Determination of Mixtures in Vegetable Oils and Milk Fat by Analysis of Sterol Fraction by Gas Chromatography

L. Alonso[a,*], J. Fontecha[b], L. Lozada[b], and M. Juárez[b]

[a]Instituto de Productos Lácteos de Asturias (CSIC), Asturias, Spain, and [b]Instituto del Frío (CSIC), Ciudad Universitaria s/n, 28040 Madrid, Spain

ABSTRACT: A rapid gas-chromatographic (GC) procedure was developed for the analysis of the total sterol fraction of vegetable oils, milk fat or mixtures, to detect possible admixtures of sunflower with olive oil and the addition of vegetable oils to milk fat. The method, which employs alkali-catalyzed transesterification with KOH/methanol, was compared with saponification procedures with and without transformation of sterols into silyl derivatives prior to analysis. Repeatability of the method was assessed, and the coefficient of variation was 6.0 and 8.0% for β-sitosterol in olive and sunflower oils, respectively. Recovery of β-sitosterol ranged from 92.6 to 95.8 for both oils. The GC method assayed in this work requires little analysis time and eliminates the need for saponification, extraction, and derivatization steps. It offers good repeatability and recovery and is thus well suited to routine use.
JAOCS 74, 131–135 (1997).

KEY WORDS: Adulteration, capillary gas-chromatographic analysis, methyl esters, milk fat, olive oil, palm oil, sterols, sunflower oil, unsaponifiable, vegetable oil.

In the study of fatty acid compositions of fats and oils, it is often impossible to detect low percentages of different oils in oil mixtures or to ascertain the origin of seed oils when they have been modified. Analysis of the composition of the minor components of the unsaponifiable fraction is useful both for identifying the sources of the various fatty acid materials and for revealing low percentages of certain oils in mixtures. The most important groups of these compounds are the sterols and triterpene alcohols. Their presence and concentrations vary over a narrower range than those of the fatty acids (1). The sterol fraction is analyzed for identification of a fat or an oil, and for detecting the addition of cheap oils to more expensive oils (2).

There is currently a great deal of interest in developing rapid methods for sterol analysis. Conventional methods involve saponification of oil or fat, with the unsaponifiable matter being extracted and analyzed directly (3–5) or after isolation of the sterols by preparative thin-layer chromatography and subsequent analysis by gas chromatography (GC) as free sterols or as trimethylsilyl derivatives (6–8). The sterol fraction also can be isolated by chromatography on a silica gel column (9), or by off-line high-performance liquid chromatography (10). Other analytical alternatives are determination of free and esterified sterol esters in oils and fats by coupled liquid chromatography–gas chromatography (1,11), or analysis of free and esterified sterols in vegetable oil by capillary GC after methanolysis under different conditions, or again by using prepacked silica columns (12–14). The sterol fraction of milk fat can also be analyzed by direct injection of fat in GC (15).

This paper deals with the application of a GC procedure for rapid analysis of the total sterol fraction of vegetable oils, milk fat, or mixtures, to detect any admixture of sunflower to olive oil and any addition of vegetable oils to milk fat. The method, which involves alkali-catalyzed transesterification with KOH/methanol, was compared with saponification procedures with and without transformation of sterols into silyl derivatives prior to analysis. Repeatability of the assay and recovery of β-sitosterol are also evaluated.

EXPERIMENTAL PROCEDURES

Standard and chemical reagent. Cholesterol, β-sitosterol (99%) and silylation reagent, hexamethyldisilazane, were supplied by Sigma Chemical Co. (St. Louis, MO). Organic solvents and potassium hydroxide were of analytical reagent grade.

Vegetable oils and milk fat. Commercial samples of sunflower and olive oil were purchased from retail stores and used to test the repeatability and the percentage recoveries of the method. Palm oil from a previous collaborative study by the International Dairy Federation (IDF) was used. Anhydrous milk fat was obtained by melting butter at approximately 50°C. When the fat was limpid, it was filtered through phobic dry paper by following the Draft IDF Standard (16).

