ACKNOWLEDGMENTS

Calvin J. Dooley operated GC-MS and MS, and Leonard S. Silbert furnished standard γ-stearolactone (100%) and participated in helpful discussions.

REFERENCES

1. Stirton, A.J., B.B. Schaeffer, A.A. Stawitzke, J.K. Weil and W.C. Ault, JAOCS 25:365 (1948).
2. Roe, E.T., W.E. Parker and D. Swern, JAOCS 36:656 (1959).
3. Eisner, A., T. Perlstein and W.C. Ault, JAOCS 40:594 (1963).
4. Ault, W.C., and A. Eisner, JAOCS 39:132 (1962).
5. Eisner, A., T. Perlstein and W.C. Ault, JAOCS 39:290 (1962).
6. Nakano, Y., and T.A. Foglia, JAOCS 61:569 (1984).
7. Barrett, F.O., and C.G. Goebel, U.S. Patent 3,074,983 (1963).
8. Sakauchi, N., and E.C. Horning, Anal. Lett. 4:41 (1971).
9. Benesi, H.A., J. Phys. Chem. 61:970 (1957).
10. Loev, B., and J.T. Massengale, J. Org. Chem. 22:988 (1957).
11. Smith, F.D., H.E. Kenney and A.J. Stirton, J. Org. Chem. 30:885 (1965).
12. Smith, F.D., A.J. Stirton and C.J. Dooley, JAOCS 45:747 (1968).
13. Smith, F.D., and A.J. Stirton, JAOCS 48:160 (1971).

[Received December 1, 1983]

♣Triglyceride Composition of Olive Oil, Cottonseed Oil and Their Mixtures by Low Temperature Crystallization and Gas Liquid Chromatography

S. SYNOURI-VRETTAKOU, Department of Food Chemistry, University of Athens, Athens, Greece, **M.E. KOMAITIS,** Department of Food Science, University of Leeds, Leeds LS2 9JT, United Kingdom and **E.C. VOUDOURIS,** Department of Food Chemistry, University of Ioannina, Ioannina, Greece

ABSTRACT

Crystallization and gas liquid chromatography (GLC) have been used to characterize the triglyceride composition of olive and cottonseed oil and their precipitates from acetone or methanol/acetone (10:90, v/v) at -2 C. The precipitate obtained after a 24 hr crystallization of a 5% (w/v) solution of the sample in acetone or methanol/acetone (10:90, v/v) at -2 C was named Precipitate I (P-I); that isolated after 2 successive crystallizations under identical conditions was named Precipitate II (P-II). In each case, the ratio of oleic to linoleic acid (O/L) was calculated and proved to be a useful index for detecting adulteration of olive oil with cottonseed oil. In olive oil, the ratio O/L increased from the original sample to its precipitates, whereas in cottonseed oil and the adulterated samples the ratio O/L was lower in the precipitates than in the original sample. For olive oil P-II, the lowest value of the ratio O/L was 8.4; for the adulterated samples it was 7.6. On the basis of this index, adulteration of olive with cottonseed oil as low as 10% can be detected. Hydrolysis of P-I by porcine pancreatic lipase and analysis of the fatty acids of the sn-2 position showed that the enrichment factor of linoleic acid varied between 1.11-1.30 for olive oil and between 1.55-1.90 for the adulterated samples. Even for adulteration with 5% cottonseed oil, the enrichment factor appears to increase (1.55-1.57) and can be used as a criterion for adulteration.

INTRODUCTION

Triglyceride analysis has proved to be important in detecting the adulteration of olive oil with other vegetable oils. As early as 1960, Mangio (1) came to some useful conclusions on the presence of animal and other vegetable fats in olive oil. The application of reversed-phase thin layer (TLC) and paper chromatography revealed distinct differences in the chromatograms of pure olive oil and of samples adulterated with vegetable oil, which can be used for detecting the presence of low concentrations (10%) of vegetable oils in olive oil (2,3,4). Galanos et al. (5,6) have shown that differences in the triglyceride composition of vegetable oils can be used for detecting adulteration of olive oil. By fractionating olive, cottonseed, soybean, sesame and corn oil and their admixtures according to their unsaturation, they concluded that the fatty acid composition of the polyunsaturated triglyceride fraction is characteristic of each oil and that the ratio of oleic to linoleic acid (O/L) of this fraction can be used as a criterion for the presence of seed oils in olive oil (6). The sensitivity of this method was further improved by a combination of column chromatography on silicic acid impregnated with silver ions and gas liquid chromatography (GLC) (7). The use of high pressure liquid chromatography (HPLC) for the separation and identification of triglycerides present in fats and oils has been increasing in recent years. Peak identification can be employed for the detection of polyunsaturated seed oils in olive oil (8).

