equipment, flakes can be produced with a minimum of denaturation or, when desired, denaturation can be controlled within practical limits.

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Estimation of Vitamin A in Margarine. III. Migration of Lecithin From the Oil to the Aqueous Phase and Its Effect on the Spectrophotometric Blank

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IN the preceding paper (1) a simple and precise spectrophotometric procedure was described for the control of vitamin A fortification of margarine made in the plant. The reliability of the method was supported by physico-chemical and biological assay data. The method involves readings taken at 328 mμ of the whole margarine oil dissolved in cyclohexane after the spectrophotometer had been set at 100% transmission with the corresponding unfortified oil at the same concentration in cyclohexane.

Two control oils were indicated (1) as essential when assays are conducted on the margarine oil before and after emulsification with the milk phase, since lecithin was found to migrate from the oil to the aqueous phase in margarine manufacture. Inasmuch as lecithin absorbs light appreciably at 328 mμ, the blank unfortified oil in the spectrophotometric assay must contain this material in testing the fortified oil prior to churning while the blank in assays of the oil separated from margarine must be free of lecithin. In the present report spectrophotometric and fluorometric data are presented, supported by phosphorus analyses and oil stabilization studies, demonstrating unequivocally that lecithin migrates completely from the oil to the aqueous phase and that this phenomenon must be taken into consideration in spectrophotometric assays of margarine oils for vitamin A content.

Spectrophotometric and Fluorometric Findings

Three commercial lecithin preparations varying in color were added, each in 0.1625% concentration, as a supplement to a margarine oil (0.13% on the margarine basis). The lecithin samples alone were first analyzed with the results obtained given in Table I. Both the Lovibond Tintometer and the Beckmann Spectrophotometer were employed in the objective color measurements. Readings with the latter instrument were taken at 328 mμ since it is at this point that esterified vitamin A absorbs maximally. Fluorometric readings were taken, using a Pfaltz and Bauer model A fluorophotometer with a Corning red ultra No. 5840 filter for the irradiating light beam and two filters in series for the fluoroescent light. The latter were a straw yellow noviol shade A, No. 038, and a light shade blue-green, No. 428. A solution of quinine sulfate in 0.1 normal sulfuric acid, 0.200 microgram per cc., was used as the reference standard for the fluorometric analyses.

TABLE I

Ultra-Violet Light Absorption and Fluorescence of Lecithin Samples

Test	Sample A	Sample B	Sample C
Visual color observation	Light orange yellow color	Deep orange brown color	Dark brown
Flavor[a]	Good	Good	Good
F.F.A.(%)	9.3	10.2	9.3
Lovibond color (1% in mineral oil)	25Y-1.0R	30Y-1.4R	35Y-2.4R
	Samples as received		
E Value[b]	36	49	58
Fluorescence[c]	184	273	307
Ratio of fluorescence to E value	5.1	5.6	5.3
	Samples after 60 hrs. at 140°F.		
E Value[b]	45	61	67
Fluorescence[c]	282	432	448
Ratio of fluorescence to E value	6.3	7.1	6.7

[a] For flavor evaluation of the commercial lecithin a 1% concentration in USP mineral oil is employed.
[b] Absorbancy × 100 of a 1% solution in cyclohexane vs. cyclohexane at 328 mμ, using a cell of 1 cm. depth.
[c] Fluorescence in cyclohexane expressed as microgram equivalents quinine sulfate per gram of sample.

Attention is directed to the fact that all three lecithin preparations were good in flavor and any one of them could have been used, despite differences in color, as a margarine oil supplement. It may be noted that absorption at 328 mμ on the samples as received paralleled fluorescence, and this is apparent from the constancy of the ratio of fluorescence to light absorption. After the lecithin preparations were held at a moderately elevated temperature (140° F.) for 60 hours, both the absorbency and fluorescence values increased appreciably. The constancy of ratio of the latter to the former value was maintained, but the average ratio was greater in the heated samples (6.7 as compared to 5.3) due to a proportionately greater increase in the fluorometric values.

