Quantitative Analysis of Food Fatty Acids by Capillary Gas Chromatography

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ABSTRACT

The superior efficiency of capillary columns is desirable for the gas chromatographic analysis of complex mixtures of fatty acids, but there have been some reservations regarding quantitation and reproducibility. This paper discusses the use of wall-coated glass capillary columns in a semiautomated system for the determination of food fatty acids. Glass columns coated with SP2340 were used for extended periods at temperatures up to 200 C without appreciable deterioration. Up to 1900 samples were analyzed on a single column over an 11-month period, with only minor changes in retention ratios, response factors and column efficiency. Quantitative precision of results, calculated either as normalized weight percentage or as absolute amounts, based on the use of an internal standard, were typically within 2% relative deviation. Difficulties encountered in obtaining acceptable chromatograms and reproducible data are discussed, and typical analyses of the fatty acids from foods presented.

INTRODUCTION

The use of capillary gas chromatography has expanded greatly in recent years. New techniques for preparing and using glass columns, plus the availability of commercial columns and accessories have contributed to this proliferation. As early as 1961 Landowne and Lipsky (1) used capillary gas chromatography to analyze fatty acids. Since that time considerable research has been done in this area, as described by two extensive reviews (2,3). Most of this work was done with metal columns, although recently the use of glass capillary columns coated with a variety of phases, including FFAP (4), SP2340 (5), and Carbowax 20 M (6), has been reported. The major interest in glass capillary gas chromatography (GCGC) has been focussed on its high separation efficiency rather than on its use for quantitative analysis. Our laboratory has for some time been using glass capillary columns coated with SP2340 for the quantitative analysis of food fatty acids. This report describes the system used and the results obtained, with emphasis on quantitative precision and accuracy.

Capillary columns were chosen instead of packed columns for several reasons. Complex prepared foods or whole meal composites may contain a wide range of fatty acids, which commonly cannot be completely separated on packed columns. It was hoped that the efficiency of capillary columns would permit the determination of all these fatty acids from one chromatogram without the need for auxilliary separations. Capillary columns also offered the possibility of providing fatty acid information not usually given by packed columns. Data on the amounts of *trans*-unsaturated fatty acids and the *cis,cis*-methylene-interrupted diene fatty acids are especially needed because of the known or suspected nutritional effects of these acids. These types of fatty acids are commonly determined by spectrometric methods, which would not be needed if the amounts of specific isomers could be determined directly by gas chromatography. A major incentive to use capillary columns was the decision to calculate the amounts of individual fatty acids in a given weight of food from an internal standard, rather than from normalized weight percentage data. Preferably, the internal standard would be a homolog of the compounds determined, and well-separated from them. Such a standard could be found for the analysis of fatty acid methyl esters (FAME) by GCGC but not by packed column GC.

MATERIALS AND METHODS

Extraction

All foods were extracted with chloroform-methanol as described by Folch (7).

Methylation

A modification of the procedure described by Metcalfe (8) was used. A sample of 20-60 mg of triglyceride plus an amount of internal standard equal to ca. 20% of the triglycerides were placed in a 16 mm x 125 mm test tube equipped with a Teflon-lined cap. Methanolic 0.5 N NaOH (1.0 ml) was added, and the tube capped and heated in a boiling water bath for 15 min. After the tube had cooled, 2 ml of BF_3/CH_3OH (14%) was added; then, the tube was recapped and heated in the boiling water bath for an additional 15 min. The tube was cooled, and 1 ml of isooctane and 2 ml of saturated aqueous NaCl were added. The tube was shaken vigorously for 1 min, then allowed to stand until the phases separated. The upper, isooctane layer was transferred with a Pasteur pipette to a 45 x 11 mm vial containing a 1 mm layer of anhydrous Na_2SO_4. The vial was capped and shaken, then allowed to stand for at least 20 min. The clear isooctane solution of FAME was transferred to a 1 ml serum bottle (Wheaton No. 223682), which was then sealed with a crimp cap lined with Viton A (Hewlett-Packard No. 5080-8730). Plastic boxes designed for storing pistol cartridges (catalog number SP1E3, Herter's, Inc., Waseca, MN) are useful for transporting and storing these vials.

Gas Chromatography

Two identical automated systems were used, each consisting of a Hewlett-Packard Model 7671A automatic liquid sampler, an HP Model 5840 gas chromatograph, and a Memodyne Model 2181W cassette tape recorder (Memodyne Corporation, 385 Elliot Street, Newton Upper Falls, MA). Since the gas chromatograph as purchased was designed for use with packed columns, it was modified slightly to accommodate capillary columns. The changes are shown schematically in Figure 1. A purifier (A) for the carrier gas was considered essential since some stationary phases, including SP2340, are sensitive to O_2. The addition of the pressure regulator (B) downstream from or in place of the flow regulator of the Model 5840 GC was necessary since capillary columns must be operated under conditions of constant pressure rather than constant flow. Reliance on the regulator on the gas tank to provide constant pressure is not advisable, since these regulators are subject to minor, often cyclic, variations. A second purifier (D), packed with molecular seive 5A, and preferably fitted with a filter, assured the removal of contaminants from the regulator, gauge and fittings. The commercial injection splitter (F), which fits any 1/4 in. injection port, provided an all-glass injection system. A length of silanized glass tubing, 1/4 in. OD x 4.4 in. long, extending into both injection port and

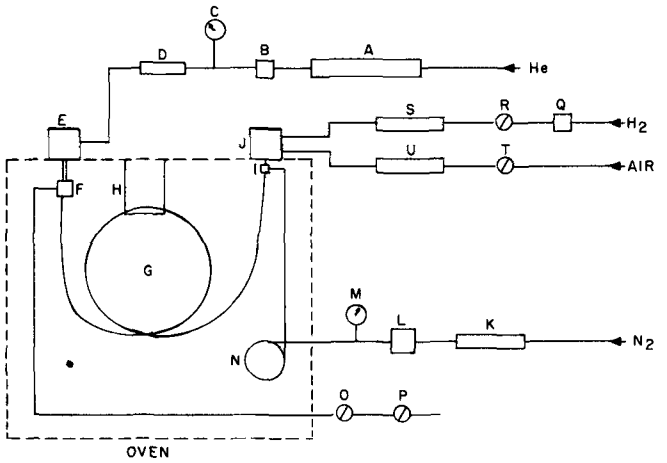

FIG. 1. Modifications to the plumbing of the HP Mod. 5840 gas chromatograph such that capillary columns could be used.
A. Carrier gas purifier to reduce O_2 and H_2O. (Supelco, Cat. No. 2-2306).
B. Pressure regulator, 0-125 psig (Cat. No. 420402-5, Airmatic-Allied, 185 Park Drive, Wilmington, OH 45277).
C. Pressure gauge, 1-160 psig (Cat. No. 270102-3; Airmatic-Allied).
D. Carrier gas purifier, 1/4" x 6", packed with Molecular Sieve 5A.
E. Injection port.
F. Splitter (Cat. No. 2-3740; Supelco. Includes valves O and P).
G. Column.
H. Column support bracket.
I. N_2 Auxilliary inlet and column exit connection. See text for details.
J. Flame ionization detector.
K. N_2 purifier, 1/2" x 8" Cu tubing packed with Molecular Sieve 5A.
L. Pressure regulator for N_2. See B.
M. Pressure gauge for N_2. See C.
N. N_2 preheating coil.
O. Toggle valve for split flow shut-off.
P. Metering valve for split flow control.
Q. Pressure regulator for H_2. See B.
R. Shut-off valve for FID H_2. Part of HP Mod. 5840 detector.
S. H_2 purifier. See K.
T. Shut-off valve for FID air. Part of HP Mod. 5840 detector.
U. Air purifier. See K.

splitter body, was constricted ca. 1.9 in. from the top. Just above the constriction, the tube was packed with about 5 mm of 10% JXR on 100-120 Supelcoport (Supelco, Inc., Supelco Park, Bellefonte, PA), sandwiched between small plugs of silanized glass wool.