KOH/MeOH method (transesterification method of vegetable oil and milk fat). The procedure is based on the method proposed by Christopherson and Glass (17) for preparation of milk fat methyl ester by alcoholysis in an essentially nonalcoholic solution. Approximately 100 mg of vegetable oil,

*To whom correspondence should be addressed at Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n 33300 Villaviciosa, Asturias, Spain.

milk fat or mixtures of both, weighed to ±0.1 mg, was dissolved in 0.8 mL of hexane. Methanolic potassium hydroxide (2 M) (0.2 mL) was added. After stirring for 1 min and reposing for 15 min, the hexane layer was separated, and 0.5 µL of the hexane fraction was injected in the GC. For quantitative analysis, 40 µL of the hexane fraction was injected in the GC. For quantitative analysis, 40 µL of cholesterol (14.5 mg/10 mL hexane) was added to vegetable oils as internal standard.

Saponification method. The vegetable oil, anhydrous milk fat or mixtures (500 mg) was saponified with 25 mL methanolic potassium hydroxide (2 M) by boiling gently on a water bath for 1 h. The unsaponifiable matter was extracted with diethyl ether by following the FIL/ISO/AOAC procedure (6) and analyzed directly by GC as in the European Communities procedure (18).

GC conditions. GC analysis of sterols was performed on a Perkin-Elmer Model AutoSytem gas chromatograph (Perkin-Elmer Co., Beaconsfield, United Kingdom), equipped with flame-ionization detector and flow splitter. Analyses were performed with a WCOT fused-silica capillary column (25 m × 0.25 mm), coated with OV 17 TRI, film thickness 0.10 µm (J&W Scientific, Folson, CA). Experimental chromatographic conditions were: He carrier gas, flow rate 29.8 cm/s; isothermal column temperature, 280°C; injector temperature, 350°C; detector temperature, 350°C. Peak identification was carried out by comparison of relative retention times with those reported in the literature (3,12) and with retention times of standards.

RESULTS AND DISCUSSION

Internal standard. For vegetable oil analysis, the standard chosen was cholesterol, which is not naturally present in vegetable oils, elutes after methyl esters of fatty acids and does not interfere with other peaks in the chromatogram that appear between α-tocopherol and campesterol (Fig. 1). The mean response factor for β-sitosterol/cholesterol mixtures with (w/w) ratios of 0.28, 0.73, and 1 was 1.09. This response factor is similar to those reported by Alonso *et al.* (15) (1.05) in determination of cholesterol in milk fat by GC with direct injection for cholesterol/5α-cholestane, and also to those reported by Ulberth and Reich (5) who used GC and Casiraghi *et al.* (19) who used liquid chromatography. However, Plank and Horber (12) reported a higher response factor (1.45) for β-sitosterol than those found in our study, possibly due to the conditions and columns employed in their GC method.

Repeatability. The repeatability of the analytical method was verified by three determinations and three injections of the vegetable oils (olive and sunflower). Table 1 gives the percentage mean values and standard deviations for individual