Enzymatic techniques have also been applied in elucidating triglyceride structure (9). Among the enzymes employed, mammalian pancreatic lipase, which preferentially catalyzes the hydrolysis of the ester linkages in the sn-1 and sn-3 positions, has been widely used to study the composition of fatty acids esterified at the sn-2 position. This technique has been applied in detecting the presence of reesterified fats in olive oil because the sn-2 position of olive-oil triglycerides is almost exclusively esterified with unsaturated fatty acids and the percentage of palmitic acid in this position should not exceed 2% (10).

Fractional crystallization from organic solvents was one of the earliest techniques used to separate natural fats into their components. Although no longer employed as a quantitative method, it is still applicable in large-scale fractionations of triglycerides. The semiquantitative nature of crystallization was immensely improved by combining it with GLC.

The solvent most extensively used has been anhydrous acetone because triglycerides having different numbers of saturated acyl-groups and double bonds exhibit different solubilities in this solvent. For separation of more polar triglycerides, methanol has proved to be a useful solvent. Systematic fractional crystallization of fats from acetone at various low temperatures and analysis of the fractions produced has been used by many workers as a means of estimating the principal classes of triglycerides (11).

In this study, an attempt was made to determine differences of the triglyceride composition of olive and cot-

tonseed oil, which may be applied in detecting the presence of cottonseed in olive oil. This was effected by using a 2-step crystallization procedure and by studying the composition of the triglyceride fractions isolated by argentation TLC and GLC. For the same purpose, pancreatic lipase hydrolysis was used as an additional means of investigating differences in the triglyceride structure of the 2 oils.

EXPERIMENTAL PROCEDURES

Materials

Samples of virgin olive oil and cottonseed oil were obtained from the chemical laboratory of the Greek Ministry of Commerce or from various factories.

Methods

Samples of the vegetable oils were dissolved in anhydrous acetone or acetone/methanol (90:10, v/v) and the mixture was cooled in a constant-temperature cabinet at 2 C for 24 hr. At the end of the cooling period, the precipitate was removed by filtration through a precooled gooch crucible 3G3 and washed twice with anhydrous acetone precooled at a temperature 5 C lower than that of the crystallization temperature. Quantitative recovery of the precipitate from the gooch crucible was achieved by dissolving it in a mixture of diethyl ether/petroleum ether (50:50, v/v).

The isolated triglycerides were fractionated according to their unsaturation by preparative TLC using glass plates layered with Silica Gel G impregnated with ammoniacal silver solution (12). The solvent systems used for the development of the plates were benzene/chloroform/ethanol (90:10:0.5, v/v/v) and benzene/diethyl ether (90:10, v/v). These solvent systems proved to be most successful in separating triglycerides containing less than 4 double bonds (see Table I). The plates were visualized under ultraviolet (UV) light after being sprayed with an ethanolic solution of 2',7'-dichlorofluorescein (0.02% w/v). Each triglyceride band was scraped off the plate and transferred quantitatively into a gooch crucible 1G3 containing 2-3 g of activated silicic acid (13). The microcolumn formed was eluted with 10 mL of diethyl ether/hexane (50:50, v/v). The triglycerides were wholly recovered in the eluant, whereas the silicic acid prevented any elution of the 2',7'-dichlorofluorescein.

After removing the solvent under a stream of nitrogen, the triglyceride content of each band was determined according to the method of Snyder and Stephens (14).

Fatty acid methyl esters were prepared according to the method of Morrison and Smith (15) and analyzed on a Perkin Elmer 900 dual column chromatograph equipped with a flame ionization detector (FID), using a 6 ft × 1/4 in. stainless-steel column packed with 20% DEGS on a 80/100 mesh Chromosorb W HMDS. Operating conditions were: carrier gas (nitrogen), 30 mL/min; column temperature, 175 C; temperature of detector and injector, 250 C. Qualitative analysis was effected by using methyl esters of lauric, myristic, palmitic, oleic, stearic and linoleic acids as standards and the relative percentage of the methyl esters was calculated from the peak areas by the integrator. The results are expressed as mol percentage.

