THE JOURNAL OF THE American Oil Chemists' Society

Volume 29 APRIL, 1952 No. 4

Estimation of Vitamin A in Margarine. II. Laboratory Control Procedures in Assaying Uncolored Margarine

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THE problem of assaying margarine for vitamin A content resolves itself into two important phases, namely, control procedures for daily routine testing in the industry and methods that can be employed by federal and state laboratories on samples obtained in the open market. In the former the procedures for routine control are made simple for the analyst because all ingredients, such as the vitamin A concentrates and unfortified margarine oils, are available. In the case of samples picked up on the open market the problem is more difficult because reliance must be placed upon a method independent of the blank control materials used in manufacture. Currently, the only official method for determining the vitamin A potency of margarine is the bio-assay (1), but the imprecision and time-consuming aspects of this method prevent its routine use.

The purpose of this paper is to describe the routine procedures used in our laboratory for assaying uncolored margarine. The applicability of the newer physico-chemical assay methods described in the United States Pharmacopoeia XIV will also be discussed. The reliability of these methods for estimating the vitamin A content of concentrates used in margarine fortification was discussed in the first paper of this series (2).

There are two physico-chemical methods applicable for controlling the vitamin A potency of margarine, a spectrophotometric method based upon the use of an identical unfortified control oil and the colorimetric procedure, which depends upon the color reaction of vitamin A with antimony trichloride. This last procedure is described by Rice, Primm, and Coombes (3). From a routine control standpoint the spectrophotometric method is preferred because of its rapidity, precision, and accuracy. However, it cannot be used by outside testing laboratories on open market samples of margarine because unfortified control oils are practically never available. For this purpose the colorimetric method may be used, despite its limitations, as will be discussed. It is also useful as a check on the spectrophotometric method in instances where there is doubt as to the validity of the unfortified control oil.

Spectrophotometric Procedure for Routine Control of Vitamin A in Fortified Oil and Margarine

Equipment: Beckman quartz spectrophotometer, model DU, equipped with tungsten lamp and glass cells (1 cm. square type).[1]

Reagents: The authors have found that cyclohexane specified as "purified for spectrophotometric use and free of extraneous ultraviolet absorption" and obtained from the Eastman Kodak Company, Rochester, N. Y., is satisfactory in most instances. Vitamin A is stable in this solvent for several days, provided that the solution is stored in the dark and the solvent itself is sufficiently pure.

It has been the authors' practice to verify the suitability of each lot of cyclohexane by spectrophotometric comparison with a sample of known purity. A sample is considered usable only if a 20% solution of unfortified margarine oil (W/V) can be readily set to 100% transmission in the spectrophotometer between the 310 and 500 mμ range. A method satisfactory for the purification of cyclohexane containing a small amount of impurities has been reported (4).

Procedure: 20% solutions (weight to volume basis) of the following samples using cyclohexane as the solvent are prepared:

I. Unfortified margarine oil containing no added ingredients whatever.

II. Unfortified margarine oil containing all added ingredients except vitamin A.

III. The fortified oil containing all added ingredients including vitamin A.

IV. Oil separated from the finished margarine produced from III. The oil is separated by melting the margarine at 50-55°C. The oil is then filtered through a No. 12 Whatman folded filter paper.

The Control Oil-I in the blank cell is used to set the Beckman spectrophotometer at 100% transmission at 328 mμ in measuring light absorption due to the vitamin A in Sample IV. The Control Oil-II is similarly used in the spectrophotometric vitamin A assay of the Fortified Oil-III, representing the oil prior to margarine manufacture. Two control oils are required for these assays because of the migration of lecithin from the oil to aqueous phase in margarine manufacture.[2] Since lecithin absorbs light appreciably at 328 mμ, a control oil not containing this ingredient must be used with oil separated from margarine. This subject will be dealt with in a subsequent paper of this series (5).

Absorbancy readings should be in the neighborhood of 0.50, i.e., if the margarine is fortified with a quality vitamin A concentrate (2) to contain not less than 15,000 USP units of vitamin A per pound. The concentration of the test solutions may be varied, if so desired, provided the readings will fall within the desired absorbancy range of 0.30-0.90.

The vitamin A potency of the sample is calculated as follows:

$$\frac{A \times 1894 \times 454 \times 0.80}{C} = \text{USP units of vitamin A per lb. of margarine}$$

Where A = Absorbancy of the fortified oil

C = Concentration in grams per 100 cc.

