Routine High-Performance Liquid Chromatographic Determination of Free 7-Ketocholesterol in Some Foods by Two Different Analytical Methods

G. Penazzi[a,*], M.F. Caboni[a], P. Zunin[b], F. Evangelisti[b], E. Tiscornia[b], T. Gallina Toschi[a], and G. Lercker[a]

[a]Istituto di Industrie Agrarie, 40126 Bologna and [b]Istituto di Analisi e Tecnologie Farmaceutiche, 16147 Genova, Italy

ABSTRACT: Two methods for routine analysis of free 7-ketocholesterol (7-k) in foods by high-performance liquid chromatography (HPLC) were compared. The analyzed samples were egg noodles, biscuits prepared with eggs, sweet snacks, grated Parmesan cheeses, and some ingredients utilized in the food industry (whole-milk and whole-egg powder). The enrichment of cholesterol oxides was carried out by solid-phase separation of the total lipids with florisil and silica cartridges for methods A and B, respectively. The 7-k analyses were run in normal-phase HPLC for method A, and a reverse-phase process was used in method B. Identification of the 7-k peak was confirmed by gas chromatography/mass spectrometry analysis and by peak purity check *via* spectral analysis with a diode array detector. The quantitation of 7-k was carried out with an internal standard for method A and with a calibration curve for method B. The limit of quantitation (LQ) was 3×10^{-9} g/injection; the limit of detection was ten times lower than the LQ for both methods. The two methods showed good recovery (99%) and good repeatability (coefficient of variation of 3.9 and 3.7% for methods A and B, respectively). These methods allow a fast, sensitive, and reliable determination of one cholesterol oxidation product, which is present at a high concentration level in the first stages of the oxidation process.

JAOCS 72, 1523–1527 (1995).

KEY WORDS: Animal fats, biscuits, cholesterol, egg noodles, grated cheese, high-performance liquid chromatography, 7-ketocholesterol, oxysterols, whole-egg powders, whole milk powder.

Cholesterol is prone to oxidation in the presence of air and energy (heat and/or ultraviolet light); in fact, the complete or partial structure of over sixty cholesterol oxidation products (COP) has been reported (1). Several COP show biological and toxicological activity, such as feedback inhibition of cholesterol biosynthesis, angiotoxicity, atherogenicity, mutagenicity, cytotoxicity, and carcinogenicity (2–4). Various analytical procedures have been set up for the determination of COP by gas chromatography (GC) (5–11) and high-performance liquid chromatography (HPLC) (12–17). However, most of these approaches are designed for research work rather than for routine analysis.

HPLC analysis eliminates the traditional acid or alkaline hydrolysis steps (18) that can lead to modifications of the oxysterol content (19,20), especially those observed in 7-ketocholesterol (7-k) due to basic hydrolysis (21–23). Cold saponification (4,10) results in a high sample grade, which is important in GC analysis (24), as well as in the release of esterified cholesterol. However, this analytical approach was ruled out *a priori* in the present study because it is a time-consuming technique.

The objective of the present study was to test two HPLC methods, one in normal-phase and the other in reverse-phase, as routine analytical procedures for the determination of the extent of cholesterol oxidation in various foods. Both approaches require the quantitation of free 7-k, which is employed as a tracer of the oxidative process in that it is one of the first COP to form and one of the oxides found in the highest amounts (7,12,25–27). The tested samples were ingredients utilized in industrial and small-scale food processing, such as whole-milk powder and spray-dried whole-egg powder, as well as mass-produced packaged foods, such as egg noodles (pasta), biscuits, sweet snacks, and grated cheeses that are considered "at risk" with regard to cholesterol oxidation (28).

MATERIALS AND METHODS

Chemicals. HPLC- or analytical-grade reagents and solvents were supplied by Carlo Erba (Milano, Italy), and anhydrous pyridine by Merck (Darmstadt, Germany). The HPLC solvents were used after filtration with an all-glass filter holder (supplied by Millipore Corp., Bedford, MA), through a 0.45-μm Phenomenex nylon filter membrane (Torrance, CA).