In Table II are given the results of similar spectrophotometric and fluorometric tests conducted on a given margarine oil not fortified with vitamin A but supplemented with each of the lecithin preparations in 0.1625% concentration. It will be noted in the tests with the freshly prepared samples that supple-

TABLE II

Comparison of Ultra-Violet Light Absorption and Fluorescence of Margarine Oils Before and After 0.1625% Lecithin Supplementation

Test	Blank Oil[a]	Margarine Oil Containing Lecithin A	Lecithin B	Lecithin C	Average Increments
	Samples Freshly Prepared				
E Value[b]					
Calculated	.....	1.86	1.88	1.89	0.077
Found	1.80	1.88	1.88	1.88	0.080
Fluorescence[c]					
Calculated	.....	4.3	4.4	4.5	0.40
Found	4.0	4.4	4.6	4.7	0.57
Ratio of found increment in fluorescence to found increment in E value	.....	5.0	7.5	8.8	7.1
	Samples After 60 Hours at 140°F.				
E Value[b]					
Calculated	.....	1.92	1.95	1.96	0.093
Found	1.85	2.08	2.08	2.08	0.230
Fluorescence[c]					
Calculated	.....	4.5	4.7	4.7	0.63
Found	4.0	4.9	4.6	4.8	0.77
Ratio of found increment in fluorescence to found increment in E value	.....	3.9	2.6	3.5	3.3

[a] Margarine oil alone, before or after supplementation with 0.32% of mixed mono- and diglycerides; no vitamin A added.
[b] Absorbancy × 2.5 of a 40.0% margarine oil solution in cyclohexane in a cell 1 cm. in depth read versus cyclohexane at 328 mμ.
[c] Fluorescence in cyclohexane expressed as microgram equivalents quinine sulfate per gram of sample.

mentation of the margarine oil with the small amount of lecithin increased light absorption at 328 mμ on the average by about 5%. Fluorescence of the unsupplemented margarine oil was increased by about 13%. The ratios of the observed increments in fluorescence over the increments in light absorption at 328 mμ were in fairly good agreement with the expected values from tests conducted on the lecithin preparations themselves (see ratios on unheated samples in Table I). This was expected because of the very good agreement between calculated and found values in the spectrophotometric and fluorometric tests conducted on the margarine oil supplemented with each of the lecithin preparations.

When the margarine oils supplemented with lecithin were held at 140°F. for 60 hours, the found increases in fluorescence were in good agreement with the values anticipated from dilution of each of the lecithin samples stored as such under similar conditions. However the values for light absorption at 328 mμ were very much greater than those calculated. Apparently during the holding of a lecithin-supplemented margarine oil, darkening and increased light absorption at 328 mμ can proceed to a greater degree than expected from observations on the absorbancy of lecithin alone following similar storage or from the magnitude of the fluorometric values. In the heated oils following supplementation with the lecithin preparations the increment in light absorption at 328 mμ over that of the freshly prepared oil solution was increased from about 5 to 16%; fluorescence was increased from 13 to only about 19%.

It should be noted that in the tests conducted on the lecithin-supplemented margarine oils, before and after the holding period at 140°F., the differences between the lecithin preparations in ultra-violet light absorption and fluorescence were largely masked. This was due to the high base values for the unsupplemented oil relative to those for the highly diluted lecithin supplement.

Similar tests conducted on the same blank margarine oil supplemented with a mixture of mono- and diglycerides in 0.32% concentration gave the same ultra-violet absorption and fluorescence values as the oil prior to supplementation. Holding the mono- and diglyceride-containing oils at 140°F. for a period of 60 hours also failed to increase these values.

The concentrations of lecithin and mono- and diglycerides in margarine oil, cited above, are characteristic of those used in margarine manufacture. The holding of a margarine oil at 140°F. for 60 hours, or some equivalent abusive handling of the oil, is not too remote a possibility. From the analytical standpoint it was desirable to include such oils in the present study since the differences in light absorption due to lecithin supplementation at 328 mμ are ordinarily small; the abusive handling of the oil magnifies the differences attributable to the lecithin supplement, thereby reducing the significance of analytical errors. In order to support further the validity of conclusions based upon small spectrophotometric differences, confirmatory data obtained by an unrelated method, the fluorometric procedure, are desirable.