A simple L-shaped bracket (H) made of sheet metal and bolted to the top of the oven supported the column. The exit end of the column was connected to the detector through a 1/16 in. x 1/8 in. Swaglok reducing adapter (I), into whose side a length of 1/16 in. stainless steel capillary was silver-soldered to serve as the inlet for the auxilliary gas (N_2). A length of 1/8 in. OD stainless steel tubing connected the split exit to a toggle valve (0) and a metering valve to control the split flow.

Before the column was installed, 5-6 in. of each end was straightened, either with a small flame (a propane brazing torch with the air-intake holes covered works well) or with an electrically heated column straightener (9). Nitrogen was passed through the column during the straightening. The head of the column was inserted into the splitter until the end was 1-1.5 cm below the constriction in the insert. Graphite ferrules (Quadrex Corp., PO Box 3881, Amity Station, New Haven, CT) were used in the 1/16 in. Swagelok fitting to assure a leak-free seal. At the exit end, the column was inserted into the detector far enough to fit into the lower end of the capillary jet tip (HP Part No. 18704-80010).

The flowrate of the auxilliary gas, ca. 40 ml/min of N_2 in our system, was chosen to provide the optimum N_2/H_2 (v/v) ratio for maximum sensitivity. Sensitivities were lower when He was used as the auxilliary gas. To facilitate setting and maintaining the proper N_2 and H_2 flows, we routed these gases through the A and B flow sensors of the Model 5840 since these are not required for the carrier gas. The nitrogen was preheated in the capillary delay coil (N) in the oven, so that it would not cool the column effluent.

Although capillary columns could be used under conditions of gas control and cleanliness less stringent than those described above, some decrease in baseline stability and increase in baseline stability and increase in baseline rise during temperature programming would be expected. Leaks were another source of trouble, especially in the carrier gas lines, since some back-diffusion of oxygen seemed to occur. Leak-testing the column connections with soap solution was undesirable; hence, for that purpose and to test for any He and H_2 leaks, a gas leak detector (Gow-Mac Model 21-100, Gow-Mac Instrument Co., 100 Kings Road, Madison, NJ) was used.

The columns used were either 60 m or 100 m x 0.25 mm ID glass (Quadrex Corp.) coated with SP2340 (Supelco, Inc.). The most useful temperature program was: 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, followed by 1 C/min to an upper temperature of 200 C, at which the oven was held for various lengths of time until all FAME were eluted. Alternatively a program of 150 C-200 C at 1 C/min was used. Other conditions were: Injection port temperature, 280 C; detector temperature, 280 C; He flow, 1 ml/min; split ratio, 1:100. The reports generated by the gas chromatograph were recorded on magnetic tape and entered into a Wang Model 2200 Programmable Calculator for consolidated report generation.

Identification of FAME

Identifications were based on GLC retention ratios of known compounds, semilog plots of retention time (isothermal) vs. FAME chain lengths, retentions of the unsaturated FAME before and after hydrogenation, TLC and argentation TLC.

Calculations

A reference standard consisting of known amounts of simple triglycerides of the major fatty acids to be determined was included with each group of samples analyzed and treated in the same way as the samples, including the addition of internal standard and methylation. This standard was chromatographed at the beginning of each group of samples, and the results used to calculated correction factors (F) for the quantitation of that group of samples. The same standard was rerun at the end of the sample run, and the results of the entire run discarded if it changed by 5% or more. Amounts of each acid could be calculated either manually from the areas or automatically by the Model 5840 processor.

Manual

Correction factors (F) were calculated from the results of the reference chromatogram as;

$$F_X = \frac{Area_{IS}/Amt_{IS}}{Area_X/Amt_X}$$

Where

$Area_{IS}$ = area of the internal standard
Amt_{IS} = amount of the internal standard in the reference standard
$Area_X$ = area of $FAME_X$
Amt_X = amount of triglyceride X in the reference standard.

Using these correction factors, the amounts of each fatty

acid in the original sample were calculated by the following equation:

$$\frac{Wt_X}{100\text{ g sample}} = \frac{F_X \cdot Area_X \cdot Amt_{IS} \cdot DF \cdot 100}{Area_{IS} \cdot Wt\text{ sample}}$$

Wt_X = grams of fatty acid X as the triglyceride
$Area_X$ = area of peak of $FAME_X$
Amt_{IS} = amount of the internal standard added (grams)
$Area_{IS}$ = area of the peak of the internal standard
DF = dilution factor (reciprocal of the fraction of the total sample extract taken for methylation).
Wt Sample = amount of food extracted, in grams.

Automatic

From the chromatogram of the initial reference standard, the amount per unit area (Amt/Area) for each component (including the internal standard) is calculated and used for all subsequent calculations.

$$\text{Calculated as: } \frac{Wt_X}{100\text{ g sample}} = \frac{Area_X \cdot (Amt_X/Area_X) \cdot Amt_{IS} \cdot DF \cdot 100}{Area_{IS}\ (Amt_{IS}/Ara_{IS})\ \text{Wt sample}}$$

The equations for manual and automatic calculations are identical, since $F_X = \frac{Amt_X / Area_X}{Amt_{IS}/Area_{IS}}$

RESULTS AND DISCUSSION

Columns of various lengths, from 10 to 120 meters, were tested, but the longer columns were preferred for routine analyses because their greater efficiency allowed us to analyze samples differing widely in fatty acid composition. Only wall-coated SP2340 columns were used for the work reported in this paper. Table I describes a 100 meter column used to collect much of the data discussed here, and gives some of its relevant characteristics. The column was in constant use for about 11 months at temperatures up to 200 C. Approximately 1900 samples were chromatographed on the column before it began to lose efficiency. SP2340 columns were very stable, with low bleed at temperatures up to 200 C, permitting single column temperature programming with only a slight baseline rise.

Many characteristics, including those in Table I, have been used to evaluate column performance (10). There is a tendency to emphasize the number of theoretical plates (n) in descriptions of commercial capillary columns, but theoretical plates alone may be misleading. It is important that a value of n be specified for a particular compound and that the capacity factor (k) for that compound also be specified. The capacity factor is related to film thickness, and is analogous to the percentage of coating on column packing, and describes, in effect, the time required for an analysis. Columns with low k values have low bleed rates and short retention times, but overload easily. We have preferred the highest k values compatible with acceptable retention times. We are now specifying columns with $k \cong 2$ for 18:0 and $k \cong 4.0$ for 18:3ω3 at 180 C, as well as a minimum number of theoretical plates for each of these compounds.