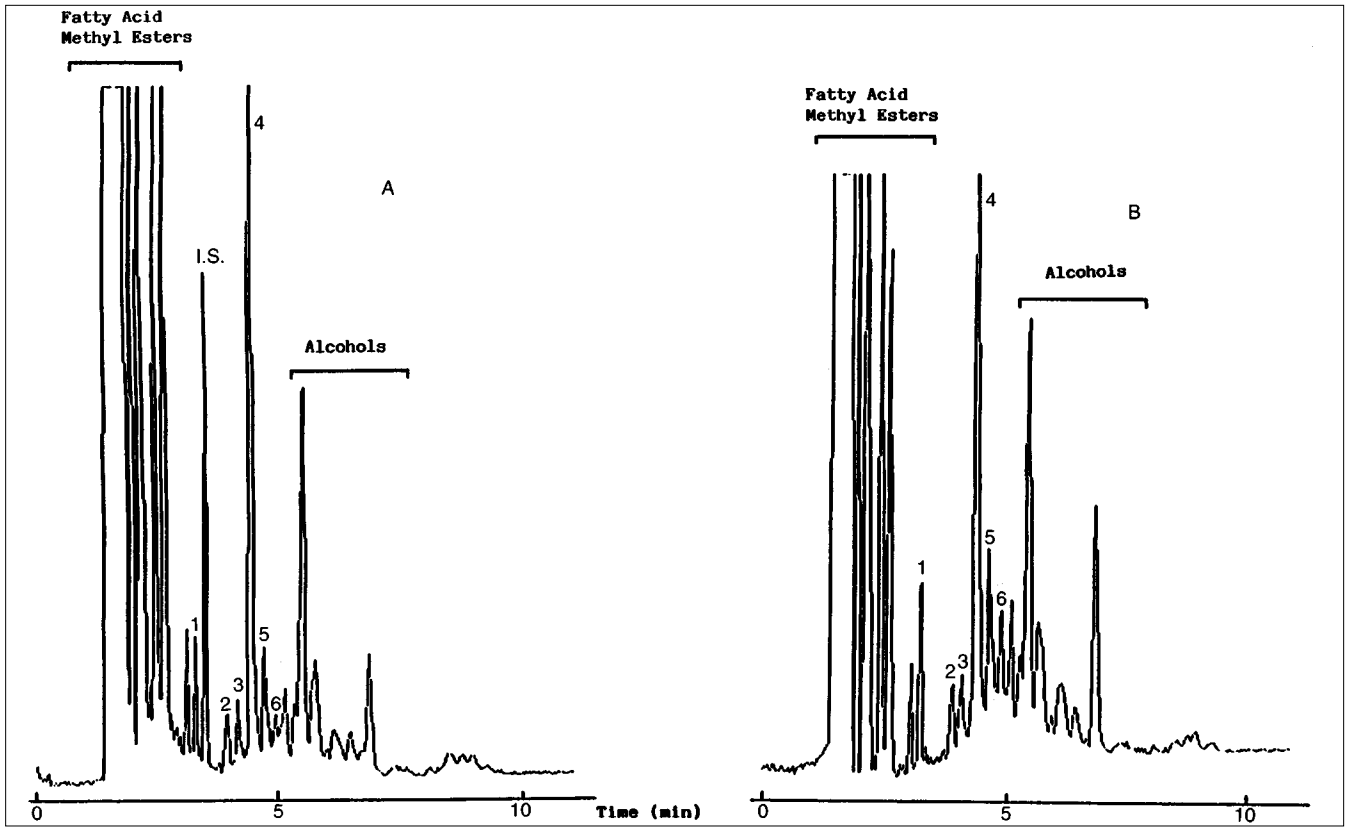

FIG. 1. Gas chromatographic analysis of olive oil (A) and olive oil with 10% of sunflower oil added (B) methyl esters with cholesterol as internal standard (I.S.). 1 = α-tocopherol; 2 = campesterol; 3 = stigmasterol; 4 = β-sitosterol; 5 = Δ^5-avenasterol; 6 = Δ^7-stigmasterol.

TABLE 1
Repeatability of the Determination of Individual Sterols[a] (as percentages) of Vegetable Oils (olive, sunflower, and palm) and Milk Fat or Mixtures by the KOH/Methanol Method