1894 = Factor for converting absorbancy to USP vitamin A units (2)

454 = Grams per pound

0.80 = Margarine is standardized to contain a minimum of 80% fat[3]

[1] Whereas the hydrogen discharge tube and quartz cells are regularly used in assays of vitamin A concentrates according to the Morton-Stubbs (10) procedure and in obtaining data for plotting absorption curves, the less costly accessories specified above give equally good results in the simple spectrophotometric method described in this report.

[2] Observation first made several years ago by C. M. Gooding of our laboratory.

[3] Very few margarine samples contain more than 81% fat. Thus the estimate of vitamin A content may be slightly greater (viz., 1%) if a fat analysis were included as part of the assay. Such precision is not required for routine control of vitamin fortification.

If the recommended concentration of 20% of oil in cyclohexane is used regularly, absorbancy × 34,400 yields directly the vitamin A unitage per pound of margarine.

Whereas isopropanol is employed as the solvent in spectrophotometric assays of vitamin A concentrates, it cannot be used as the solvent in assays of margarine oils. Twenty per cent concentrations of margarine oil in isopropanol cannot be prepared due to the fact that the alcohol is a poor fat solvent. On the other hand, margarine oil is readily soluble in cyclohexane; indeed, there is no difficulty in preparing 40% (W/V) solutions when cyclohexane is the solvent. Insofar as the spectrophotometric evaluations are concerned, E values on the whole oils (i.e., of the vitamin A concentrates) are the same whether isopropanol or cyclohexane is used as the solvent.

In the previous paper in this series (2) it was demonstrated through collaborative assay that a valid and precise estimate of the vitamin A potency of quality vitamin A concentrates can be obtained by spectrophotometric assay of the whole oil. The data in Table I of the present paper indicate that the spectrophotometric assay of the whole oil separated from margarine, as already described, is a valid means for controlling the vitamin A fortification of this product. In Table I the extinction ratios of the vitamin A concentrates and the oils separated from the corresponding margarines are listed for the region of 310 mμ to 350 mμ.[4] Identical values are obtained on the fortified margarine oils prior to manufacture of the margarines in the plant. It is evident that essentially the same ultraviolet absorption curves are obtained for the fortifying concentrates and the fortified margarine oils when read versus the proper control solutions in setting the spectrophotometer to 100% transmission. In other words, the solution of the concentrate in the margarine oil, the churning of the oil with the added milk component, the subsequent chilling, working, and packaging do not significantly affect the ultra-violet absorption characteristics of the vitamin A added. Hence the data previously presented (2) on the reliability of the simple spectrophotometric method in assays of quality vitamin A concentrate apply equally well to the assays of oils separated from margarines fortified with such concentrates.

In further support of this conclusion are the data presented in Table II on assays of the unsaponifiable extract of another vitamin A concentrate (blend of food fish liver oils of high potency), of the fortified margarine oil prior to churning, and of the oil separated from the margarine. For reference purposes the findings obtained in spectrophotometric analyses of the respective whole oil samples are also included. In both series the absorbancy of the vitamin A concentrate was determined versus the solvent used to set the instrument at 100% transmission while the absorbancy of the fortified margarine oil was determined versus the proper unfortified oil of the same concentration in the indicated solvent. The unsaponifiable extract of the vitamin A concentrate was prepared according to the USP XIV procedure (1), and that of the margarine oils by the method described by Rice and associates (3).

It will be noted that, in the spectrophotometric tests of either the whole oil or unsaponifiable extract, the

TABLE I

Extinction Ratios of the Vitamin A Concentrates, and the Oils Separated From the Fortified Margarines in the Region 310 to 350 mμ

Wave Length	Fish Liver Oil Used[a]	Oil from Margarine Fortified with Fish Liver Oil[b]	Distilled Vitamin A Esters[c]	Oil From Fortified Margarine[d]
mμ				
310	0.79	0.77	0.76	0.74
315	0.88	0.86	0.86	0.85
320	0.93	0.92	0.92	0.91
325	0.99	0.97	0.97	0.95
328	1.00	1.00	1.00	1.00
330	0.99	1.00	1.00	1.00
335	0.94	0.96	0.94	0.96
340	0.85	0.88	0.86	0.85
345	0.75	0.78	0.74	0.76
350	0.64	0.66	0.63	0.65

[a] High potency food fish liver oil (approximately 210,000 USP units of vitamin A per gram). 0.00600% solution (weight to volume basis) was used, and this gave an absorbancy of 0.66 at 328 mμ, read against the cyclohexane solvent.