Retention Ratios

The retention ratios (RR) of a number of FAME are given in Table II, for separations both at 180 C isothermally and with a temperature program found to work well with food samples. Typical chromatograms of a standard fatty acid mixture and of FAME from a typical food sample are shown in Figures 2 and 3. Isothermal operation was not satisfactory for quantitative analysis, but was used for identification via semilog plots, for evaluation of column performance, and for specific separations. Not all FAME were separable under a given set of conditions, in spite of the high column efficiencies, but in many instances different column temperatures gave the desired separations. For example, the temperature program described in Table II did not separate 16:1ω7c from anteiso 17:0, but the pair were well separated isothermally at 180 C. With the temperature program, 18:3ω3 was not well separated from the subsequently eluted 20:1ω9. Isothermally at 180 C, the compounds not only were separated, but the elution order was reversed, (Fig. 2, 3). On the other hand, temperature programming improved the separations between 18:1 ω7c/18:1ω9c, 20:0/18:2ω6cc, 19:1ω9c/18:2ω6cc, 22:0/20:2ω6, and 20:3ω3/22:1ω9c. Other isothermal temperatures would doubtless be as good as or better than the temperature program for a limited range of FAME, but we avoided extremely short or extremely long retention times.

Retention data such as those in Table II can be used to predict the separability of specific compounds, or to predict the efficiency required for a particular separation. It can be shown* that the relation between the number of

TABLE I

Characteristics of a Glass Capillary Column Used For Fatty Acid Analysis[a]

Phase (wall-coated)		SP2340
Length (m)		100
Inner diameter (mm)		0.25
Inlet pressure (psig)		46
Linear velocity (cm/sec)		18-25
Solvent holdup time (min)		9.2
Capacity factor (k)[b] for 18:0		1.95
Theoretical plates (n)[c] 18:0		328,869
"Real plate number" (n_{real})[d]		144,147
Separation number (TZ)[e]	18:0/18:1ω9c	16
	18:1ω9c/19:1ω9c	36

[a]All calculations were made from samples chromatographed isothermally at 180 C, with the injector and FID detector both held at 280 C.

[b]$k = \frac{RT\text{-}ST}{ST}$, where RT = retention time of compound, ST = solvent holdup time.

[c]$n = 16\left(\frac{RT}{BW}\right)^2$, where BW = base width.

[d]Effective theoretical plates for k = 10, calculated from saturated straight chain fatty acids. See Kaiser (11).

[e]See Kaiser (11).

*The number of effective theoretical plates required for the baseline separation of two adjacent peaks, a and b, may be estimated from their corrected retention times (RT'), if $RT'_b > RT'_a$, and it is assumed that $BW_a \cong BW_b = RT'_b - RT'_a$:

1) $N_{eff} = 16\left(\frac{RT'_b}{BW_b}\right)^2$;

2) $N_{eff} = 16\left(\frac{RT'_b}{RT'_b - RT'_a}\right)^2$;

3) $N_{eff} = 16\left(\frac{RT'_b/RT'_b}{RT'_b/RT'_b - RT'_a/RT'_b}\right)^2$;

4) $N_{eff} = 16\left(\frac{1}{1 - RT'_a/RT'_b}\right)^2$;

5) $N_{eff} = 16\left(\frac{1}{\frac{RT'_a/RT'_{18:0}}{RT'_b/RT'_{18:0}}}\right)^2$;

6) $N_{eff} = 16\left(\frac{1}{1 - RR_a/RR_b}\right)^2$

TABLE II

Retention Ratios of Fatty Acid Methyl Esters on SP2340 (18:0 = 1.00)[a]

Peak No.[b]	Fatty acid		Retention ratios (18:0 = 1.000) 180 C	Temperature program 150-200 C[c]
1		4:0	---	0.011
2		6:0	0.032	0.026
3		8:0	0.059	0.054
4	anteiso	9:0	0.072	0.067
5		9:0	0.079	0.075
6	iso	10-0	0.090	0.088
7		10:0	0.107	0.105
8	anteiso	11:0	0.129	0.133
9		11:0	0.142	0.145
10	iso	12:0	0.163	0.182
11		12:0	0.188	0.199
12	anteiso	13:0	0.230	0.247
13		13.0	0.250	0.271
14	iso	14:0	0.284	---
15		14:0	0.330	0.362
16	iso	15:0	0.371	0.416
17	anteiso	15:0	0.401	0.440
18		14:1ω5c	0.428	0.457
19		15:0	0.436	0.479
20	iso	16:0	0.497	0.546
21		16:0	0.578	0.628
22	iso	17:0	0.648	0.704
23	anteiso	17:0	0.684	0.742
24		16:1ω7c	0.704	0.737
25		17:0	0.757	0.803
26		17:1	0.910	0.914
27	iso	18:0	0.865	0.895
28		18:0	1.000	1.000
29		18:1ω12t	1.120	1.085
30		18:1ω9t	1.128	1.091
31		18:1ω?t	1.139	1.098
32		18:1ω7t	1.146	1.105
33		18:1ω?t	1.163	1.121
34		18:1ω12c	1.176	1.123
35		18:1ω9c	1.200	1.133
36		18:1ω?c	1.232	1.139
37		18:1ω7c	1.224	1.151
38	anteiso	19:0	1.212	1.162
39		18:1ω?c	1.242	1.169
40		18:1ω?c	1.266	1.189
41		19:0	1.316	1.239
42		18:2ω6tt	1.383	1.267
43		18:2ω6ct	1.463	1.313
44		18:2ω6tc	1.497	1.337
45	iso	20:0	1.510	1.379
46		18:2ω6cc	1.547	1.362
47		19:1ω9c	1.568	1.392
48		20:0	1.735	1.500
49		18:3ω6	1.873	1.532
50		20:1ω9c	2.049	1.635
51		18:3ω3	2.078	1.630
52	anteiso	21:0	2.112	1.691
53		21:0	2.292	1.757
54		20:2ω6	2.643	1.846
55	iso	22:0	2.646	1.882
56		22:0	3.027	1.964
57		20:3ω6	3.149	1.984
58		22:1ω9t	3.380	2.065
59		20:3ω3	3.517	2.089
60		22:1ω9c	3.518	2.094
61		20:4ω6	3.600	2.105
62	anteiso	23:0	3.679	2.137
63		23:0	3.996	2.209
64		20:5ω3	4.810	2.337
65	iso	24:0	5.030	2.318
66		24:0	5.288	2.438
67		24:1ω9c	6.061	2.579
68		22:5ω3	8.280	2.961
69		22:6ω3	9.306	3.114

[a]Obtained by chromatographing a number of different mixtures of FAME, each mixture containing the internal standard, 21:0. These are typical values only and may differ for other columns coated with SP2340.

[b]Refers to number on peaks in illustrative chromatograms.