Cholesterol (5-cholesten-3β-ol) and 7-k (5-cholesten-3β-ol-7-one) standards were supplied by Sigma (St. Louis, MO), and 7-ketopregnenolone (5-pregnen-3β-ol-7,20-dione) by Steraloids (Wilton, NH). The solid-phase extraction (SPE)

*To whom correspondence should be addressed at Istituto di Industrie Agrarie, via S. Giacomo 7, 40126, Bologna, Italy.

columns were packed with florisil and silica purchased from Analytichem International-Varian (Harbor City, CA). The sorbent mass of both types of cartridges was 500 mg.

Samples. The following mass-produced packaged foods were purchased in an Italian supermarket and analyzed in groups of three samples each: egg noodles, biscuits containing eggs, sweet snacks, and grated Parmesan cheeses. Three samples of whole-milk powder and three of whole-egg powder, used as ingredients in the small- and large-scale food industries, were supplied by the processor.

Lipid extraction. The method by Folch *et al.* (29) was used for lipid extraction from biscuits, snacks, and cheeses. The powdered products were extracted as suggested by Bligh and Dyer (30), after addition of water to the samples.

Method A: sample preparation. The enrichment of cholesterol oxides was carried out on SPE florisil cartridges. 7-Ketopregnenolone (1.5 μg) was added to 50–60 mg of the extracted lipids. This mixture was then dissolved in 0.5 mL of 2-propanol/*n*-heptane (2%, vol/vol) and loaded onto an SPE florisil cartridge, which had been previously washed with *n*-heptane. The cartridge was then washed with 4 mL of 2-propanol/*n*-heptane (2%, vol/vol) to remove triacylglycerols, together with most of the cholesterol. The cholesterol oxides were eluted with 5 mL acetone. The solvent was subsequently evaporated under a nitrogen stream at <50°C, and the residue was dissolved in 1 mL of 2-propanol/*n*-hexane (7%, vol/vol).

Normal-phase HPLC. HPLC was performed with a Perkin-Elmer (Norwalk, CT) Series 400 liquid chromatograph with Rheodyne 7125 injector (Cotati, CA) and a Hewlett-Packard 1050 diode array detector (Waldbronn, Germany). The column was a μPorasil type (Water Associates, Milford, MA), with 30 cm × 3.9 mm i.d. and 10-μm particle size (12).

HPLC elution was carried out under isocratic conditions with a mobile phase of 2-propanol/*n*-hexane (7%, vol/vol) at a 1-mL/min flow rate. The wavelength for 7-k determination was 233 nm.

Method B: sample preparation. The enrichment of cholesterol oxides was carried out on SPE silica cartridges. Lipids (100 mg) were dissolved in 0.5 mL of *n*-hexane/diethyl ether (8:2, vol/vol) and applied onto a silica cartridge which had been prewashed with 3 mL *n*-hexane. The elution was carried out with 3 mL of *n*-hexane/diethyl ether (8:2, vol/vol), 4 mL of *n*-hexane/diethyl ether (1:1, vol/vol), and 3 mL of methanol (31). Chromatography was accelerated with vacuum. The last two fractions were pooled, the solvent was evaporated under a low-temperature nitrogen stream at <50°C, and the residue was dissolved in 1 mL acetonitrile.

Reverse-phase HPLC. HPLC was carried out with an S5 C6 column (15 cm × 4.6 μm i.d., 5-μm spherical particles; Spherisorb Phase Sep, Deeside, United Kingdom), a chromatograph connected to a Knauer pump (Berlin, Germany), and a Rehodyne 7125 injector, a Knauer variable ultraviolet wavelength spectrophotometer, and a Spectra Physics SP 4270 integrator (San Jose, CA).

HPLC elution was accomplished under isocratic conditions with a mobile phase of acetonitrile/water (4:1, vol/vol) at a 0.8-mL/min flow rate. The ultraviolet-wavelength for 7-k detection was 245 nm.

GC/mass spectrometry (MS). The HPLC fraction matching the 7-k peak was collected, the solvent was eliminated, and the residue was treated by silanization as suggested by Sweeley *et al.* (32). Analyses were run on a Carlo Erba (Rodano, MI, Italy) QMD 1000 GC/MS system. The column was a capillary fused-silica unit coated with SE 52 (25 m × 0.32 mm i.d., 0.10-μm film thickness; Mega, Milano, Italy). Temperature was programmed from 60 to 260°C at 25°C/min and from 260 to 310°C at 2°C/min. The carrier gas (helium) flow rate was 2 mL/min. The transfer line temperature was 250°C, and the source temperature was 200°C. Ionization energy was 70 eV. Mass spectra were scanned within mass range *(m/z)* 40–600; scan speed was 0.9 scan s^{-1}.