Triglycerides were hydrolyzed with porcine pancreatic lipase (E.C. 3.1.1.3.) by the procedure of Luddy et al. (16). The 2-monoglycerides produced were separated from the other hydrolysis products by TLC on Silica Gel G using petroleum ether/diethyl ether/acetic acid (60:40:1.6, v/v/v). The 2-monoglyceride band was visualized with 2',7'-dichlorofluorescein and extracted 3 times with diethyl ether.

TABLE I

Fatty acid and triglyceride Composition of the Precipitates Obtained by Crystallization of Olive and Cottonseed Oil

Sample	Fatty acid composition (mol %)								Triglyceride[a] composition (mol %)				
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	S/O+P_0+L	O/L	F_0	F_1	F_2	F_3	F_4
Olive oil														
Original sample	trace	14.3	1.2	2.5	75.4	6.2	0.4	0.20	12.2	—	6.1	27.2	49.5	17.2
Precipitate (acetone)[b]				No precipitate obtained										
Precipitate (methanol/acetone [10:90, v/v])[b]	trace	28.5	0.7	4.9	61.6	4.3	trace	0.50	14.3	—	23.4	25.3	39.8	11.5
Cottonseed oil														
Original sample	1.1	29.7	0.4	1.5	19.5	47.8	—	0.48	0.41	—	7.7	18.5	18.6	55.2
Precipitate (acetone)[b]	1.1	56.1	0.2	2.9	10.8	28.9	—	1.51	0.37	4.9	19.0	44.0	13.5	18.6
Precipitate (methanol/acetone [10:90, v/v])[b]	1.1	55.3	0.2	3.9	11.1	28.4	—	1.52	0.39	4.5	18.1	43.0	14.6	9.8

[a]Triglycerides were fractionated according to their unsaturation by argentation TLC. GLC of Fractions 0, 1, 2, 3, 4 showed that Fraction 0 (F_0) contained trisaturated TG, Fraction 1 (F_1) contained TG with 1 double bond, Fraction 2 (F_2) contained TG with 2 double bonds and Fraction 3 (F_3) contained TG with 3 double bonds. Triglycerides containing more than 4 double bonds were not separated satisfactorily and therefore Fraction 4 (F_4) corresponds to TG containing 4, 5 and 6 double bonds.
[b]Precipitates were obtained after 24 hr crystallization of a 5% (w/v) solution of the sample in the solvent indicated at -2 C.

RESULTS AND DISCUSSION

Triglyceride analyses of olive oil (17-23) and of cottonseed oil (21,22, 24-28) have revealed distinct differences in their composition. Thus, cottonseed oil contains considerable amounts of the SLS, SSL and SLL triglycerides, which are either absent or found only in traces in olive oil. These differences appear to be particularly pronounced in the triglyceride fraction containing less than 2 double bonds. The major constituents of this fraction in olive oil are SSO, SOS and SOO, whereas in cottonseed oil significant amounts of SLS and SSL triglycerides as well as traces of SSS are found. The purpose of this work was the study of certain properties because of the presence of the aforementioned triglycerides, their determination and the application of these findings in the detection of adulteration of olive oil with cottonseed oil. Low temperature crystallization has been used as the preliminary step of obtaining fractions enriched in the more saturated triglycerides of the oil because this technique, in addition to its simplicity and low cost, has the advantage of giving satisfactory separations of the more saturated triglycerides and, moreover, does not alter their molecular structure.