[c]Column, see Table I; temperature program; 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min-200 C, hold at 200 C until all FAME eluted.

effective theoretical plates (N_{eff}) required to give baseline separation between two compounds a and b, and their retention ratios RR_a and RR_b is approximated by

$$1)\ N_{eff} = 16\left(\frac{1}{1-RR_a/RR_b}\right) \text{ where } RR_b > RR_a.$$

For example, if N_{eff} = 160,000, using the above equation one can calculate that the maximum RR_a/RR_b ratio that will give baseline separation is $\leqslant$ 0.9900. By this criterion at 180 C under isothermal conditions 18:1ω12t and 18:1ω9t, with an RR ratio of 0.9929, would not be completely separated. The 18:2ω6ct and tc isomers, with an RR ratio of 0.9773 at 180 C would be well separated, but 20:3ω3 and 22:1ω9c, with a ratio of 0.9997, not at all. The latter separation would require 1.78 x 10^8 effective theoretical plates.

Retention times and retention ratios were highly reproducible on the same column under both isothermal and temperature-programmed conditions for short periods. When 24 samples were chromatographed over a 31-hr period, the RT for 10 FAME (12:0 to 21:0) changed only 1.20%. Over longer periods RT changes were greater, but

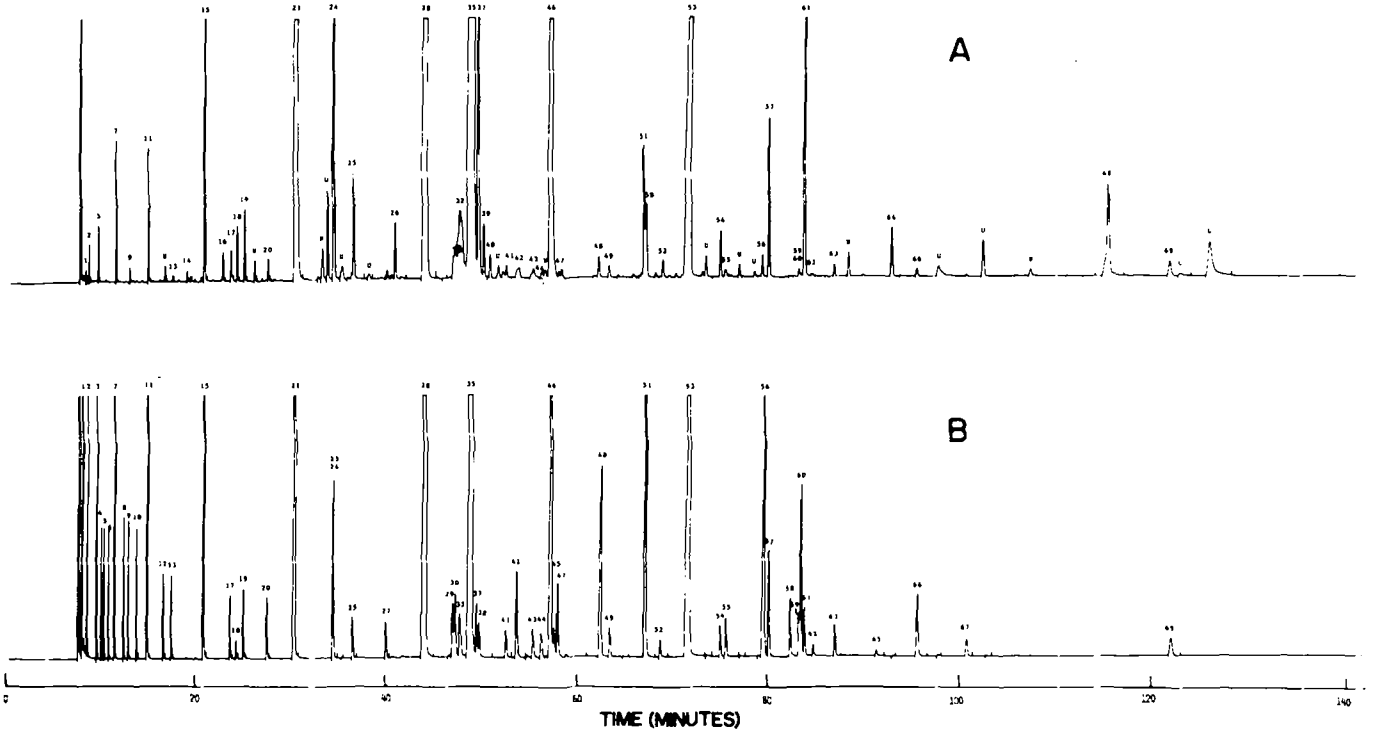

FIG. 2. FAME separations with temperature programming. (A) Lipid from a baby food composite; (B) mixture of pure standards. Column, see Table I: temperature program; 150 C-170 C at 0.5 C/min, the 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted. Identities of numbered peaks are given in Table II.

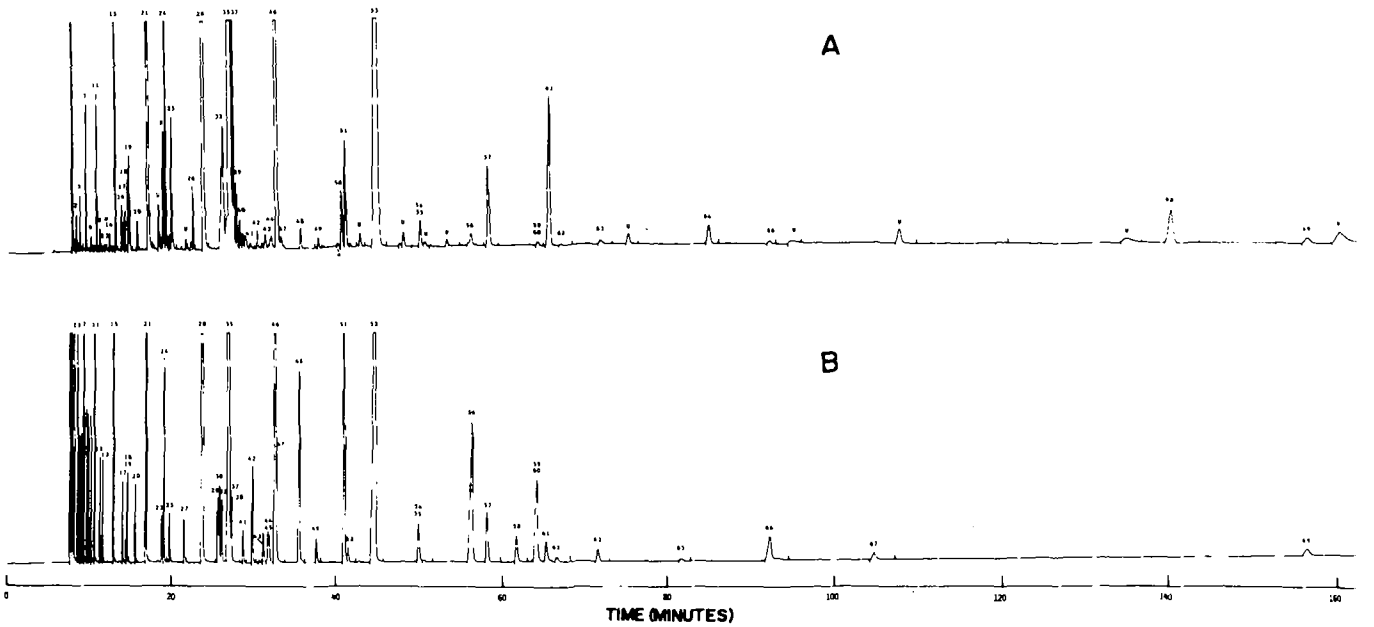

FIG. 3. FAME separations at 180 C, isothermal. (A) Lipid from a baby food composite; (B) mixture of pure standards. Column, see Table I. Numbers of peaks refer to identities given in Table II.