[b] 20.0% solution (weight to volume basis) was used, which gave an absorbancy of 0.55 at 328 mμ, read against the proper unfortified oil of the same concentration in cyclohexane.

[c] Distilled vitamin A esters (approximately 205,000 USP units of vitamin A per gram). 0.00800% solution (weight to volume basis) was used, which gave an absorbancy of 0.87 at 328 mμ, read against the cyclohexane solvent.

[d] 20.0% solution (weight to volume basis) was used, which gave an absorbancy of 0.52 at 328 mμ, read against the proper unfortified oil of the same concentration in cyclohexane.

[4] By extinction ratio is meant the quotient obtained by dividing the absorbancy at each wave length by the absorbancy obtained at 328 mμ for the same concentration. The value of plotting extinction ratios rather than extinction coefficients or absorbancy readings has been treated elsewhere (6).

TABLE II

Extinction Ratios of the Whole Oils and Unsaponifiable Extracts of the Vitamin A Concentrate, the Fortified Margarine Oil, and the Oil Separated From Margarine

Wave Length	Tests on Whole Oils				Tests on Unsaponifiable Extracts		
	Vitamin A Concentrate		Margarine Oil in Cyclohexane		Vitamin A Concentrate	Margarine Oil in Isopropanol	
	In Isopropanol[a]	In Cyclohexane[b]	Fortified Oil Prior to Churning[c]	Separated Oil From Margarine[d]	In Isopropanol[e]	Fortified Oil Prior to Churning[f]	Separated Oil From Margarine[g]
mμ							
305	.69	.67	.67	.67	.71	.75	.75
310	.82	.79	.77	.78	.84	.85	.84
315	.89	.88	.86	.86	.89	.89	.90
320	.94	.93	.92	.92	.95	.95	.96
325	1.00	.99	.98	.97	1.00	1.00	1.00
328	1.00	1.00	1.00	1.00	.99	.98	.98
330	.98	.99	1.00	1.00	.96	.96	.96
335	.93	.94	.96	.96	.87	.88	.87
340	.83	.85	.88	.88	.78	.79	.78
345	.73	.75	.78	.78	.66	.66	.65
350	.62	.64	.67	.66	.53	.54	.52

[a] 0.00600% solution; absorbancy at 328 mμ = 0.66; vitamin A content = 208,400 USP units per gram.
[b] 0.00600% solution; absorbancy at 328 mμ = 0.66; vitamin A content = 208,400 USP units per gram.
[c] 20.0% solution; absorbancy at 328 mμ = 0.51; vitamin A content = 17,500 USP units per lb. margarine.
[d] 20.0% solution; absorbancy at 328 mμ = 0.515; vitamin A content = 17,700 USP units per lb. margarine.
[e] 0.00720% solution (original oil basis); absorbancy at 325 mμ = 0.79; vitamin A content = 207,500 USP units per gram.
[f] 20.0% solution (original oil basis); absorbancy at 325 mμ = 0.49; vitamin A content = 16,900 USP units per lb. margarine.
[g] 20.0% solution (original oil basis); absorbancy at 325 mμ = 0.49; vitamin A content = 16,900 USP units per lb. margarine.

ultra-violet absorption curves obtained with the fortifying concentrate and the fortified oils are practically identical when the same solvent is used. Thus there would appear to be no need to conduct the spectrophotometric test on the unsaponifiable extract. The absorbancy at 328 mμ of like concentrations of the whole vitamin A concentrate is the same when isopropanol or cyclohexane is used as the solvent. No significant difference (less than 1%) is noted in assays of the whole oil and the unsaponifiable extract of the vitamin A concentrate. On the other hand, the assay values of the unsaponifiable extracts of the margarine oils are 0-6% less than those of the respective whole oils. (For more data on this point, see Table VI.) Since the absorption curves of the unsaponifiable extracts of the vitamin A concentrate and of the fortified margarine oils are the same, it can be concluded that the lower values on the unsaponifiable extracts of the margarine oils are due to mechanical losses in preparing these extracts. Vitamin A losses of this small magnitude are not surprising since the procedure used for preparing the unsaponifiable extract calls, of necessity, for saponification of a large test sample, 10 grams.