Method viability test. The recovery trials were run by loading the SPE columns with known amounts of the 7-k standard, eluting as described previously and determining the amount of recovered 7-k by GC. The stability of 7-k was also determined by running known amounts of its standard through all experimental procedures and analyzing the results with GC.

GC. GC analyses were run on a Carlo Erba model 4160 with a flame-ionization detector and a Spectra Physics SP 4270 integrator. The capillary fused-silica column was coated with SE 52 (25 m × 0.32 mm i.d., 0.10-μm film thickness; Mega). Temperature was programmed from 220 to 310°C at 4.5°C/min; the injector and detector temperature was 350°C.

RESULTS AND DISCUSSION

There are several differences between various steps of the two tested methods. Sample preparation and COP enrichment by SPE were carried out with florisil cartridges in method A, and silica cartridges were used in method B. HPLC analysis in method A was performed in normal-phase, and reverse-phase was utilized in method B. The 7-k quantitation in method A was done with 7-ketopregnenolone as the internal standard; for method B, the amount of 7-k was calculated from the calibration curve.

Both types of cartridges gave more than 99% on the 7-k recovery tests, resulting in a similar efficiency; the recovery obtained with the silica cartridge agrees with the data published by Chen and Chen (15). Analysis also showed that 7-k did not form during sample purification, and that it was neither decomposed nor transformed during the experimental steps, the latter being a rather important feature in other types of sample treatment (18). The coefficients of variation of the methods (CV%) were calculated on four replicates of the same sample, giving 3.90 and 3.75% for methods A and B, respectively.

Table 1 shows the amounts of 7-k (ppm in lipids) found in the foods analyzed by the two HPLC method; this table also lists the per-sample SD and the CV% for the values recorded

TABLE 1
Amounts of 7-Ketocholesterol Found in Various Foods Analyzed by the Two High-Performance Liquid Chromatography Methods

Sample	7-Ketocholesterol (ppm in lipids)			
	Method A	Method B	SD	CV%[a]
Egg noodles (pasta)				
1	16.1	16.5	0.3	1.7
2	19.4	21.6	1.6	7.6
3	15.1	14.4	0.5	3.4
Biscuits				
1	3.8	3.6	0.1	3.8
2	3.5	3.7	0.1	3.9
3	26.8	27.1	0.2	0.8
Sweet snacks				
1	3.5	3.8	0.2	5.8
2	1.8	2.0	0.1	7.4
3	4.4	4.7	0.2	4.7
Grated cheeses				
1	2.6	2.2	0.3	11.8
2	1.3	1.6	0.2	14.6
3	1.5	1.5	0.0	0.0
Whole-milk powders				
1	3.2	2.9	0.2	7.0
2	1.2	1.1	0.1	6.1
3	2.5	2.8	0.2	8.0
Whole-egg powders				
1	20.9	22.2	0.9	4.3
2	20.2	23.4	2.3	10.4
3	58.0	61.8	2.7	4.5

[a]CV% means coefficient of variation.

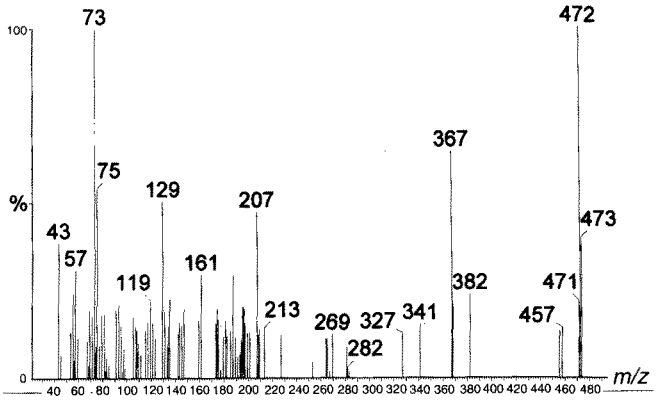

FIG. 1. Mass spectrum of 7-ketocholesterol found in a biscuit sample. Ionization energy was 70 eV. Mass spectra were scanned within mass range *(m/z)* 40–600; scan speed was 0.9 scan s^{-1}.

by each method. The two data sets indicate that the results for the tested samples, which differed by nature and by complexity of the lipid matrix, are in good agreement; the CV% ranged from a minimum of 0 to a maximum of 14.6. The grated-cheese data are quite different from those reported by other authors (28,33), in which 7-k was found in one out of six samples. This difference was probably due to a loss of 7-k during saponification because other cholesterol oxides were detected in those samples. On the other hand, the 7-k values for whole-egg powders are similar to those obtained in other studies (28,34), as well as to those found in the egg noodle samples; it is possible that these egg noodles were prepared with egg powder because cholesterol oxides have not been found in fresh eggs (35).

