	2·54
12:0	3·23	2·88	2·77	3·21	3·23	3·00
14:0	10·86	10·18	9·89	10·49	11·17	9·16
16:0	26·62	26·51	24·73	25·44	30·18	24·24
18:0	10·76	12·10	14·10	14·12	10·29	10·64
18:1	24·78	25·26	25·07	23·57	23·03	25·44
18:2	2·11	2·30	2·14	1·10	1·83	2·22
18:3	1·16	1·44	2·29	1·62	1·54	1·90

[a] Average of 1974 and 1982 data.
[b] Compiled from the literature.
[c] Average of 112 samples of Australian butter collected over a 12-month period.
[d] Average of 14 samples collected throughout one dairy season.
[e] Average of June–December samples from 19 Finnish dairies.
[f] Average of Belgian butters.

TABLE 4
Fatty Acids[a] Present in Trace Elements Amounts in Milk Fat Determined as Methyl Esters

Saturated		*Monosaturated*		*Polyenoic*	
11:0	0·20	10:1	0·15	20:2	0·07
13:0	0·19	12:1	0·06	20:3	0·10
15:0	1·48	13:1	0·03	20:4	0·14
17:0	0·60	14:1	0·40	20:5	0·09
19:0	0·15	17:1	0·36	22:2	0·04
20:0	0·35	19:1	0·16	22:3	0·07
21:0	0·04	20:1	0·32	22:4	0·03
22:0	0·20	21:1	0·04	22:5	0·04
23:0	0·12	22:1	0·06	22:6	0·01
24:0	0·14	23:1	tr[b]		
25:0	0·03	24:1	tr		
26:0	0·06				
Branched					
13:0i[c]	0·03				
14:0a[d]	0·02				
15:0i	0·40				
15:0a	0·44				
16:0i	0·40				
17:0i	0·50				
17:0a	0·52				
18:0i	0·16				
19:0i	0·10				
20:0i	tr				
22:0i	tr				
Multibranched					
16:0	tr				
19:0	tr				
20:0	tr				

[a] Weight per cent.
[b] tr = $<0{\cdot}01$.
[c] i = iso.
[d] a = anteiso.

The *trans*-oleate known to be present was not adequately separated from the *cis*-oleate for quantitation. While the C-19 unsaturated fatty acids are undoubtedly present, their positive identification is difficult. When the fraction was hydrogenated, the C-19 saturated components appeared, thus verifying the presence of the C-19 unsaturates. Identifications are similar to those obtained by Strocchi & Holman (1971). The long-chain *trans*-isomers were not quantitated in this study because the separation of *cis*- and *trans*-isomers decreases with increasing chain length. This is a problem that most workers have not been able to overcome. A total of forty-six fatty acids present at low concentrations were identified in this study. The relative amounts of the minor components are given in Table 4. At low levels there is more variation in quantitation than at higher levels. The data obtained for the minor fatty acids compare favorably with those found by previous investigators.

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