The data in Table III demonstrate why an identical control oil must be used in the spectrophotometric assay of a given margarine oil. Absorbancy values on six samples of unfortified whole margarine oil and

TABLE III

Absorbancy Values on Unfortified Control Oils[a]—20.0% Oil Solutions in Cyclohexane Versus Cyclohexane

Sample	Absorbency at 328 mμ	
	Whole Oil	Unsaponifiable
1	0.32	0.23
2	0.36	0.18
3	0.48	0.23
4	0.42	0.24
5	0.43	0.28
6	0.58	0.37

[a] Margarine oils without any additives.

their unsaponifiable fractions are listed. It should be noted that in the case of the unfortified whole oils, a spread as great as 0.26 in absorbancy values, corresponding to an apparent vitamin A content of approximately 8,940 units per pound of margarine, is obtained. The spread in absorbancy values when the test is conducted on the unsaponifiable fractions is somewhat less, but still very much significant, corresponding to an apparent vitamin A content of approximately 6,540 units per pound of margarine. It is therefore evident that no one oil can be used as a universal or standard control in the spectrophotometric assay for vitamin A in margarine.

Colorimetric ($SbCl_3$) Procedure for Vitamin A Assay of Margarine

Forty samples of margarine were analyzed for vitamin A content, using the colorimetric method (3) and the spectrophotometric procedure described in this paper. For the colorimetric assays the unsaponifiable extract of the separated margarine oil was assayed with no correction made for irrelevant chromogenic material. The blue color developed was translated into vitamin A unitage, using a vitamin A reference or calibration curve rather than the increment or internal standard procedure (7). That margarines contain substances which interfere with the reaction between vitamin A and antimony trichloride has been adequately demonstrated by Rice and associates (3). The latter authors point out that the inhibitory effect of impurities in the unsaponifiable material is about the same as the colorimetric value on the unfortified margarine oil. This has led to "the tacit assumption by some analysts that the blank compensates for inhibition of color formation and that values may be calculated directly from a calibration curve without consideration of the blank." In the present study this simplified version of the colorimetric test was evaluated.[5]

In Table IV are presented the results of the assays of the 40 margarine samples. It will be noted that mean colorimetric value is 590 USP units of vitamin

TABLE IV

Comparison of Colorimetric and Spectrophotometric Assay Values on Oils Separated from Margarine in Estimating Vitamin A Content

Sample	Colorimetric Test	Spectrophotometric Test
	USP Units Vitamin A per lb. Margarine	
1	19,500	17,000
2	18,400	17,000
3	17,700	17,000
4	17,600	16,700
5	16,900	17,300
6	18,900	17,900
7	17,200	17,200
8	17,500	16,800
9	17,200	17,600
10	19,100	17,300
11	18,200	16,400
12	18,200	17,000
13	17,500	16,800
14	17,400	17,400
15	17,200	17,200
16	17,700	16,700
17	18,500	17,000
18	20,000	19,700
19	17,600	17,500
20	17,000	17,200
21	19,400	18,900
22	17,800	17,500
23	19,000	18,000
24	17,200	18,000
25	17,800	16,800
26	17,200	17,800
27	17,500	16,400
28	17,900	18,000
29	17,400	17,500
30	18,300	17,200
31	17,800	17,800
32	18,000	17,200
33	17,800	17,700
34	17,900	16,600
35	17,800	18,000
36	19,800	17,100
37	17,200	17,500
38	17,400	16,400
39	17,800	17,700
40	18,400	18,400
Mean	17,970	17,380
Standard Deviation	±775	±672

A per pound of margarine in excess of the mean spectrophotometric figure. Had the spectrophotometric test been conducted on the unsaponifiable extract, values about 700 USP units per pound of margarine less than those found in assays of the whole separated margarine oil would have been obtained due to mechanical losses of the vitamin A in preparing the unsaponifiable extracts. This was discussed earlier (further supporting data are presented in Table VI). It should follow therefore that similar losses of vitamin A should have occurred in preparing the

[5] In justification of our selection of the simplified colorimetric method, it should be pointed out that the compensating errors mentioned are of relatively small magnitude, from 500 to 800 USP units of vitamin A per pound of margarine, containing not less than 15,000 USP units, and that these errors cannot be additive but are always self neutralizing.

unsaponifiable extract for colorimetric assay. Nevertheless for the difference between the mean values noted in Table IV "t" = 3.63, which is highly significant since the t value for p = 0.001 is 3.55. It is of interest to note that colorimetric values on the unsaponifiable extract can exceed appreciably the spectrophotometric figures derived from assays of the whole oils. Values of 1,000 USP units and more above the spectrophotometric estimates are not uncommon (15 of the 40 samples).