TABLE III

Comparison of Ultra-Violet Light Absorption and Fluorescence of Various Margarine Oils Before and After Emulsification With the Aqueous Milk Phase

Test	Blank Oil[a]	Margarine Oil Containing 0.1625% Lecithin A	Lecithin B	Lecithin C
	Margarine Oil Freshly Prepared			
E Value[b]				
Oil prior to churning	1.70	1.75	1.75	1.75
Oil separated from margarine	1.70	1.70	1.70	1.70
Fluorescence[c]				
Oil prior to churning	3.7	4.5	4.3	4.4
Oil separated from margarine	3.7	3.8	3.9	3.8
	Margarine Oil Held for 60 Hours at 140°F.			
E Value[b]				
Oil prior to churning	1.75	2.00	2.05	2.00
Oil separated from margarine	1.75	1.77	1.77	1.77
Fluorescence[c]				
Oil prior to churning	3.7	4.6	4.3	4.5
Oil separated from margarine	3.7	3.8	3.8	3.8

[a] Margarine oil alone, before or after supplementation with 0.32% of mixed mono- and diglycerides; no vitamin A added.
[b] Absorbancy × 2.5 of a 40.0% oil solution in cyclohexane in a cell 1 cm. in depth read versus cyclohexane at 328 mμ.
[c] Fluorescence in cyclohexane expressed as microgram equivalents quinine sulfate per gram of sample.

TABLE IV

Stability of Margarine Oils Held at 40-42°F.

Oil Sample	Peroxide Values Fresh	4 Weeks	8 Weeks	18 Weeks
	m.e. per kilo			
1. No lecithin added	2.0	2.6	3.4	6.4[a]
0.16% lecithin added	2.0	1.4	2.0	1.6
Separated from margarine	2.2	3.6	4.0	6.4[a]
2. No lecithin added	0.6	0.6	0.6	2.0
0.16% lecithin added	1.0	1.0	0.8	0.6
Separated from margarine	1.2	1.6	2.4	4.6
3. No lecithin added	1.8	2.8	3.6	6.0[a]
0.16% lecithin added	3.0	2.0	2.4	3.6
Separated from margarine	3.2	4.0	4.6	8.0[a]
4. No lecithin added	0.4	0.8	0.6	1.0
0.16% lecithin added	0.6	0.6	0.6	0.6
Separated from margarine	0.6	0.8	1.2	2.2
5. No lecithin added	1.8	2.8	3.6	6.6[a]
0.16% lecithin added	2.0	2.2	2.4	3.8
Separated from margarine	2.2	3.6	3.8	6.6[a]
6. No lecithin added	0.4	0.8	1.2	2.2
0.16% lecithin added	0.6	0.8	0.8	0.6
Separated from margarine	1.6	2.0	2.6	4.8

[a] Faintly positive Kreis test.

In Table III are presented data on the ultra-violet light absorption and fluorescence of various margarine oils before and after emulsification with the aqueous milk phase. Data obtained on the oils with and without the lecithin supplements are included. For these tests no vitamin A was added to the margarine oils.

It is apparent from the data in Table III that increased light absorption at 328 mμ and increased fluorescence occurred as a result of the lecithin additions to the margarine oil. However when the same tests were run on the oils separated from the margarines, both light absorption at 328 mμ and fluorescence were practically the same as noted in analyses of the oils prior to lecithin supplementation. The tests conducted on the margarine oils with and without lecithin, held at 140°F. for 60 hours, gave the same results noted above. However on the margarine oils subjected to abusive storage, the findings are more dramatic as a result of the greater increments, particularly in light absorption, attributable to lecithin supplementation.

Confirmatory Chemical Studies

The experimental findings summarized in Table III indicate that lecithin is not present in the oil separated from margarine and used in the spectrophotometric assay for vitamin A content (1). Further proof for this conclusion was obtained in tests designed to evaluate the resistance of margarine oils to oxidative deterioration.

In Table IV are presented the results of accelerated holding tests on margarine oils with and without the lecithin supplement and on the oils separated from the margarines made with the lecithin-supplemented oils. It will be noted that the stabilizing effect of the lecithin supplement is absent when the tests are conducted on the separated margarine oils. Essentially the same results are obtained when margarine oils are subjected to the A.O.M. stability test (2, 3); illustrative data are listed in Table V.