Various samples were subjected to crystallization at 2 C for 25 hr from 5% (w/v), 7% (w/v), 10% (w/v) and 15% (w/v) solutions in anhydrous acetone or various mixtures of acetone/methanol. At the end of the cooling period, the precipitates formed were separated from the mother liquid as described above and their fatty acid and triglyceride composition were subsequently examined. The fatty acid and triglyceride analysis showed that the precipitates were most enriched with mono- and disaturated triglycerides when 5% (w/v) solutions in acetone or methanol/acetone (10:90, v/v) were used. This agrees with previous findings (29) and confirms that the best separations of triglycerides by fractional crystallization are obtained from dilute solutions of the fatty material. As shown in Table I, the precipitates are highly enriched with saturated fatty acids compared with the original oil. This is clearly illustrated by the ratio $S/O+P_0+L$, which has spectacularly increased in the precipitates of both the olive and cottonseed oil. The concentration of triglycerides with 1 double bond (SOS, SSO) has significantly increased, whereas the concentration of triglycerides with 3 or more double bonds (OOO, SOL, SLO, OOL, OLL) has decreased. Saturated triglycerides, which exist only in traces in cottonseed oil, have significantly increased in its precipitate. Moreover, whereas the more insoluble triglycerides (SSS, SSO, SOS, SLS, SSL, OSO) account for the 26.2% of the cottonseed oil triglycerides, they form 65.6-67.9% of the triglycerides in the precipitate. Another major difference is that, although the disaturated triglycerides in olive oil exist almost entirely in the SOO and OSO form (17, 18, 19, 20, 23), in cottonseed oil the major disaturated triglyceride is SLS (12.0-17.0% of the total triglycerides) (24, 27, 28). The difference in the triglyceride composition of the 2 oils may be that while in both vegetable oils the concentration of unsaturated fatty acids appears to decrease in the precipitate, in olive oil the relative decrease of linoleic acid is more pronounced than that of oleic acid, resulting in an increase of the ratio O/L from 12.2 (original sample) to 14.3 (precipitate). In cottonseed oil, the ratio O/L decreases from 0.41 to 0.37-0.39 (precipitate).

In all cases, the cooling period lasted 24 hr. At 2 C, olive oil from 5% (w/v) solutions in acetone or methanol/acetone (10:90, v/v) failed to produce any precipitate for cooling periods shorter than 15 hr. Accurate control of the cooling period is important in obtaining reproducible results because the amount of precipitate and its composition are influenced by a number of factors including supercooling, mutual solubility effects and soforth, which are partially dependent on the duration of the cooling period.

Temperatures between +5 C and -27 C were tested for the effect of the crystallization temperature on the enrichment of the precipitates in the more insoluble triglycerides. The precipitates isolated from cottonseed oil proved to be particularly rich in triglycerides with 2 or less double bonds (72.0-75.0% of the total triglycerides of the precipitate) at temperatures between +5 C and 0 C. However, under the same experimental conditions, none of the 12 olive-oil samples tested gave any precipitate at all. Triglyceride fractions obtained at temperatures lower than -4 C contained appreciable quantities of triglycerides with more than 3 double bonds; this is probably caused by coprecipitation of the more unsaturated triglycerides at these temperatures. Based on the above results, all further experiments were carried out from 5% (w/v) solutions of the samples in acetone or methanol/acetone (10:90, v/v) at 2 C for 24 hr.

Study of Precipitates I and II

Samples of olive oil, cottonseed oil and various mixtures of these 2 were crystallized from 5% (w/v) solutions in acetone or methanol/acetone (10:90, v/v), as described previously. After the first crystallization and removal of the formed precipitate (P-I), a portion was removed and subjected to a second crystallization under identical conditions. The second precipitate was called P-II. As seen in Table II, the weight of P-I is much higher for cottonseed oil, whereas in the mixtures of olive and cottonseed oil, the amount of P-I is related to their content in cottonseed oil. Youngs and Sallans (30) have shown that low-temperature crystallization separates triglycerides mainly on the basis of the number of the saturated acyl-groups rather than the degree of their unsaturation. The fact that in cottonseed oil, the percentage of triglycerides with 2 saturated acyl-groups varies between 17-23% (21,22,24,27,31), whereas in olive oil it varies between 0.0-7.5% (19-22) may explain the differences in the weight of P-I isolated in each case. The possibility that differences in the amount of precipitate may be a result of the presence of various other minor constituents (waxes, alcohols and soforth) in cottonseed oil, which act as crystal nuclei during the cooling period, was excluded by obtaining pure triglycerides by column chromatography (32); crystallization of the pure triglycerides of olive and cottonseed oil gave results similar to those of the original samples.

Triglyceride analysis showed that trisaturated triglycerides are completely absent from the olive oil as well as from the olive-oil precipitates, in spite of the fact that the latter have been enriched in the most insoluble triglycerides (Table II). On the contrary, the concentration of trisaturated triglycerides has significantly increased in the precipitates of cottonseed oil and the mixtures of olive and cottonseed oil.