Another group of 20 margarines were assayed colorimetrically, preparing the test solutions by two different methods. In one series the unsaponifiable extracts of the whole margarine samples were tested; in the other series the unsaponifiable extracts of the separated margarine oils were assayed. The difference in assay values was only 150 USP units of vitamin A per pound of margarine and was not statistically significant ("t" = 0.27). Hence either procedure for preparing the test solution for colorimetric assay may be used.

On the basis of the values reported in Table IV, it is evident that the colorimetric assay is useful in checking on the reliability of the spectrophotometric results obtained in plant control. At times low spectrophotometric values have been obtained in checking plant operations. Colorimetric assays have shown in these cases that proper fortification had been made. Colorimetric assays of the supposedly unfortified oils have revealed the presence of significant amounts of vitamin A in such oils. It was shown that whenever this had occurred, unfortified oil had been pumped into a storage tank not completely drained of previously fortified oil. Samples of this composite, prior to fortification of the added oil, were then submitted to the laboratory as the blank control for the spectrophotometric test.

The data in Table IV also indicate that the colorimetric test is useful for screening open-market samples of margarines by state and federal laboratories prior to scheduling biological assays. These tests would serve to indicate maximal vitamin A potencies. If the discrepancy between colorimetric and spectrophotometric assay of the degree noted can occur in assays of margarines fortified with quality vitamin A concentrates, it would follow that the use of fortifying oils of high ratio of colorimetric to USP XIV spectrophotometric values can increase substantially the size of the discrepancy (2).

It was shown in the previous paper (2) that the colorimetric value in assaying the unsaponifiable extract of quality vitamin A concentrates is only slightly greater, if at all, than the spectrophotometric estimate based on assay of the whole concentrate. However in testing margarine fortified by such concentrates by the simplified version of the colorimetric test, it is not uncommon to note a substantial discrepancy between the results obtained by the two assay methods; the colorimetric values being the greater of the two despite use of the unsaponifiable extract as the test solution. Parallel colorimetric and spectrophotometric assays conducted on the unsaponifiable extracts of a) the unfortified margarine oils, b) the vitamin A concentrates, c) the fortified margarine oils, d) the oils separated from the finished margarine, e) the cultured milk phase prior to churning, f) the whole margarine, and g) the separated aqueous phase, using both a vitamin A calibration curve and the increment (internal standard) procedure in the colorimetric assays, failed to reveal what factors were responsible for the somewhat higher colorimetric estimates. It is tentatively suggested that some conversion of vitamin A to the unsaturated hydrocarbon, anhydro vitamin A, occurs in margarine manufacture and storage. The pH of margarine made with cultured milk is 5.0. During churning some benzoic acid[2] (from the added sodium benzoate) and lactic acid migrate into the oil phase. In the presence of acid, vitamin A in a non-aqueous medium loses a molecule of water to form anhydro vitamin A. This derivative is practically free of biological activity (8), has an absorbancy at 328 mμ, about one-half that of vitamin A, but reacts with antimony trichloride to almost the same degree as vitamin A itself (9). Hence the formation of such a compound in margarine could readily explain the lower spectrophotometric values relative to those obtained colorimetrically, despite equivalence in assays of the concentrates. In support of this hypothesis are the results of studies in progress on less acid margarines (pH 5.5-6.0). The agreement between colorimetric values and those obtained by the recommended spectrophotometric procedure is excellent in assays of the freshly prepared margarines. However, following storage, viz., one month at 75°F., there is a measurable decrease in vitamin A content by spectrophotometric assay of about 8% while the apparent loss of vitamin A on colorimetric assay is somewhat less than one-half the true loss.