Sample	Campesterol	Stigmasterol	β-Sitosterol	Δ^5-Avenasterol	Δ^7-Stigmasterol
A: Olive oil	2.8 ± 0.6	2.4 ± 0.3	87.8 ± 5.9	6.9 ± 1.3	0.3 ± 0.1
B: Sunflower oil	9.3 ± 1.9	11.4 ± 2.6	60.3 ± 4.8	4.1 ± 1.1	14.9 ± 2.9
A + 5% B	3.0 ± 0.5	4.0 ± 0.8	86.3 ± 5.9	4.3 ± 0.8	2.4 ± 0.5
A + 10% B	4.0 ± 0.9	3.5 ± 0.4	83.1 ± 4.7	5.3 ± 1.1	4.2 ± 0.3
	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol	
C: Milk fat	100				
D: Palm oil	3.8 ± 0.4	23.3 ± 2.6	14.5 ± 1.5	58.6 ± 4.1	
C + 5% D	97.8 ± 6.5	0.6 ± 0.2	0.4 ± 0.1	1.2 ± 0.4	
C + 10% D	95.9 ± 6.8	1.3 ± 0.4	0.8 ± 0.3	2.0 ± 0.4	

[a]See Figure 1 for identification of sterols.

sterols (campesterol, stigmasterol, β-sitosterol, Δ^5-avenasterol, and Δ^7-stigmasterol). The overall mean value and standard deviation for β-sitosterol, which is the major sterol, was 87.8% ± 5.9 in olive oil and 60.3 ± 4.8 in sunflower oil, with coefficients of variation (CV) of 6.7 and 8.0, respectively. These results are comparable to those obtained by saponification and extraction of the unsaponifiable matter with direct analysis by GC without silylation: 90.7% ± 3.8 (CV: 4.2) for olive oil and 59.5% ± 3.1 (CV: 5.2) for sunflower oil (Table 2). Again, the results were similar when the samples of vegetable oils or mixtures of palm oil/milk fat were silylated and analyzed as trimethylsilyl (TMS) derivatives, although some artifacts appeared to interfere with the analysis (results not shown). Bortolomeazzi *et al.* (14) reported lower CV percentages for repeatability than in this paper, but they used a procedure where the extracted lipids were applied to a silica Sep-pak cartridge to obtain the sterol fraction analyzed as TMS derivatives. Nota *et al.* (13), who used the same methylation method (KOH/methanol), did not report CV results.

Recovery of GC sterol method. Recovery of β-sitosterol was carried out on sunflower and olive oils. Prior to this analysis, it was necessary to calculate the amounts (mg/kg) of β-sitosterol present in the oil samples, with cholesterol as internal standard. Once the β-sitosterol content of the oils had been determined, the recovery percentages were determined by adding to the oil samples a constant amount of β-sitosterol, approximately 50 and 100% with respect to the initial amounts of β-sitosterol. Recoveries were performed by three different addition assays and three replicate injections for each assay. Table 3 shows the initial β-sitosterol amounts, as determined for the transmethylation method, and the amounts of β-sitosterol added to the olive oil and sunflower oils for the recovery test. The mean percentage recoveries calculated according to the formula given by Ulmann's Encyclopedia (19) for 100% addition of β-sitosterol to the original vegetable oils were 95.8 ± 3.6 for olive oil and 94.4 ± 3.0 for sunflower oil. These results are slightly lower than in a recovery study of cholesterol added to sunflower and soya oils by Ulberth and Reich (5), who reported 102.7 ± 1.8% and 100.8 ± 2.1% for a direct saponification GC method. These slight differences in recoveries could be attributed to the different methods used to analyze the sterol fractions. In any event, greater accuracy could be achieved by applying response factors.

Application of the procedure. Quantitative comparison between these chromatograms indicates that the method can be used to determine the presence of sunflower oil in olive oil or of palm oil in milk fat, down to as low as 5%.