Further evidence for the migration of lecithin from the oil to the aqueous phase of margarine is presented in Table VI. The phosphorus content was determined of three samples of margarine oil prior to margarine manufacture and containing 0.16% of each of three commercial lecithins described earlier in this report. Phosphorus analyses were likewise carried out on the oils separated from the margarines. The assay method used was a modification of the semi-micro colorimetric procedure described by Gerritz (4). The results on the oils prior to margarine manufacture are in good agreement with the amount of commercial lecithin added. The results on the separated margarine oil confirm the conclusion that no lecithin is found in the oil separated from the margarine.

The data presented thus far do not necessarily indicate that lecithin is hydrated during margarine manufacture. It is conceivable that separation of the oil from margarine, requiring gentle warming to break the emulsion, may have contributed to the hydration of the lecithin. Actually, however, phosphorus analyses of the oil separated from the emulsion at the churn, just prior to chilling, have demonstrated that the hydration of the lecithin and migration into the aqueous phase occurs at this stage of margarine manufacture.

It may be concluded that no antioxidant activity can be attributed to lecithin in margarine. When tests were conducted on the oils separated from the margarines, listed in Table IV, 18 weeks after manufacture and held at 40-42°F. during that period, the peroxide values in every case were greater by about 3 m.e. per kg. of fat than the figures obtained in assays of the oils separated from the freshly prepared margarines and then stored for the same period.

TABLE V

A.O.M. Tests on Margarine Oils

	Sample	A.O.M. Test[a]
A	Margarine oil before lecithin addition	60
	Margarine oil + 0.16% lecithin	71
	Oil separated from margarine made with lecithin-supplemented oil	47
B	Margarine oil before lecithin addition	69
	Margarine oil + 0.16% lecithin	72
	Oil separated from margarine made with lecithin-supplemented oil	42

[a] Hours to 100 peroxide value.

TABLE VI

Phosphorus Content of Margarine Oils Before and After Separation From Margarine

Sample	Oil Before Margarine Manufacture		Oil Separated from Margarine	
	Phosphorus	Commercial Lecithin[a]	Phosphorus[b]	Commercial Lecithin
	%	%	%	%
Lecithin A 0.16% added to oil prior to margarine manufacture	0.003	0.14	Less than 0.0005	Less than 0.02
Lecithin B 0.16% added to oil prior to margarine manufacture	0.003	0.14	Less than 0.0005	Less than 0.02
Lecithin C 0.16% added to oil prior to margarine manufacture	0.003	0.14	Less than 0.0005	Less than 0.02

[a] Based on commercial lecithin containing an average of 2.2% phosphorus (equivalent to 55% lecithin where % lecithin = % P × 777/31).

[b] No measurable blue color developed in each of the systems tested. 0.0005% phosphorus is the lower limit of concentration detectable under the conditions of test.

TABLE VII

Apparent and True Vitamin A Content of Margarine Oils Determined Spectrophotometrically[a]

Sample	Oil Before Margarine Manufacture			Oil Separated from Margarine		
	Versus margarine oil prior to lecithin addition (A)	Versus margarine oil after lecithin addition (B)	A-B	Versus margarine oil prior to lecithin addition (C)	Versus margarine oil after lecithin addition (D)	C-D
	USP Units Vitamin A per lb. Margarine					
1	18,000	17,500	500	17,400	16,700	700
2	17,900	17,400	500	16,900	16,700	200
3	18,000	17,400	600	16,300	15,600	700
4	18,400	17,700	700	18,400	17,700	700
5	17,900	17,500	400	17,200	16,700	500
6	17,700	17,000	700	17,700	17,000	700
7	17,400	17,000	400	17,500	16,900	600
8	18,400	17,500	900	17,900	17,000	900
9	17,500	16,700	800	18,000	17,400	600
10	17,500	16,700	800	17,500	16,700	800
11	17,900	17,400	500	17,400	16,700	700
12	18,400	17,700	700	17,900	17,100	800
13	18,400	17,700	700	17,500	16,900	600
Average	18,000	17,400	600	17,500	16,850	650

[a] See reference (1) for details of assay procedure.