Assay of Margarines for Vitamin A Content, Using the USP XIV or Morton-Stubbs Correction Procedure

The applicability of the Morton-Stubbs (10) correction to spectrophotometric assays of the unsaponifiable extract of fortified margarine oils has also been evaluated. In the absence of unfortified oil blanks the Morton-Stubbs correction yields impossible values (see Table V). Hence the unsaponifiable extracts of the fortified oils were read against the unsaponifiable extracts of the corresponding unfortified oils. The spectrophotometric data were then "corrected"

TABLE V

Inapplicability of the Morton-Stubbs Correction of Spectrophotometric Estimates of the Vitamin A Content of Margarines When Assays Are Conducted in the Absence of the Unfortified Oil Blanks

Margarine No.	True Vitamin A Content[a]	Apparent Vitamin A Content; Margarine Unsap. Extract vs. Isopropanol	
		Uncorrected	M-S Corrected[b]
	USP units/lb.	Apparent USP Units/lb.	
1	17,200	25,500	−19,300
2	17,500	26,900	−22,100
3	16,800	26,200	−24,800
4	18,000	22,700	− 6,200
5	17,500	24,800	− 6,200
Average	17,400	25,200	−15,700

[a] According to the spectrophotometric method described in this paper, involving $E^{1\%}_{1cm.}$ 328 mμ readings on the separated margarine oils versus the corresponding unfortified oil blanks thereby correcting for irrelevant absorption.

[b] Note negative values following Morton-Stubbs correction. The failure of the Morton-Stubbs procedure to give reliable values in these tests is due to the fact that the irrelevant absorption between the 310 and 334 mμ fixation points is not a straight line. This is apparent from the following typical extinction ratios for the unsaponifiable extract of the unfortified margarine oil: E 310/325 mμ = 2.38, E 325/325 mμ = 1.00, E 334/325 mu = 0.83.

TABLE VI

Application of the Morton-Stubbs Correction to Spectrophotometric Assays of the Unsaponifiable Extracts of Separated Margarine Oils in Estimating Vitamin A Content

Margarine No.	Potency of Vitamin A Concentrate		Vitamin A in Margarine		Apparent Vitamin A in Margarine; Assays on Unsaponifiable Extract		
	Whole Oil $E^{1\%}_{1cm.}$ 328 mμ Value (A)	Unsaponifiable Extract After Morton Stubbs Correction (B)	Spectrophotometric Assay Whole Oil[a] (C)	Calculated: C×B/A= (D)	Spectrophotometric[b] (E)	Morton-Stubbs Corrected[c] (F)	Colorimetric (G)
	USP units per gram		USP units per pound		USP units per pound		
1	211,000	180,900	17,200	14,800	16,500	15,100	18,300
2			17,500	15,000	16,500	14,400	17,800
3	212,000	179,400	16,800	14,200	16,800	14,400	17,800
4	213,000	177,500	18,000	15,000	17,200	13,800	17,200
5			17,500	14,600	16,500	13,800	17,800
Average	212,000	179,300	17,400	14,700	16,700	14,300	17,800

[a] Based upon the spectrophotometric method described in this paper involving $E^{1\%}_{1cm.}$ 328 mμ readings on the separated margarine oils versus the corresponding unfortified oil blanks thereby correcting for irrelevant absorption.

[b] Based upon $E^{1\%}_{1cm.}$ 325 mμ readings of the unsaponifiable extracts of the margarine oils versus the unsaponifiable extracts of the corresponding unfortified oil blanks. Mechanical losses of vitamin A in preparing the unsaponifiable extracts have been shown to be responsible for the average 4% lower value when compared with the average value in assays of the whole oils (Column C).

[c] $E^{1\%}_{1cm.}$ 325 mμ corrected = (7 × $E^{1\%}_{1cm.}$ 325 mμ) − (2.625 × $E^{1\%}_{1cm.}$ 310 mμ) − (4.375 $E^{1\%}_{1cm.}$ 334 mμ).

for irrelevant absorption. The results are presented in Table VI.