TABLE 2
Repeatability of the Determination of Individual Sterols[a] (as percentages) of Vegetable Oils (olive, sunflower, and palm) and Milk Fat or Mixtures by the Saponification Method

Sample	Campesterol	Stigmasterol	β-Sitosterol	Δ^5-Avenasterol	Δ^7-Stigmasterol
A: Olive oil	3.0 ± 0.6	1.3 ± 0.4	90.7 ± 3.8	4.5 ± 0.7	0.5 ± 0.2
B: Sunflower oil	8.7 ± 1.4	8.1 ± 1.9	59.5 ± 3.1	4.1 ± 1.7	19.7 ± 2.9
A + 5% B	3.5 ± 0.8	1.6 ± 0.4	87.5 ± 4.9	4.9 ± 1.6	2.1 ± 0.4
A + 10% B	4.2 ± 0.7	3.3 ± 0.6	84.9 ± 4.1	4.2 ± 1.1	3.4 ± 0.5
	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol	
C: Milk fat	100				
D: Palm oil	4.3 ± 0.6	23.2 ± 3.1	14.1 ± 1.2	58.4 ± 4.9	
C + 5% D	98.4 ± 6.2	0.4 ± 0.1	0.2 ± 0.1	1.0 ± 0.3	
C + 10% D	96.8 ± 6.4	0.8 ± 0.2	0.7 ± 0.2	1.7 ± 0.4	

[a]See Figure 1 for identification of sterols.

TABLE 3
Recovery of β-Sitosterol Added to Olive and Sunflower Oils After KOH/Methanol Method by Gas Chromatography

	β-Sitosterol concentration (mg kg^{-1} fat)							
	Initial	Amount added[a]		Calculated total		Recovered		
Sample	amount	a	b	a	b	a	b	(%)[b]
Olive oil	877 ± 21	459	918	1336	1795	1310 ± 48	1756 ± 62	94.3/95.8
Sunflower oil	1267 ± 35	515	1029	1782	2296	1754 ± 54	2248 ± 66	92.6/94.4

[a]a: Amount added ~50% initial amount. b: Amount added ~100% initial amount.
[b]Percentage calculated according to the formula given by Ulmann's Encyclopedia (Ref. 20).

Table 1 shows the results for the individual sterols (campesterol, stigmasterol, β-sitosterol, Δ^5-avenasterol, and Δ^7-stigmasterol) in different mixtures (5 and 10% of sunflower oil in olive oil and 5 and 10% of palm oil in milk fat), by using the transesterification method. As expected, Δ^7-stigmasterol was the individual sterol that provided the best discrimination for detection of sunflower oil in olive oil (Fig. 1). For 5 and 10% mixtures of sunflower in olive oil, the percentages of total Δ^7-stigmasterol with respect to the initial percentages for olive oil 0.27 ± 0.1 were 2.44 ± 0.5 and 4.17 ± 0.7. In milk fat with 5–10% added palm oil (Fig. 2), the percentage of cholesterol decreased 97.76 ± 6.5 and 95.88 ± 6.8 and vegetable sterols increased. Comparable results were obtained by saponification and direct GC analysis of the individual sterols of the unsaponifiable fraction (Table 2).

The GC method assayed in this work for analysis of sunflower mixed with olive oil and of vegetable oils added to milk fat offers good repeatability and recovery and is thus suitable for routine use as a rapid procedure, eliminating the need for saponification, extraction and derivatization steps and requiring only a short time for analysis.

ACKNOWLEDGMENTS

The authors acknowledge financial support from the research projects, FICYT (PA-AGR 94-02) and CICYT (ALI-95 0046-C02-01).