Regarding the whole-milk powder samples, the amount of 7-k found are lower than those reported for 12-month-old whole-milk powders (25).

The correlation coefficient of 7-ketopregnenolone, method A's internal standard for 7-k quantitation, was close to 1 (r^2 = 0.9984); this coefficient was calculated by multiple-level calibration.

The calibration curve of the 7-k standard, whose concentration ranged from 4×10^{-9} to 12×10^{-8} g/injection, was the quantitation procedure followed in method B; in this case, the linear coefficient was also close to linearity (r^2 = 0.9996).

Identification of the 7-k peak was confirmed by GC/MS analysis and by peak purity check *via* spectral analysis. Figure 1 shows the mass spectrum of 7-k found in a biscuit sample, which agrees with that reported by Osada *et al.* (36). Because cholesterol is present in small amounts in the lipid fraction of foods from animal origin, COP were found only in trace amounts. Given its sensitivity and selectivity, HPLC with ultraviolet detection proved to be useful in determining small amounts of 7-k in foods because the latter's limit of quantitation (LQ) was 3×10^{-9} g/injection, and its limit of detection was ten times lower than its LQ for both methods. Its high selectivity derives from the fact that one can use relatively selective wavelengths (233, 245 nm). The same selectivity cannot be achieved with most COP that form in the first stages of oxidation because they absorb at lower wavelengths (around 210 nm), thereby increasing the background noise (12).

A sensitivity range in the order of 10^{-12} g has been achieved with techniques such as GC/MS; however, sample preparation steps to isolate sterols, such as cold saponification, are more time-consuming and laborious than those reported in the present study. HPLC analysis simplifies sample preparation because it uses SPE, which is an easier and faster method than cold saponification. Nevertheless, it is important to control each type of matrix by structural analysis (i.e., GC/MS) or diode array detector to ensure the absence of any compound with a similar HPLC elution time as that of the 7-k peak.

The two methods proposed here only quantitate free 7-k; the free sterol fraction is present in a much higher level than the esterified one, and their ratio varies according to the matrix (12). However, if the esterified fraction is to be quantitated, a cold saponification must be carried out.

Figures 2 and 3 show the HPLC traces of a sample of egg noodles analyzed by method A and a sample of biscuits analyzed by method B. Good separation of the 7-k peak was achieved with both methods.

The overall determination in the present study with the two methods takes about 45 min from lipid extraction to final data. This analytical approach allows a faster quantitation of a large number of samples than more complex methods, and gives good sensitivity and reproducibility levels.