It was previously shown that the ultra-violet absorption curve for the separated margarine oil (read versus the corresponding unfortified oil blank) is essentially the same as that for the vitamin A concentrate. Thus justification exists for multiplying the spectrophotometric assay value on the margarine (Column C of Table VI) by the ratio of the Morton-Stubbs to the $E^{1\%}_{1cm.}$ 328 mμ whole oil value on the concentrate in order to obtain a theoretical Morton-Stubbs value for the margarine (Column D of Table VI). The found Morton-Stubbs value, following correction of the spectrophotometric figure obtained in testing the unsaponifiable extract of the separated margarine oil versus the unsaponifiable extract of the corresponding unfortified oil blank, is in very good agreement with the calculated value (compare figures in Column F with those in Column D). The average calculated figure is only 400 USP units per pound of margarine (3%) above that actually found. This too supports the conclusion previously drawn that in preparing the unsaponifiable extract from 0 to 6% of vitamin A is lost. Here also it may be noted that the average spectrophotometric assay value on the unsaponifiable is somewhat less (4%) than the value obtained in testing the whole margarine oil (compare values in Column E with those in Column C). The tendency of the colorimetric procedure to overestimate the vitamin A content of margarine is again noted.

Biological Confirmation

The preceding paper (2) and the data presented in this report have emphasized the reliability of the spectrophotometric assay of the whole margarine oil by the procedure recommended for estimating biologically-active vitamin A unitage. Multiple level biological assays on two margarine samples demonstrated (2) that the Morton-Stubbs procedure gives erroneously low values in estimating vitamin A content. In Table VII are presented the results of nine more biological assays. Here again it is noted that very good agreement is obtained between the values obtained by spectrophotometric test and those derived from biological assay. When corrected by the Morton-Stubbs procedure, the spectrophotometric values average about 14,400 USP units, a value which is obviously too low. Not one of the biological assay values indicates a sample containing less than 15,000 USP units of vitamin A per pound despite the poor precision of the biological assay. The standard deviation of the average bio-assay value obtained in testing the margarines listed in Table VII—all of the same vitamin A content as noted from the spectrophotometric findings—is ± 2,100 USP units per pound or ± 12%. The tendency of the colorimetric method to overestimate vitamin A content is again noted.

The excellent correlation noted above between the results obtained in spectrophotometric assay of the whole margarine oil by the recommended procedure is obtainable only when the fortifying concentrates

TABLE VII

Reliability of Non-Biological Assay Procedures in Estimating the Vitamin A Content of Uncolored Margarine

Sample No.	Non-Biological Assay of Separated Oil		Biological Assay of Margarine[c]
	Spectrophotometric[a] (Whole Oil)	Colorimetric ($SbCl_3$)[b] (Unsaponifiable Extract)	
	USP Units of Vitamin A per lb. of Margarine		
1	18,000	18,100	15,100
2	17,500	18,800	17,700
3	17,500	19,200	17,500
4	17,000	16,700	15,500
5	17,500	18,800	15,900
6	17,200	18,900	17,100
7	17,400	19,200	18,600
8	17,600	19,000	15,600
9	17,200	18,100	21,300
Average	17,400	18,500	17,100

[a] $E^{1\%}_{1cm.}$ 328 mμ value of cyclohexane solution × 1,894 with the Beckmann Spectrophotometer set at 100% transmission with the appropriate unfortified control oil; calculations on the basis of 80% fat in margarine (see text).

[b] Essentially the same values were obtained following saponification of the whole margarine.

[c] Estimate obtained by interpolating the average growth response of the assay animals (one group) on the average log-dose response curve obtained with feedings at two levels of the USP Reference Oil.

are of high quality such as those evaluated in the previous report (2). A manufacturer using a concentrate of lower quality, i.e., one containing more irrelevant light-absorbing materials at 328 mμ and more irrelevant chromogenic materials in the $SbCl_3$ test, must establish for himself a correlation between biological and non-biological assay results before using the latter methods exclusively in controlling the vitamin A fortification of his product.

Summary

A simple and precise spectrophotometric procedure has been described for the control of vitamin A fortification of margarine made in the plant. This method involves readings taken at 328 mμ of the whole margarine oil dissolved in cyclohexane. The spectrophotometer was set at 100% transmission with the corresponding unfortified oil at the same concentration in cyclohexane. Physico-chemical and biological assay data have been presented demonstrating that the spectrophotometric method is the most reliable procedure for assaying margarine for vitamin A following fortification with quality vitamin A concentrates. It is far more precise than the biological assay method and equally as specific for vitamin A when the fortifying concentrates used satisfy the requirements set forth in the preceding paper (2).