Effect of Lecithin Migration

In Table VII are shown the results of spectrophotometric assays conducted on margarine oils before and after margarine manufacture. The two types of unfortified control oils previously described (1) were

employed in the spectrophotometric assays of the margarine oils at the two stages of interest in controlling vitamin A fortification. From the data presented in this report the figures obtained in Column B are the true values for the fortified margarine oil prior to churning. If the margarine control oil containing no lecithin were used in assaying such fortified oils, the estimates of vitamin A content would be erroneously high by 600 USP units per pound of margarine. The proper blank oil to be used in assays of the separated margarine oil is that containing no lecithin; the true estimates of the vitamin A content of the margarines are given in Column C of Table VII. If the margarine blank oil containing lecithin were used in assays of the separated margarine oils, the estimates of vitamin A content would have been erroneously low by 650 USP units per pound of margarine.

The difference between apparent and true vitamin A content in either instance is small, equivalent to 3.5% of the true vitamin A content. This 3.5% figure is of real significance economically and can be of very great importance should estimates of vitamin A content border on the minimal declared potency of 15,000 USP units of vitamin A per pound.

The difference between apparent and true vitamin A content of the oils listed in Table VII is obviously a real one. Statistical analyses are unnecessary since the figures for A-B and C-D are positive in every case.

The figures in Table VII are characteristic of those obtained in control assays of products made by one manufacturer and undoubtedly represent the smallest magnitude of discrepancy between apparent and true vitamin A content following spectrophotometric assays. Other manufacturers not aware of the significance of the lecithin supplement in spectrophotometric assays may find much larger differences between apparent and true vitamin A content. Discrepancies as large as 1,500-2,400 USP units of vitamin A per pound of margarine have been noted in similar tests conducted on oils kindly furnished by another manufacturer. On the basis of the results obtained with the three lecithin preparations used in the present study, large variations between apparent and true vitamin A content are attributable more to excessive heating of the lecithin-supplemented oils rather than to differences in the light absorbancy of the lecithin preparations themselves. Calculations indicate that assays of the margarines made with the oils supplemented with any one of the three lecithin preparations and held for 60 hours at 140°F. would yield vitamin A figures in error by 1,600 USP units per pound.

Summary

On the basis of light absorption measurements at 328 mμ, fluorometric tests, stability of oils to oxidative deterioration, and phosphorus analyses it has been shown that the oil separated from margarine contains no lecithin. Phosphorus analyses demonstrated that this migration of added lecithin from the oil to the aqueous phase occurs during the churning operation and prior to chilling the emulsion.

For the spectrophotometric vitamin A assay of the fortified margarine oil prior to margarine manufacture, the control oils used to set the spectrophotometer at 100% transmission must contain the added lecithin. For the assay of the oil separated from the margarine the proper blank oil should contain no lecithin.

Use of improper control oils would be responsible for estimates of vitamin A content which are either erroneously high or low by about 3.5%. Abusive handling (storage at elevated temperatures) of lecithin-supplemented oils and use of improper control oils can be responsible for differences as much as 10% between apparent and true vitamin A content.

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Wesson Loss as a Measure of the Degree of Refining

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THE time-honored Wesson method (1) of estimating the neutral oil content of crude oils or, strictly speaking, of estimating the constituents responsible for loss during alkali refining, had until several years ago a more theoretical than practical importance since refining losses in practice were usually a multiple of the Wesson loss. Only after the introduction of the Clayton (2) and other continuous refining processes did plant losses become sufficiently low to approach the Wesson or "ideal" loss within a few tenths of a percent (3). This greatly enhanced the applicability of the method in the refining industry.

Recently however the value of the Wesson method has been subjected to criticism from two independent sources, but for diametrically different reasons. Linteris and Handschumaker (4) expressed the opinion that the method, apart from being tedious and time-consuming, tends to overestimate the neutral oil content, probably owing to decomposition of phosphatides by alcoholic potassium hydroxide used in the first step of the Wesson loss determination. They suggested a chromatographic technique based on the

[1] The removal of non-glycerides by the Wesson method requires the presence of soap and therefore reacidification of the neutralized oils is essential.

[2] This probably explains the results claimed for the De Laval Shortmix process according to which inter alia a coconut oil showing a Wesson loss of 7.20% has been alkali refined with an overall plant loss of 6.74% (7).