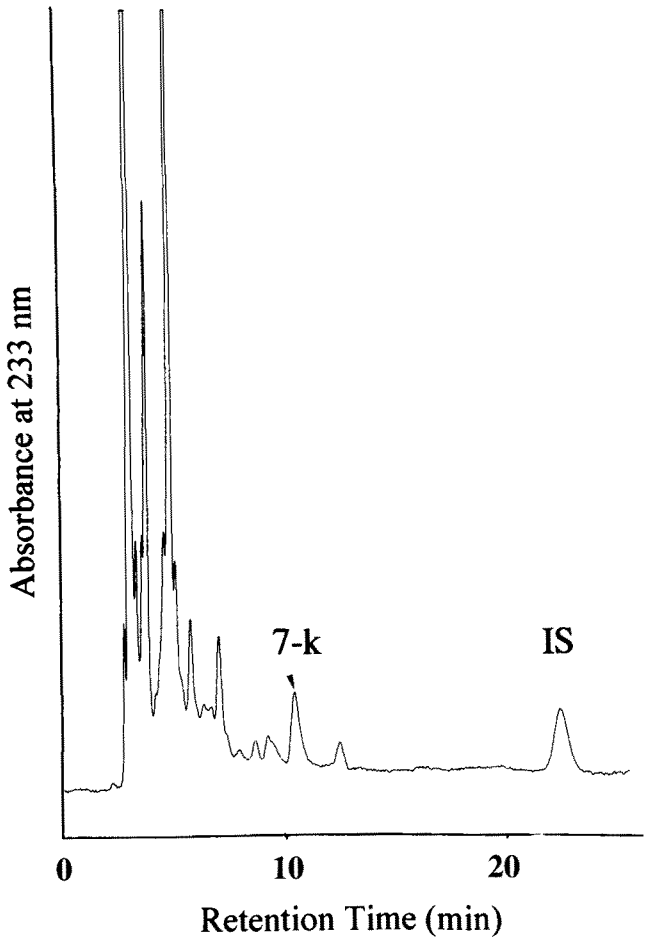

FIG. 2. High-performance liquid chromatographic trace of a sample of egg noodles (pasta) analyzed by method A. Conditions: column b, μPorasil (30 cm × 3.9 mm i.d., 10-μm particle size); elution, 2-propanol/*n*-hexane (7%, vol/vol) under isocratic conditions; flow, 1 mL/min; diode array detector at 233 nm. Abbreviations: 7-k, 7-ketocholesterol; IS, internal standard.

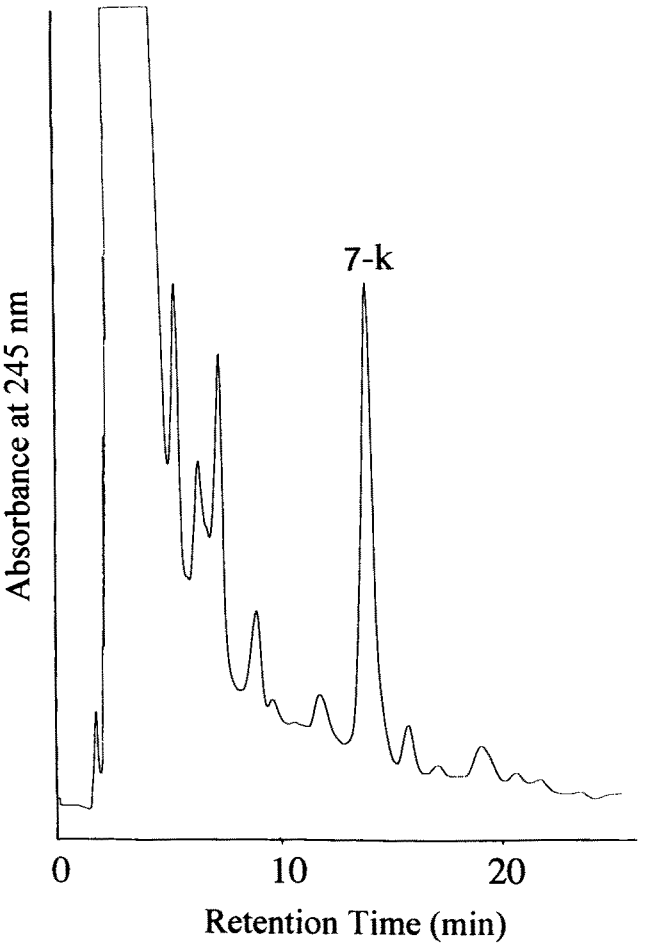

FIG. 3. High-performance liquid chromatographic trace of a sample of biscuits analyzed by method B. Conditions: column, S5 C6 (15 cm × 4.6 mm i.d., 5-μm spherical particles); elution, acetonitrile/water (4:1, vol/vol) under isocratic conditions; flow, 0.8 mL/min; ultraviolet-wavelength spectrophotometer at 245 nm. Abbreviations as in Figure 2.