The colorimetric procedure, involving the reaction of vitamin A in the unsaponifiable extract of margarine with antimony trichloride, tends to overestimate on the average by about 600 USP units per pound the true vitamin A content of the margarine. However it is not at all unusual for the overestimates to exceed 1,000 USP units. The colorimetric method is useful as a screening test on open-market samples for estimating maximal vitamin A potencies and as a check on the reliability of the unfortified oil blanks used in the spectrophotometric assay. Reasons were given for the belief that some conversion of vitamin A to the biologically-inactive anhydro vitamin A occurs in margarine and that the presence of this derivative is responsible in large part for the discrepancy between colorimetric and spectrophotometric estimates.

Acknowledgments

The technical assistance of Miss Helen Zmachinski and Miss Mary Kiernan of The Best Foods Laboratory in the course of these studies is greatly appreciated. Grateful acknowledgment is also extended to Harry J. Deuel Jr., University of Southern California, Los Angeles, and to Bernard L. Oser, Food Research Laboratories Inc., Long Island City, N. Y., for the biological assay contributions.

REFERENCES

1. Pharmacopoeia of the United States, Fourteenth Revision, 784-792, 1950.
2. Melnick, D., Luckmann, F. H., and Vahlteich, H. W., J. Am. Oil Chem. Soc., *29*, 104-108 (1952).
3. Rice, E. E., Primm, E., and Coombes, A. I., J. A.O.A.C., *31*, 621-633 (1948).
4. Neal, R. H., Haurand, C. H., and Luckmann, F. H., I. & E. C., Anal. Ed., *13*, 150-154 (1941).
5. Luckmann, F. H., Gooding, C. M., and Melnick, D., J. Am. Oil Chem. Soc. (in press).
6. Oser, B. L., Melnick, D., and Pader, M., I. & E. C., Anal. Ed., *15*, 717-724 (1943).
7. Oser, B. L., Melnick, D., and Pader, M., I. & E. C., Anal. Ed., *15*, 724-729 (1943).
8. Shantz, E. M., J. Biol. Chem., *182*, 515-524 (1950).
9. Shantz, E. M., Science, *108*, 417-419 (1948).
10. Morton, R. A., and Stubbs, A. L., Biochem. J., *42*, 195-203 (1948).

[Received August 30, 1951]

Iodine Number Determinations by Dead-Stop Titrimetry

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THE methods for the determination of iodine numbers indicating the degree of unsaturation of fats and oils are well established analytical procedures. The routine procedure in our laboratory is that by Hanus (1, 2). The Wijs method (3) is recommended by the American Oil Chemists' Society. A recent development (4) modifies the Rosenmund-Kuhnhenn procedure by using a pyridine sulfate dibromide solution in conjunction with mercuric acetate as a catalyst. This reduces the reaction times to one minute for nonconjugated and 30 to 120 minutes for highly conjugated oils (5). The starch endpoint is employed throughout.

It was thought desirable to apply the amperometric (dead-stop) titration procedure (6) to the above methods and compare the results obtained, using a variety of fatty acids and oils which differ in their degrees of unsaturation.

Apparatus

The instrument used in these determinations was the one previously described (6), modified in such a way that arbitrary readings on the galvanometer indicated the potential at the electrodes (see Figure 1). This secured a uniform potential and served to check the correct functioning of the instrument. A Fisher Model A galvanometer with a sensitivity of 0.10 microamps per mm. was employed.

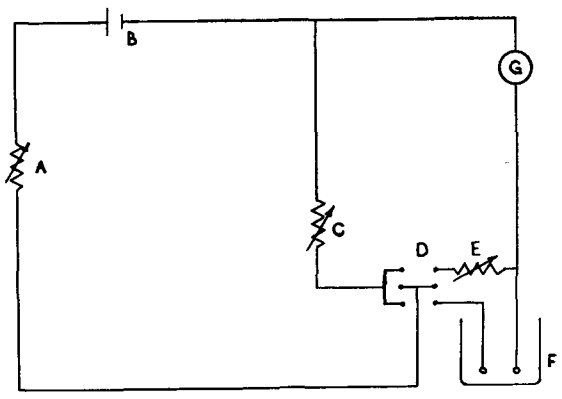

FIG. 1. Dead-stop titration apparatus.
A. 2 K-ohm variable resistor.
B. 1.5 volt dry cell.
C. 20-ohm variable resistor.
D. Double pole throw switch.
E. 5 K-ohm variable resistor.
F. Pt. electrodes in cell (coiled platinum wire).
G. Galvanometer.

[1] Communication No. 249.