In olive oil, the relative decrease of oleic acid is less pronounced than that of linoleic acid. Therefore, the ratio O/L increases from the original sample to P-II, whereas in the case of cottonseed oil and its admixtures, the concentration of monoenoic fatty acids has decreased more markedly than that of linoleic acid, resulting in a decrease of the ratio O/L from the original sample to P-II. In P-II of cottonseed oil, palmitoleic acid is absent. Such differences can be attributed to the differences in the triglyceride composition of olive and cottonseed oil. The P-II of the cottonseed oil is particularly enriched in triglycerides with 2 double bonds, which account for the 46.5-48.9% of the triglyceride content of this fraction (Table II). The fatty

TABLE II

Fatty Acid and Triglyceride Composition of Olive Oil, Cottonseed Oil, their Mixtures and Precipitates I and II

Sample			Yield	Fatty acid composition (mol %)								Triglyceride composition (mol %)				
Olive oil (% w/w)	Cottonseed oil (% w/w)		Precipitate (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	O/L	F_0	F_1	F_2	F_3	F_4
100	0			trace	14.3	1.2	2.5	75.4	6.2	0.4	12.2	–	6.1	27.2	49.5	17.2
100	0	P-I[a]	0.8	trace	28.5	0.7	4.9	61.6	4.3	trace	14.3	–	23.4	25.3	39.8	11.5
100	0	P-II[a]		trace	30.9	0.5	4.9	61.2	2.5	–	24.5	–	26.0	31.0	34.8	8.2
0	100			1.1	29.7	0.4	1.5	19.5	47.8	–	0.41	trace	7.7	18.5	18.6	55.2
0	100	P-I[a]	12.0	1.1	55.3	0.2	3.9	11.1	28.4	–	0.39	4.5	18.1	43.0	14.6	19.8
0	100	P-II[a]		1.0	62.0	–	2.8	8.4	25.8	–	0.33	7.7	21.7	46.5	7.7	16.4
90	10			0.1	15.9	0.6	1.8	71.5	10.1	trace	7.08	–	6.3	26.6	45.5	21.6
90	10	P-I[a]	7.5	trace	20.7	0.3	3.4	65.9	9.7	trace	6.79	–	20.5	30.1	33.9	15.5
90	10	P-II[a]		trace	32.5	0.5	8.1	48.8	10.1	trace	4.83	–	26.8	35.8	26.8	10.6
80	20			0.2	17.5	0.8	1.6	65.1	14.8	trace	4.40	–	6.5	25.5	43.4	24.6
80	20	P-I[a]	4.5	0.2	28.4	0.2	3.9	54.0	13.3	trace	4.06	22.6[b]	22.6[b]	28.7	32.1	16.6
80	20	P-II[a]		0.2	39.4	0.2	6.0	42.5	11.7	trace	3.63	25.2[b]	25.2[b]	31.1	30.1	13.6
70	30			0.3	19.5	0.8	1.9	59.3	18.2	–	3.26	–	6.6	24.2	40.3	28.9
70	30	P-I[a]	5.9	0.3	27.6	0.3	3.8	51.0	17.0	–	3.00	18.9[b]	18.9[b]	29.7	33.2	18.2
70	30	P-II[a]		0.3	39.6	0.2	7.8	37.5	14.6	–	2.57	25.7[b]	25.7[b]	31.8	28.6	13.9
60	40			0.3	19.3	0.8	2.5	52.2	24.9	–	2.10	–	6.8	23.5	37.3	32.4
60	40	P-I[a]	7.5	0.5	27.9	0.3	4.3	44.2	22.8	–	1.94	18.9[b]	18.9[b]	27.2	28.0	25.9
60	40	P-II[a]		0.6	37.1	0.3	6.7	35.1	20.2	–	1.74	27.1[b]	27.1[b]	31.1	25.4	16.4

[a]P-I was obtained after a 25-hr crystallization at -2 C of a 5% (w/v) solution of the original sample in methanol/acetone (10:90, v/v) and P-II after crystallization of P-I under similar conditions.
[b]These numbers represent the combined triglycerides of Fractions 0 and 1.

acid composition of Fraction 2 (triglycerides with 2 double bonds) of contonseed oil and its precipitates showed that the latter are enriched in saturated and linoleic acids, while the oleic acid concentration has decreased (Table III). This may be attributed to the fact that SLS triglycerides, although having the same degree of unsaturation with SOO triglycerides, are less soluble in acetone because of their greater number of saturated acylo groups (30). Similarly, the increase of saturated fatty acids in the polyunsaturated triglyceride fraction (Fraction 4) of cottonseed oil P-I and P-II may be caused because SLL, having one saturated acylo-group, coprecipitates to a greater extent in P-I and P-II of cottonseed oil than the other polyunsaturated triglycerides (Table III).