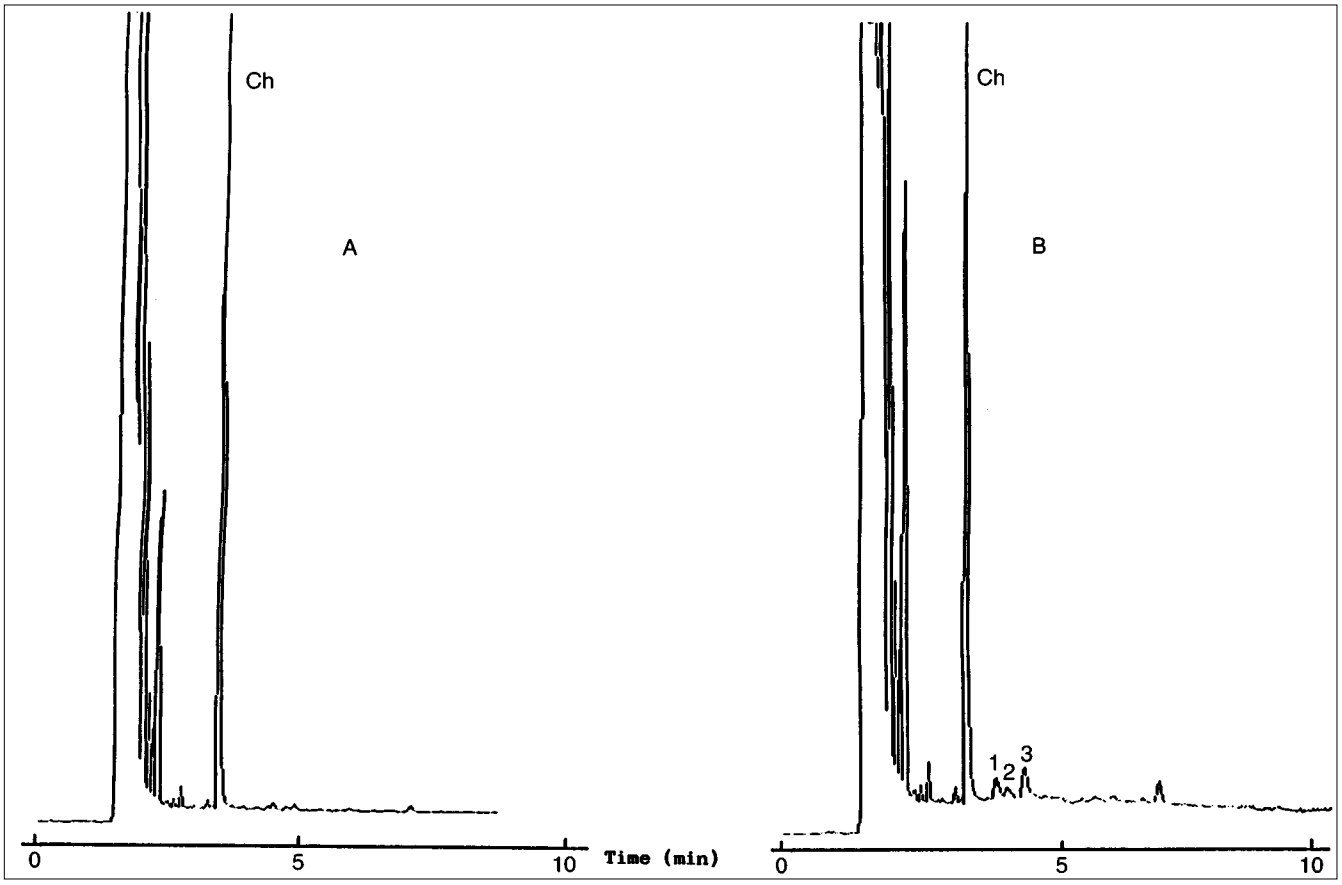

FIG. 2. Gas chromatographic profile of sterol fraction of milk fat (A) and milk fat with 10% of palm oil added (B) methyl esters. Ch = Cholesterol; 1 = campesterol; 2 = stigmasterol; 3 = β-sitosterol.