TABLE III

Correction Factors: Mean and Variation During One Month of Continuous Operation[a]

FAME	$\bar{X}$	SD	CV (%)
10:0	0.958	0.016	1.7
12:0	0.913	0.012	1.3
14:0	0.910	0.010	1.1
16:0	0.930	0.016	1.7
18:0	1.000	---	---
18:1ω9c	0.994	0.013	1.3
18:2ω6cc	0.993	0.015	1.5
20:0	1.054	0.008	0.8
18:3ω3	1.020	0.018	1.8
22:0	1.134	0.009	0.8
24:0	1.310	0.029	2.2

[a]Triglyceride standard chromatographed on 16 different days during 1 month. For 24:0 N=9. The splitter system used is described in the Materials and Methods section. Column, see Table I temperature program; 150C to 170C at 0.5C/min, then 0.2C/min for 16 minutes, then 1C/min to 200C, hold at 200C until all FAME eluted.

TABLE IV

Correction Factors: Typical Values[a]

Fatty acid	F	Fatty acid	F	Fatty acid	F
4:0	4.822	18:2ω6cc	0.996	22:1ω9t	1.165
6:0	1.923	19:1ω9c	1.069	22:1ω9c	1.228
8:0	1.187	20:0	1.055	20:3ω3	1.152
10:0	0.980	18:3ω3	1.051	20:4ω6	1.180
12:0	0.915	20:1ω9c	1.109	24:0	1.348
14:0	0.908	21:0	1.111	24:1ω9c	1.313
16:0	0.930	20:2ω6	1.033	22:6ω3	1.593
18:0	1.000	22:0	1.160		
18:1ω9c	1.004	20:3ω6	1.064		

[a]The splitter system used is described in the Materials and Methods section. Column, see Table I; temperature program; 150 C to 170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted.

not enough to shift RR more than 0.23% per 4 months, even during temperature programming. In the course of a year, there were greater changes in RR, possibly caused by the loss of liquid phase and the interaction with the glass surface, which in WCOT columns is never completely inert (12).

Correction Factors

In all types of GC analysis empirically determined correction factors (F) are used to compensate for differences in response and for losses caused by the entire analytical process. In FAME analysis the variation due to chromatography has been attributed to irreversible adsorption on the column and surfaces of the GC system, to destruction, and to differences in response of the flame detector to the different FAME (13). For capillary GC with split injection fractionation of the sample mixture by the splitter is also a possibility (14).

The relative variation of correction factors for those fatty acids most frequently found in foods was 2% or less over a short time period (Table III). In contrast to the variations given in Table III, the F values differed by as much as 10% with different injection inserts and columns during the course of a year. The reasons for the fluctuation in F values is now known. Splitless injection was tried, but the results were even more variable. Because of the variability, both reference standards and control samples were required to assure accuracy and precision, as discussed in a later section. Correction factors for 25 standards calculated

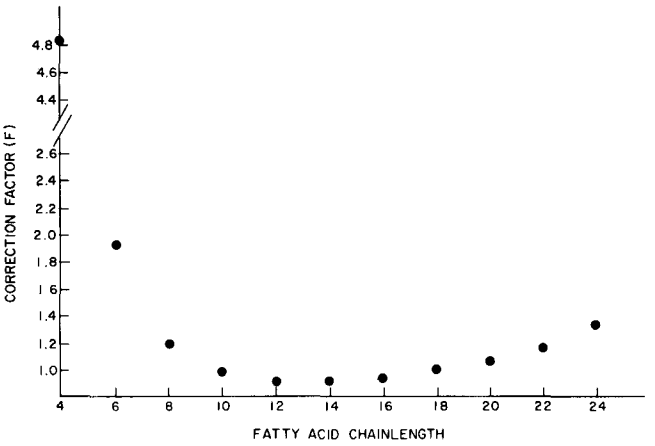

FIG. 4. Separation of FAME derived from beef lipid under conditions of temperature programming with no internal standard. Column, see Table I; temperature program: 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted. Numbers on peaks refer to identities tiven in Table II.

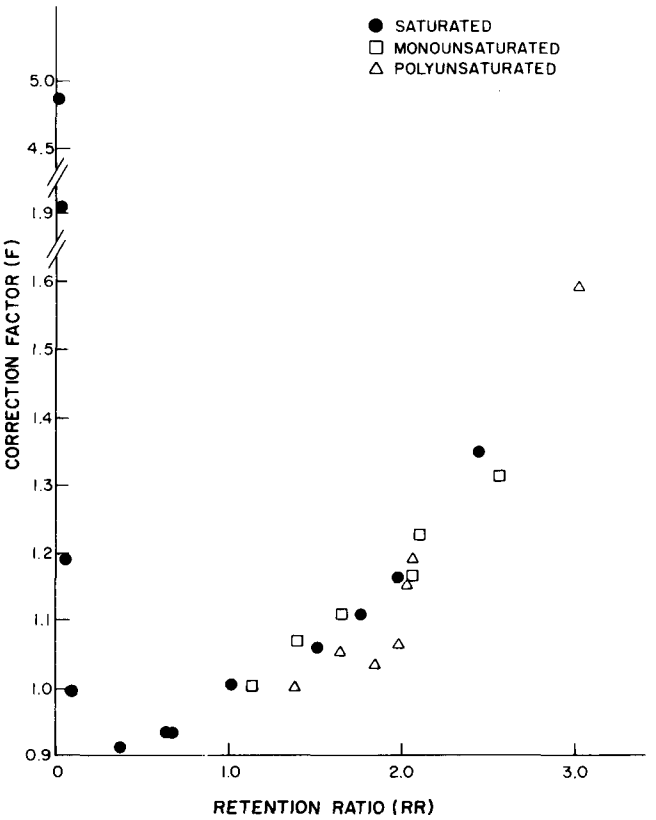

FIG. 5. Temperature programmed separation of FAME derived from lipid extracted from Zweiback Toast (Nabisco). Column, see Table I, ,temperature program; 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted. Numbers on peaks refer to identities given in Table II.

to 18:0 are given in Table IV. These values, obtained by chromatographing three different reference mixtures consecutively on the same column, are typical of the ranges found. The F values in Table IV for the saturated fatty acids and for 18:1ω9, 18:2ω6 and 18:3ω3 were determined by chromatographing triglyceride standards; other unsaturates for which pure triglycerides were not available were chromatographed as methyl esters. Triglycerides were preferred since they more closely resemble the samples

TABLE V

Variation of Correction Factors with Amount of FAME (= Response Linearity)[a]

FAME					
10:0		14:0		18:1ω9c	
mg FAME 2.1770 mg 21:0	Correction factor[b]	mg FAME 2.1770 mg 21:0	Correction factor	mg FAME 2.1770 mg 21:0	Correction factor
17.0315	0.910	18.941	0.899	14.926	0.988
8.5158	0.895	9.4705	0.891	7.4630	0.976
1.8924	0.884	2.1046	0.855	1.6584	0.940
0.8110	0.890	0.9020	0.850	0.7108	0.943
0.2317	0.892	0.2577	0.847	0.2031	0.952
0.1159	0.916	0.1289	0.847	0.1015	1.057
0.0579	0.902	0.0644	0.846	0.0508	1.009
0.0290	0.884	0.0322	0.869	0.0254	c
0.0097	0.838	0.01073	0.931	0.00849	c

[a]Obtained by chromatographing approximately 1.5 μl of the mixture of FAME + 21:0 (internal standard) dissolved in 1 ml. isooctane. Column – See Table I; temp. program; 150-170 C at 0.5°/min, then 0.2°/min. for 16 min., then 1°/min. to 200 C, hold at 200 C until all FAME eluted; split ratio 1:100.