The relative concentration of polyunsaturated triglycerides (Fraction 4) is much lower in olive oil (17.2%) than in cottonseed oil (55.2%), whereas in the mixtures, the concentration of polyunsaturated triglycerides appears to increase according to the content of cottonseed oil (Table II). This finding agrees with previous results (6,7) and may be used as a preliminary indication of the adulteration of olive oil with cottonseed oil.

Change of the Ratio O/L

Twelve samples of olive oil, 3 samples of cottonseed oil and various mixtures of them were subjected to crystallization from acetone or methanol/acetone (10:90, v/v). Ten of the olive-oil samples had a comparatively high oleic and relatively low linoleic acid content; the opposite occurred with the rest. In all olive-oil samples, the value of the ratio O/L was higher in P-I (6.1-20.3) than that of the original sample (4.0-19.1) and it has further increased in P-II (8.4-29.4). On the contrary, in the cottonseed-oil samples the ratio O/L has decreased from 0.39-0.42 (original sample) to 0.32-0.43 (P-I) and 0.28-0.36 (P-II). Similarly, in all the samples of adulterated olive oil, the ratio O/L has decreased from 2.1-8.3 (original sample) to 1.9-8.3 (P-I) and 1.6-7.6 (P-II).

Thus, changes in the values of the ratio O/L from the original sample to P-I and P-II can prove a useful index for detecting the presence of cottonseed oil in olive oil up to 10%. In all mixtures, the value of the ratio O/L in P-II does not exceed 7.6, whereas its lowest value in virgin olive oil is 8.4.

Although the method involves 2, 24-hour crystallizations, it does not require a lot of labor and is not time-consuming if applied for a series of samples. Moreover, it is simple, cheap and gives reproducibles results (standard deviation 1.8%).

Pancreatic Lipase Hydrolysis

P-I isolated from olive oil, cottonseed oil and their mixtures were hydrolyzed in the presence of porcine pancreatic lipase. The term "enrichment factor" (ratio of a fatty acid concentration in the 2-monglycerides to that in the triglycerides) was used to express differences in the distribution of fatty acids in sn-2 position (33). The enrichment factor of linoleic acid was much lower in olive oil (1.11-1.30) than in cottonseed-oil precipitates (2.00-2.18). Marked differences also occurred between the values of the enrichment factor of linoleic acid in olive-oil precipitates and those of the adulterated samples (1.57-1.90). Such differences can be associated with the fact that P-I isolated from cottonseed oil and its admixtures were enriched in SLS, SLL triglycerides and the like. Even in the presence of small quantities of cottonseed oil (adulteration 5%), the enrichment factor of linoleic acid appeared to increase (1.55-1.57), indicating that this index can prove particularly useful for the detection of adulteration of olive oil with small quantities of cottonseed oil.

REFERENCES

1. Mangio, F., Boll. Lab. Chim. Prov. 11:12 (1960).
2. Sliwiok, J., Mikrochim. Acta 2:294 (1964).
3. Kaufmann, H.P., and M. Aparicio, Anales Inst. Invest. Vet. (Madrid) 11:179 (1961).
4. Kaufmann, H.P., and M. Aparicio, Fette. Seifen. Anstrichm. 61:768 (1959).
5. Galanos, D.S., and V.M. Kapoulas, JAOCS 42:815 (1965).
6. Galanos, D.S., V.M. Kapoulas and E.C. Voudouris, Ibid. 45:825 (1968).
7. Kapoulas, V.M., and S. Passaloglou-Emmanouilidou, Ibid. 58:694 (1981).
8. Dong, M.W. and J.L. DiCesare, Ibid. 60:788 (1983).
9. Litchfield, C., Analysis of Triglycerides, Academic Press, New York and London, 1972, pp. 173-187.
10. Official Journal of the European Communities 20:L128 ed., Office for Official Publications of the European Communities, Luxembourg, 1977.
11. Hilditch, T.P., and P.N. Williams, The Chemical Constitution of Natural Fats, 4th ed., Chapman and Hall, London, 1964.
12. Wood, R., and F. Snyder, JAOCS 43:53 (1966).
13. Litchfield, C., M. Farquhar and R. Reiser, JAOCS 41:588 (1964).
14. Snyder, F. and N. Stephens, Bioch. Biophys. Acta 34:244 (1959).
15. Morrison, W.R., and L.M. Smith, J. Lipid Research 5:600 (1964).
16. Luddy, R.F., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, JAOCS 41:693 (1964).
17. Coleman, M.H., Ibid. 38:685 (1961).
18. Vioque, E., M.P. Mara and M. Calderon, Grasas Aceites 15:173 (1964).