REFERENCES

1. Grob, K., and M. Lanfranchi, Determination of Free and Esterified Sterols and of Wax Esters in Oils Fats by Coupled Liquid Chromatography Gas Chromatography, *J. Chromatog. 471*:397–405(1989).
2. Homberg, E., and B. Bielefeld, Freie und Gebundene Sterine in Rohen und Raffinierten Palmolen, Teil I: Gehalt und Zusammensetzung der Freien Sterine, Sterinester, Freien und Acylierten Steringlucoside, *Fette Seifen Anstrichm. 84*:141–146 (1982).
3. Bonaga, G., A. Antonelli, L.S. Conte, and G. Lercker, Determinazione Contemporanea del Contenuto Assoluto di Steroli e di Dialcoli Triterpenici Negli Oli Alimentari, *Riv. Delle Sostanze Grasse 63*:583–590 (1986).
4. Frega, N., F. Bocci, and G. Lercker, Direct Gas Chromatographic Analysis of the Unsaponifiable Fraction of Different Oils with a Polar Capillary Column, *J. Am. Oil Chem. Soc. 69*:447–450 (1992).
5. Ulberth, F., and H. Reich, Gas Chromatographic Determination of Cholesterol in Processed Foods, *Food Chem. 43*:387–391 (1992).
6. International Dairy Federation, Milk Fat and Milk Fat Products. Determination of Cholesterol Content, Provisional FIL-IDF Standard, Brussels, 159, 1992.
7. Sciancalepore, V., and A. de Giglio, The Content and Composition of Free and Esterified Sterols in Sunflower Seed Oil, *Industrie Alimentari 19*:503–506 (1980).
8. Lercker, G., and M.F. Caboni, Analisi Degli Insaponificabili Mediante GLC, *Riv. Ital. Sostanze Grasse 62*:193–198 (1985).
9. Wothington, R.E., and H.L. Hitchcock, A Method for the Separation of Seed Oil Steryl Esters and Free Sterols: Application to Peanut and Corn Oils, *J. Am. Oil Chem. Soc. 61*:1085–1088 (1984).
10. Iatrides, M.C., J. Artaud, and M. Derbesy, Automated Preparation of Analytical Columns for Liquid Chromatography. Application to Natural Substances: Anethol, Vanillin, Sterols, *Analysis 12*:205–208 (1984).
11. Scott, R.P.W., and S. Traiman, *J. Chromatogr. 196*:193 (1980).
12. Plank, C., and E. Lorbeer, Analysis of Free and Esterified Sterols in Vegetable Oil Methyl Esters by Capillary GC, *J. High Res. Chromatogr. 16*:483–487 (1983).
13. Nota, G., S. Spagna Musso, R. Romano, D. Naviglio, and C. Impronta, Idrolisi Rapida degli Esteri Degli Steroli nei Grassi, *Riv. Ital. Sostanze Grasse 72*:315–316 (1995).
14. Bortolomeazzi, R., N. Frega, and G. Lercker, Rapid Determination of Free and Esterified Sterols Using Prepacked Silica Columns, *Ital. J. Food Sci. 4*:265–268 (1990).
15. Alonso, L., L. Lozada, J. Fontecha, and M. Juárez, Determination of Cholesterol in Milk Fat by Gas Chromatography with Direct Injection and Sample Saponification, *Chromatographia 41*:23–28 (1995).
16. International Dairy Federation, Method for the Extraction of Lipids and Liposoluble Compounds from Milk and Dairy Products, Group E49, Draft FIL-IDF Standard, Brussels, 1990.
17. Christopherson, S.W., and R.L. Glass, Preparation of Milk Fat Methyl Esters by Alcoholysis in an Essentially Nonalcoholic Solution, *J. Dairy Sci. 52*:1289–1290 (1969).
18. *Official Journal of the European Communities,* Establishing a Reference Method for the Determination of Sitosterol and Stigmasterol in Butteroil, Commission Regulation EEC Nº3942, 29-38, 1992.
19. Casiraghi, E., M. Lucisano, C. Pompei, and C. Dellea, Cholesterol Determination in Butter by High-Performance Chromatography, *Milchwissenchaft 49*:194–196 (1994).
20. *Ulmann's Encyclopedia of Industrial Chemistry: Analytical Methods I,* Vol. 35, 5th edn., edited by B. Elvers, S. Hawkins, and W. Russey, VCHY, Weinheim, 1994, p. 54.

[Received April 1, 1996; accepted September 23, 1996]