[b]Correction Factor = $\frac{\text{Weight of FAME}}{\text{Weight of internal standard}} \Big/ \frac{\text{Area of FAME}}{\text{area of internal standard}}$

[c]Not integrated.

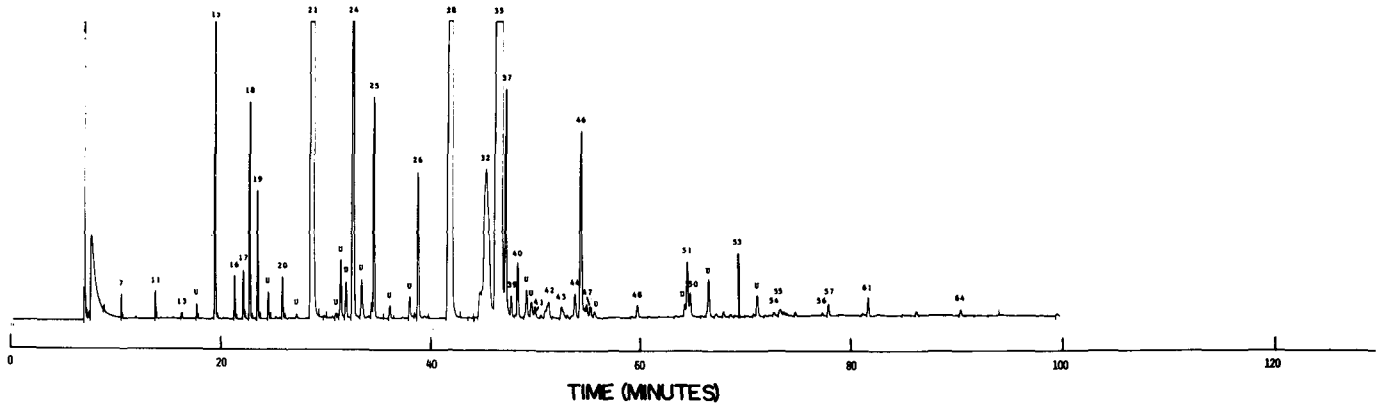

FIG. 6. Correction factors for saturated fatty acids vs. chain-length.

TABLE VI

Fatty Acids in Beef Lipid: GLC Precision of Absolute Amounts (g/100 g of sample) and of Normalized Weight Percent (N=7)[a]

Fatty acid	Absolute amounts (g/100 g)			Normalized weight percent		
	Mean	SD	CV	Mean	SD	CV
14:0	2.076	0.045	2.19	3.366	0.027	0.81
14:1ω5c	0.575	0.011	2.00	0.927	0.009	0.97
16:0	15.477	0.291	1.88	25.097	0.068	0.27
16:1ω7c	2.593	0.050	1.95	4.203	0.014	0.33
17:0	0.745	0.014	1.85	1.208	0.010	0.83
17:1ω?	0.519	0.011	2.02	0.842	0.003	0.38
18:0	9.131	0.141	1.54	14.806	0.050	0.34
18:1ω?t	0.981	0.054	5.54	1.591	0.078	4.90
18:1ω7t	0.986	0.042	4.24	1.598	0.069	4.33
18:1ω9c	22.687	0.365	1.61	36.789	0.083	0.23
18:1ω7c	0.920	0.017	1.82	1.492	0.005	0.35
18:2ω6cc	0.963	0.019	1.93	1.562	0.009	0.58
All other (<0.5%)	4.018	0.110	2.74	6.515	0.085	1.31
Σ All fatty acids	61.669	1.088	1.76	—	—	—
Σ *trans* monoene	2.262	0.038	1.70	3.668	0.015	0.42
Σ *trans* PUFA	0.316	0.020	6.42	0.512	0.029	5.58
Σ *trans*	2.578	0.053	2.04	4.180	0.028	0.68
Σ *cis* PUFA	1.329	0.046	3.47	2.136	0.044	2.07

[a]Column, see Table I; temperature program: 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted.

TABLE VII

Precision of Entire Fatty Acid Analytical Procedure[a]

FAME	$\bar{X}$ (g/100 g)	SD	CV (%)
10:0	0.039	0.002	5.13
12:0	0.049	0.002	4.08
14:0	2.058	0.063	3.06
16:0	15.274	0.412	2.70
16:1ω7c	2.553	0.072	2.82
18:0	8.968	0.186	2.07
18:1ω9c	22.431	0.254	1.13
18:2ω6cc	0.973	0.026	2.67
20:0	0.056	0.002	3.57
18:3ω3	0.261	0.010	3.83

[a]Represents total variation. 24 Beef samples were methylated and chromatographed on 24 different days. Column, see Table I; temperature program; 150 C-170 C at 0.5 C/min, then 0.2 C/min for 16 min, then 1 C/min to 200 C, hold at 200 C until all FAME eluted.

analyzed, and should yield more valid correction factors. A comparison of correction factors derived from triglyceride standards with those from methyl ester standards found that the differences were less than 1%, except for 4:0, 6:0 and 8:0. For these three acids, the ratios of F (FAME): F (TG) were 7.4:10, 7.6:10, and 9.1:10, respectively (n=3).

In Table IV both short chain and long chain FAME have high F values. Since F is inversely proportional to unit response, both short chain and long chain FAME clearly suffered some loss. A plot of correction factors vs. chain length (Fig. 4) gave a fairly smooth curve, with a minimum at 14:0. Since the relative contributions of the many variables which can affect individual correction factors are not known, F values should be obtained by chromatographing standards. Unfortunately, all fatty acids are not available either as triglycerides or as the methyl esters. Even for those which are available, it is not always practicable to include all of them in a standard mixture. A plot of F vs. RR (Fig. 5) suggested that fatty acids for which standards are not available could be assigned the average of the nearest proceeding and following known standards. This gives a reasonable approximation, and is certainly preferable to assigning an F value of unity.

Linearity

Because of the variations in the amounts of individual fatty acids in foods, it is imperative that gas chromatographic response be linear with amount. We have evaluated response linearity by chromatographing mixtures containing a constant amount of the internal standard and varying amounts of the individual FAME. The correction factors determined in this way for 10:0, 14:0 and 18:1ω9c, over a range in amount of 1760:1, are given in Table V. When 75 nanograms or more were injected (0.05 mg/2.1770 mg. 21.0), consistent correction factors were obtained. Below 75 nanograms, the results were somewhat erratic, due in part to the difficulty of measuring such small peaks.