REFERENCES

1. Smith, L.L., in *Cholesterol Autoxidation,* Plenum Press, New York, 1981.
2. Addis, P.B., and G.J. Warner, in *Free Radicals and Food Additives,* edited by O.I. Aruoma and B. Halliwell, Taylor and Francis Ltd., London, 1991, p. 77.
3. Bösinger, S., W. Luf, and E. Brandl, in *International Dairy Journal,* Vol. 3, edited by P.F. Fox and P. Jelen, Elsevier Science Publishers Ltd., England, 1993, p. 1.
4. Addis, P.B., H.A. Emanuel, S.D. Bergmann, and J.H. Zavoral, *Free Radical Biology & Medicine 7*:179 (1989).
5. Morgan, J.N., and D.J. Armstrong, *J. Food Sci. 54*:427 (1989).
6. Park, S.W., and P.B. Addis, *Anal. Biochem. 149*:275 (1985).
7. Pie, J.E., K. Spahis, and C. Seillan, *J. Agric. Food Chem. 38*:973 (1990).
8. Park, S.W., and P.B. Addis, *Ibid. 34*:653 (1986).
9. Park, S.W., and P.B. Addis, *J. Food Sci. 51*:1380 (1986).
10. Zhang, W.B., P.B. Addis, and T.P. Krick, *Ibid. 56*:716 (1991).
11. Emanuel, H.A., C.A. Hassel, P.B. Addis, S.D. Bergmann, and J.H. Zavoral, *Ibid. 56*:843 (1991).
12. Park, S.W., and P.B. Addis, *Ibid. 50*:1437 (1985).
13. Sugino, K., J. Terao, H. Murakami, and S. Matsushita, *J. Agric. Food Chem. 34*:36 (1986).
14. Saari Csallany, A., S.E. Kindom, P.B. Addis, and Joo-Hee Lee, *Lipids 24*:645 (1989).
15. Chen, B.H., and Y.C. Chen, *J. Chromatogr. 661*:127 (1994).
16. Smith, L.L., *J. Liquid Chromatography 16*:1731 (1993).
17. Sevanian, A., R. Seraglia, P. Traldi, P. Rossato, F. Ursini, and H. Hodis, *Free Radical Biology & Medicine 17*:397 (1994).
18. Park, S.W., and P.B. Addis, in *Biological Effects of Cholesterol Oxides,* edited by M.D.S.-K. Peng and R.J. Morin, CRC Press, Boca Raton, 1992, p. 33.
19. Chicoye, E., W.D. Powrie, and O. Fennema, *Lipids 3*:335 (1968).
20. Tsai, L.S., K. Ijichi, C.A. Hudson, and J.J. Meehan, *Ibid. 15*:124 (1980).
21. Bergstrom, S., and O. Wintersteiner, *J. Biol. Chem. 141*:597 (1941).
22. Chicoye, E., W.D. Powrie, and O. Fennema, *Lipids 3*:551 (1968).
23. Maerker, G., and J. Unruh Jr., *J. Am. Oil Chem. Soc. 63*:767 (1986).
24. Caboni, M.F., T. Gallina Toschi, G. Lercker, and P. Capella, *Riv. Ital. Sost. Grasse 71*:243 (1994).
25. Nourooz-Zadeh, J., and L.-A. Appelqvist, *J. Food Sci. 53*:74 (1988).

26. Caboni, M.F., C. Zullo, O. Boschelle, G. Lercker and P. Capella, in *Proceedings of the Fifth European Conference on Food Chemistry,* FECS event no. 150, INRA Ed. Paris, Versailles, 1989, p. 131.
27. Pie, J.E., K. Spahis and C. Seillan, *J. Agric. Food Chem. 39*:250 (1991).
28. Sander, B.D., P.B. Addis, S.W. Park and D.E. Smith, *J. Food Prot. 52*:109 (1989).
29. Folch, J., M. Lees and G.H. Sloane-Stanley, *J. Biol. Chem. 726*:497 (1957).
30. Bligh, E.G., and W.J. Dyer, *Can. J. Biochem. Physiol. 37*:911 (1959).
31. Bortolomeazzi, R., N. Frega and G. Lercker, *Ital. J. Food Sci. 4*:265 (1990).
32. Sweeley, C.C., R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc. 85*:2497 (1963).
33. Finocchiaro, E.T., K. Lee and T. Richardson, *J. Am. Oil Chem. Soc. 61*:877 (1984).
34. Missler, S.R., B.A. Wasilchuk and C. Merritt, Jr., *J. Food Sci. 50*:595 (1985).
35. Nourooz-Zadeh, J., and L-A. Appelqvist, *Ibid. 52*:57 (1987).
36. Osada, K., T. Kodama, K. Yamada and M. Sugano, *J. Agric. Food Chem. 41*:1198 (1993).

[Received December 2, 1994; accepted September 4, 1995]