TABLE III

Fatty Acid Composition of Fractions 2 and 4 in Cottonseed Oil and Its Precipitates

	Fatty acid composition (mol %)					
	14:0	16:0	16:1	18:0	18:1	18:2
Fraction 2[a]						
Cottonseed oil (18.3%)[c]	1.0	53.3	0.2	3.8	18.2	23.5
Cottonseed oil P-I (44.0%)[c]	1.1	56.3	0.2	4.2	12.4	25.8
Cottonseed oil P-II (48.9%)[c]	1.1	57.3	0.2	4.5	9.5	27.4
Fraction 4[b]						
Cottonseed oil (55.2%)[c]	0.4	15.5	0.2	1.6	12.0	70.3
Cottonseed oil P-I (18.6%)[c]	0.4	19.1	0.1	1.8	8.8	69.8
Cottonseed oil P-II (14.7%)	0.4	21.6	–	2.0	8.6	67.4

[a]Fraction 2 corresponds to triglycerides containing 2 double bonds.
[b]Fraction 4 corresponds to triglycerides containing 4, 5 and 6 double bonds (polyunsaturated triglycerides).
[c]The numbers in parentheses indicate the relative percentage of Fraction 2 or Fraction 4 in the sample.

19. Chobanov, D., R. Tarandjiska and R. Chobanova, JAOCS 53:48 (1976).
20. Privett, O.S. and M.L. Blank, Ibid. 40:70 (1963).
21. Youngs, C.G. and M.R. Subbaram, Ibid. 41:218 (1964).
22. Subbaram, M.R., and C.G. Youngs, Ibid. 41:595 (1964).
23. Fedeli, E., and G. Jacini, Riv. Ital. Sost. Grasse 44:393 (1967).
24. Gunstone, F.D., and M. Ilyas Qureshi, JAOCS 42:961 (1965).
25. Hilditch, T.P., and L. Maddison, J. Soc. Chem. Ind. 59:162 (1940).
26. Barrett, C.B., M.S.J. Dallas and F.B. Padley, JAOCS 40:580 (1963).
27. Jurriens, G., and A.C. Kroesen, Ibid. 42:9 (1965).
28. Vereschagin, A.G., Biokhimiya 27:866 (1962).
29. Doerschuk, A.P., and B.F. Daubert, JAOCS 25:425 (1948).
30. Youngs, C.G., and H.R. Sallans, Ibid. 35:388 (1958).
31. Vereschagin, A.G., S.V. Skortsova and N.I. Ishkhakov, Biokhimiya 28:868 (1963).
32. Quinlin, P., and H.J. Weiser, JAOCS 35:325 (1958).
33. Gunstone, F.D., R.J. Hamilton, F.B. Padley and M. Ilyas Qureshi, Ibid. 42:965 (1965).

[Received September 9, 1983]

Phytate Removal from Soybean Proteins

J.R. BROOKS and **C.V. MORR**, Department of Food Science, Clemson University, Clemson, SC 29631

ABSTRACT

The ability of Ca++, Ba++ and Zn++ ions to complex with and precipitate phytate ions was investigated as a possible approach for producing phytate-reduced soy-protein isolates. The treatments involved adding divalent cation reagents to mildly alkaline soy-protein extracts, or using mildly alkaline solutions of the cation reagent as protein extractant. Treatments used up to 5% (w/v) divalent cation reagent in dilute NaOH at pH 8-9 at room temperature with stirring for 1 hr. The proteins that remained soluble, or that were extracted following the above treatments, were further fractionated by dialysis against distilled water and then adjusted to the isoelectric pH of 4.5 with HCl. All treatments generally precipitated 15-90% of the total extracted proteins. The resulting phytate-reduced fractions had their total P reduced by ca. 30-74%, but their yields were reduced to only 11-85% of the total extract proteins. On the basis of these findings and on other adverse factors, e.g., toxicity, possible off-flavor and reduced functionality from residual precipitant ions, the divalent cation-fractionation method is not recommended for producing phytate-reduced soy-protein isolates for food use.