Quantitation

Fatty acid data, to be useful for nutritional purposes, are best expressed as grams of each fatty acid per 100 grams of food. Although the use of an internal standard increased the number of analytical problems, these are preferable to the difficulties of translating normalized weight percentage data into g/100 g. These difficulties have been reviewed by Kinsella et al. (15) and are, in summary: (a) the total weight of fatty acids must be known, either from a separate analysis for total fatty acid or calculated from the total lipid by the use of factors for different food types (16); (b) the chromatographic peaks for all the fatty acids must be measured; and (c) normalized data, by the nature of the calculation, are not independent, and an error in one component will produce compensating errors in others.

The most commonly used internal standards have been saturated fatty acids with an odd number of carbon atoms in the chain. In complex mixtures of food fatty acids the choices are limited, as illustrated by a chromatogram of the FAME of high fat ground beef (Fig. 6). All straight chain saturated fatty acids from 4:0 to 24:0 except 21:0 and 23:0 were present in the sample. Available branched chain acids are not suitable, since they are either found in samples or are not adequately resolved. In this work 21:0 was used as the internal standard. The fatty acid composition of the beef referred to in Figure 6 is given in Table VI, both as absolute amounts based on 21:0 as the internal standard and as normalized weight percentages. The coefficients of variation were greatest for the *trans* isomers, because a number of them were poorly resolved. The precision of the absolute amounts, although less than that for the normalized data, varied from 1-2% for FAME $>$ 0.5% of the total. This level of precision is typical of the gas chromatographic results for repetitive runs.

To obtain acceptable day to day precision, we included a standard mixture and a control sample with each group of samples. An adequate number of aliquots of a reference triglyceride standard approximating the composition of the samples to be analyzed were dispensed at the same time (to minimize errors due to volume measurement) and refrigerated. One of the aliquots was included with each group of samples. In addition a control sample from a similar food extract was included. The triglyceride reference standard allowed us to determine the correction factors to be used with that group of samples. The results for the control sample were compared with the mean values from previous analyses as a check on the overall accuracy and precision.

During a typical month of operation, 24 control samples were analyzed on 24 different days. The variations found (Table VII) were ascribed to volume measurements (both

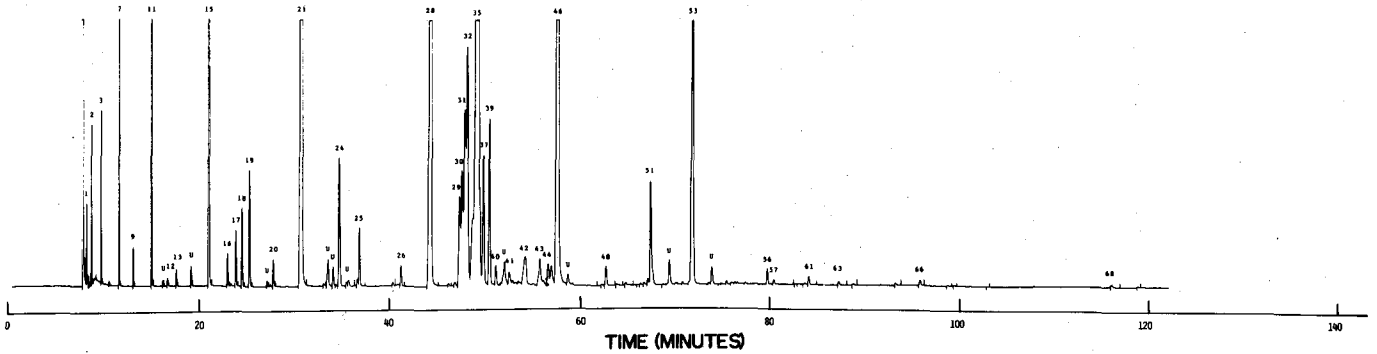

FIG. 7. Correction factors for fatty acids vs. corrected retention ratios.

TABLE VIII

Precision of Replicate GLC Analyses: Shortening Fatty Acids (N=8)[a]

FAME	$\bar{X}$ (g/100g)	SD	CV (%)
12:0	0.117	0.004	3.49
14:0	0.219	0.009	4.16
16:0	15.220	0.259	1.70
18:0	11.846	0.136	1.15
18:1ω12t	1.460	0.065	4.44
18:1ω9t	1.883	0.094	5.02
18:1ω?t	4.632	0.137	2.96
18:1ω?t	3.697	0.105	2.83
18:1ω12c	1.683	0.098	5.81
18:1ω9c	22.956	0.304	1.32
18:1ω?c	0.738	0.016	2.20
18:1ω7c	1.941	0.022	1.12
18:1ω?c	6.119	0.068	1.11
18:2ω6tt	1.002	0.046	4.55
18:2ω6ct	0.976	0.040	4.12
18:2ω6tc	0.496	0.004	0.81
18:2ω6cc	17.830	0.227	1.27
20:0	0.373	0.018	4.78
18:3ω3	1.113	0.026	2.33

[a]The FAME from a Crisco shortening sample was chromatographed eight times. Replicate injections were made on a 60 m SP2340 column and temperature programmed from 150 C at 0.5 C/min for 35 min, then 0.2 C/min for 10 min, the 1 C/min to an upper temperature of 200 C, then held at 200 C until all FAME eluted.

sample and internal standard), methylation, normal chromatographic variations and day to day changes in correction factors. These variations were about 1% more than the short term variations shown in Table VI for one of the control samples chromatographed seven times consecutively. This difference was ascribed principally to the nonchromatographic operations involved, and to the day to day shifts in correction factors. Without the use of the daily reference standard, the variations would have been much higher.

Determination of Isomers

The four geometric isomers of 18:2ω6 – *cc, ct, tc,* and *tt* – were separable, but the 18:1 geometric and positional isomers were only partially so. This is illustrated in Figure 7, which shows the fatty acids from a commercial Zweiback Toast. The peaks for the *trans* isomers of 18:1 are symmetrical and at least partially separated in Figure 7 but not Figure 6. The *trans* region in Figure 5 is characteristic of meat fats, and suggests that many more 18:1 isomers are present in meat than in hydrogenated oils.

The isomers were identified on the basis of GCGC and TLC data. An example of the precision with which the 18:1 isomers were determined is shown by the data in Table VIII for Crisco shortening.

Accuracy

Two commercial reference mixtures were analyzed, and the accuracy of the normalized weight percentages was evaluated as suggested by Herb and Martin in their report of a collaborative study of GLC accuracy (17). The deviations between the percentages we found and the known compositions were summed, and the sum was compared with that obtained by the collaborators (Table IX). The summed deviations for reference mixture B were 1.96, as compared to the collaborators' 2.87. The second reference mixture used in the collaborative study is not now available; a more complex commercial mixture with a composition similar to peanut oil was substituted (Mixture A). The summed deviations were 2.43, which may be compared to the collaborators' total for peanut oil of 3.06. These results indicate that the quantitative accuracy of our capillary system compares well with the accuracy of the laboratories in the collaborative study, over a wide range of fatty acids.

Sensitivity

The smaller the sample size the better the resolution. We preferred to chromatograph the largest sample size consonant with good peak shape and resolution in order to maximize the integrator area counts and, hence, precision for small peaks. Samples of 20-60 micrograms of FAME in ca. 1.5 microliters of isooctane were injected; with a split ratio of 1:100, ca. 200-600 nanograms reached the column. The more complex mixtures required larger sample sizes. No effort was made to calibrate the system absolutely, but we estimated that each area count with the HP 5840 integrator corresponded to 2-3 picograms. Peaks of 25-50 area counts were regularly detected, with low precision, and corresponded to 50-150 picograms on the column, or 5-15 nanograms injected.

TABLE IX

Accuracy of Analyses of Standard Reference Materials

	Reference mixture A[a]			Reference mixture B[b]		
Fatty acid	Known	Found	Difference	Known	Found	Difference
			Percent			
14:0	1.0	0.90	0.10			
16:0	4.0	4.11	0.11	6.0	5.88	0.12
18:0	3.0	3.10	0.10	3.0	2.94	0.06
18:1	45.0	44.71	0.29	35.0	34.20	0.80
18:2	15.0	15.07	0.07	50.0	50.65	0.65
20:0	3.0	2.80	0.02	3.0	3.02	0.02
18:3	3.0	2.99	0.01	3.0	3.31	0.31
22:0	3.0	2.77	0.23			
22:1	20.0	20.92	0.92			
24:0	3.0	2.63	0.37			
Sum			2.43			1.96
Found by collaborators[c]:			3.06[d]			2.87

[a]Fat and oil reference mixture No. 3 (Applied Science Laboratories Inc.).
[b]Fat and oil reference mixture No. 1 (Applied Science Laboratories Inc.).
[c]See text.
[d]For peanut oil.

TABLE X

Representative Food Fatty Acid Data Determined by Glass Capillary Gas Chromatography[a,b]

FAME[c]	Shortening (Crisco)	Baby food: strained beef liver (Gerber)	Baby food: strained lamb broth (Heinz)	Baby food: chicken stew toddler (Gerber)	Filet of fish: w/cheese sandwich (McDonald's)	Egg McMuffin: (McDonald's)
	grams fatty acid (as triglyceride)/100 grams of food					
6:0					0.040	0.090
8:0				0.040	0.038	0.053
10:0	0.065		0.009	0.009	0.063	0.108
11:0					0.006	0.012
12:0	0.508	0.001	0.007	0.011	0.072	0.126
A13:0		0.001				
13:0						0.005
U		0.001	0.005			0.004
14:0	0.379	0.035	0.197	0.053	0.380	0.425
I15:0		0.007	0.018		0.010	0.007
A15:0		0.004	0.020	0.002	0.017	0.017
14:1ω5c		0.004	0.005	0.006	0.040	0.034
15:0		0.012	0.048	0.007	0.043	0.040
I16:0		0.004	0.017		0.011	0.008
16:0	15.948	0.605	1.814	0.774	2.915	2.329
U		0.015	0.041	0.029	0.005	
U		0.010	0.036		0.009	0.036
U		0.011	0.056			
16:1ω7c		0.050	0.106	0.140	0.185	0.231
A17:0		0.008				
U		0.001				
17:0	0.088	0.032	0.026	0.010	0.076	0.031
U			0.012			
17:1ω?		0.011	0.049	0.006	0.033	0.011
18:0	11.692	0.648	1.756	0.241	1.585	0.907
18:1ω12t	1.221		0.013		0.017	0.009
18:1ω9t	2.539		0.018	0.004	0.026	0.012
18:1ω?t	4.615				0.056	0.016
18:1ω7t	2.987	0.052	0.261		0.098	0.044
18:1ω?t		0.007				0.018
18:1ω?c						
18:1ω9c	24.217	0.523	2.784	1.639	4.260	2.940
18:1ω7c	1.870	0.028	0.054	0.074	0.229	0.175
18:1ω?c	5.253	0.003	0.014		0.017	0.016
18:1ω?c	0.287	0.003	0.008	0.002	0.035	
U		0.003	0.040		0.017	0.007
19:0		0.006	0.034	0.001		
U	0.764					
U		0.002				
			0.008			
U		0.001	0.033			
18:2ω6tt	0.862		0.006			
18:2ω6ct	0.778		0.029	0.004	0.025	
18:2ω6tc	0.446				0.020	
U		0.006	0.039	0.002	0.041	
18:2ω6cc	20.882	0.197	0.169	0.816	5.346	0.965
19:1ω9c			0.007			
U	0.588					
20:0	0.370	0.001	0.011	0.004	0.047	0.009
U		0.005		0.007	0.045	
U					0.009	
U				0.003	0.044	
U			0.004	0.013	0.009	0.028
18:3ω3	1.592	0.065	0.094	0.030	0.709	0.045
20:1ω9c						0.028
U				0.003		
U		0.006	0.023		0.032	
U			0.004			
U		0.008	0.026	0.002	0.117	0.007
20:2ω6		0.001		0.002	0.049	0.013
U					0.108	
U		0.004	0.007			
22:0	0.425	0.004	0.006	0.004	0.121	
20:3ω6		0.076		0.003	0.100	0.012
20:4ω6		0.135	0.018	0.016	0.111	0.097
U					0.007	
U		0.024	0.004			
20:5ω3		0.040				
24:0					0.021	
U	0.642					
22:5ω3		0.059				
22:6ω3		0.009				
Summations:						
All fatty acids	95.831	2.731	8.036	3.921	17.197	8.915
All known F.A.	93.831	2.634	7.888	3.862	16.754	8.833
Satd. F.A.	29.475	1.368	4.063	1.149	5.453	4.167

TABLE X (continued)

Representative Food Fatty Acid Data Determined by Glass Capillary Gas Chromatography[a,b]

FAME[c]	Shortening (Crisco)	Baby food: strained beef liver (Gerber)	Baby food: strained lamb broth (Heinz)	Baby food: chicken stew toddler (Gerber)	Filet of fish: w/cheese sandwich (McDonald's)	Egg McMuffin: (McDonald's)
	grams fatty acid (as triglyceride)/100 grams of food					
cis Monoene F.A.	31.627	0.622	3.027	1.867	4.799	3.435
trans Monoene F.A.	8.175	0.052	0.292	0.004	0.197	0.081
Total monoene F.A.	39.802	0.733	3.319	1.871	4.996	3.534
cis PUFA[d]	22.474	0.582	0.281	0.871	6.315	1.132
trans PUFA	2.086	0	0.035	0.004	0.045	0
Total PUFA	24.560	0.582	0.316	0.865	6.360	1.132
trans (%)[e]	10.707	1.904	7.566	0.204	1.407	0.909
P/S[f]	0.762	0.425	0.069	0.758	1.158	0.272
Total fat, (%)[g]		4.10	9.33	4.02	19.78	11.23

[a]For conditions, see text.

[b]Average of two analyses.

[c]U = unknown, A = anteiso, I = iso.

[d]PUFA = polyunsaturated fatty acid.

[e] $$trans\ (\%) = \frac{trans\ \text{monoene} + trans\ \text{PUFA}}{\text{All fatty acids}}$$

[f] $$\text{P/S} = \frac{cis\ \text{PUFA}}{\text{Saturated fatty acids}}$$

[g]Folch extraction.

Data

Some typical analytical results are shown in Table X, illustrating the information that may be derived from a single GCGC analysis. More than 90 individual components were found in these and other samples, and ca. 65% of them identified (Table II).

ACKNOWLEDGMENT

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