INTRODUCTION

Phytate, the hexaphosphate salt of myo-inositol (1), is the principal storage form of phosphorus (P) in the soybean (2). Hydrogenated and monovalent salts of phytic acid are water soluble, whereas its divalent and trivalent metal salts are quite insoluble (3). For example, in the presence of magnesium ion, phytate precipitates at pH values of 5.2 or greater, and in the presence of the more active calcium ions, precipitation occurs at pH values of 4.2 or greater, where phytate exists as its tetrahydrogenated, octa-anion form (4). However, in the presence of soy protein, calcium-magnesium phytate remains soluble above pH 10, presumably because of complex formation with the proteins, but coprecipitates with the protein at acid pH values (5,6).

Coprecipitation and recovery of proteins and phytate during commercial preparation of soy-protein isolates results in a final product containing ca. 2-3% phytate (2,5). This relatively high concentration of phytate has led to concerns regarding the bioavailability of soy proteins, per se, as well as zinc, iron and other trace minerals in diets containing soy protein (1,2,6,7). A clear indication of the stability of these soy-protein–mineral–phytate complexes is demonstrated by the relative inability of various workers to remove more than 70-80% of the phytate from soy-protein extracts (2,6,8-11). A recent procedure using a sequential, 2-stage cation and anion exchange treatment, was developed to remove 95% or more of the phytate from soy-protein extracts (5).

McKinney et al. (12) reported on efforts to selectively precipitate and remove phytate from soy-protein extracts by adding $Ca(OH)_2$ and $Ba(OH)_2$ at concentrations of 0.1-1.0% and at pH 8.4 and 9.2. Because protein–mineral–phytate complexes are presumed to be associated mainly through ionic bonding, dissociating the above complexes should be possible by adding excess divalent cations. This paper describes several experiments conducted to investigate the possibility of using this approach for precipitating phytate from aqueous soy-protein extracts and for extracting soy proteins from defatted flakes to prepare soy-protein isolates with low phytate content.

MATERIALS AND METHODS

Low temperature, hexane-extracted soy flakes with high protein solubility were obtained from Ralston Purina Company (St. Louis, MO). The hollow fiber ultrafiltration (UF) unit was an Amicon Model DC-2 unit equipped with a H1P10-8 hollow fiber cartridge with a 10,000 MW cutoff. Dialysis tubing was Spectrapore No. 1, with a 32 mm flat diameter and a 6,000-8,000 MW cutoff, from Fisher Scientific, Fairlawn, NJ. All chemicals were purchased from Fisher Scientific except 2,4-diaminophenol dihydrochloride, which was purchased from Eastman Kodak Company, Rochester, NY. Demineralized, distilled water was prepared by passing glass-distilled water through a Barnstead Model BD-2 demineralizer.

All centrifugation treatments were performed at 25,860 × *g* for 20 min at 20 C and dialysis was against demineralized, distilled water for 18 hr at 0-5 C. Soy extracts and their fractions were freeze-dried before analysis.

Protein determination was by the micro-Kjeldahl procedure (13) following previously described modifications (5) and using a conversion factor of 5.70 (14). Total phosphorus (P) was determined by the method of Allen (15). The molybdenum blue complex was assayed at 640 nm in a Coleman Jr. II Spectrophotometer (5).

Details of the procedure used to prepare and treat soy-protein extracts with $CaCl_2$, $BaCl_2$ and Zn acetate are presented in Figures 1-3. In Trial 1, aliquots of soy-protein extract were treated with 5% (w/v) solutions of each of the above reagents and stirred for 1 hr at room temperature (20-25 C). The treated extracts were then centrifuged to remove the precipitate (cation-insoluble fraction) and the supernatants were concentrated 4:1 by hollow fiber UF and dialyzed against distilled water for 18 hr at 0-5 C. Dialyzed supernatants were centrifuged to remove the precipitate that had formed during dialysis (dialysis-insoluble fraction) and the resulting supernatant was then acidified to pH 4.5 with HCl and recentrifuged to recover the precipitate (dialysis-soluble fraction). In addition, an aliquot of the the untreated extract was concentrated 4:1 by hollow