Estimation of Vitamin A in Margarine. I. Collaborative Study of Assay Methods for Estimating the Potency of the Vitamin A Concentrates

DANIEL MELNICK, FREDERICK H. LUCKMANN, and HANS W. VAHLTEICH,
Research Laboratories, Best Foods Inc., Bayonne, New Jersey

SINCE the advent of fortification of margarine with vitamin A, blends of food fish liver oils and concentrates prepared from such oils have been used for this purpose. The potency of these vitamin A enriching materials was based on the results of bio-assay until the major change with respect to vitamin A estimation was adopted in the fourteenth revision of the United States Pharmacopoeia (1). This change consists in a spectrophotometric determination of the potency with a quantitative identification test based on the color reaction of vitamin A with antimony trichloride. The correction of the observed absorbancy at 325 mμ of the vitamin A for irrelevant absorbancy of the non-vitamin A materials is the prime feature of the spectrophotometric method. This correction is based upon the procedure published by Morton and Stubbs (2).

The replacement of the biological method for the determination of vitamin A in fish liver oil preparations and in pharmaceutical products by the U.S.P. spectrophotometric method has proved satisfactory for the high-potency products described in the U. S. Pharmacopoeia. However this method cannot be employed for estimating the vitamin A content of foods normally containing vitamin A and those fortified with this vitamin, such as margarine. Although margarine is not included in the U. S. Pharmacopoeia, it is still required according to federal standards and state laws to have its vitamin A fortification based on U.S.P. units. Thus the biological assay must be retained as the basic reference method for determining vitamin A in margarine.

Since the U.S.P. spectrophotometric method is applicable to the assay of the vitamin A concentrates used to fortify margarine, there would seem to be little justification for buying and using such concentrates on a biological assay basis. However the margarine manufacturer is responsible for the potency of the end-item expressed in biological, not in spectrophotometric units. Hence it is mandatory that a correlation be established between results obtained by biological and spectrophotometric assays. One such study has been completed (but the results as yet not published) by the Informal U.S.P. Committee for the Estimation of Vitamin A under the chairmanship of Sereck H. Fox of the R. P. Scherer Corporation, Detroit, Michigan. This collaborative study was of necessity restricted in scope since it involved multiple-level biological assays. The study was limited to five natural fish liver oils, varying in potency from 1,300 to 130,000 U.S.P. units per gram, and to three dilutions of crystalline vitamin A acetate. The purpose of the present report is to present more data on this important subject obtained in six laboratories[1] testing five different vitamin A concentrates all of a potency (about 200,000 U.S.P. units per gram) characteristic of that of the oils frequently employed by margarine manufacturers in fortifying their products.

[1] The following participated in the collaborative study:
Physico Chemical Assays: R. W. Lehman of Distillation Products Industries, Rochester, N. Y.; R. W. Harrison of Halibut Liver Oil Producers, Seattle, Wash.; and the authors of this paper at The Best Foods Inc.
Multiple (three-level) Biological Assays: B. L. Oser, Food Research Laboratories Inc., Long Island City, N. Y.; K. G. Falk, Laboratory of Industrial Hygiene Inc., New York, N. Y.; and H. J. Deuel Jr., University of Southern California, Los Angeles. Appreciation is expressed to these collaborators.

TABLE I
Comparison of Values Reported by Collaborators in Assaying Vitamin A Concentrates Employed in Fortifying Margarine

Method of determination[a]	Sample[e] 1	2	3	4	5	Average
Spectrophotometric whole oil,[b] U. S. P. units per gm.						
A	202,800	201,500	203,600	205,000	199,500	202,500
B	201,100	204,100	204,200	205,000	199,200	202,700
C	201,300	201,800	206,100	206,000	199,600	203,000
Morton-Stubbs,[c] U. S. P. units per gm.						
A	186,200	192,100	195,200	188,600	189,300	190,300
B	180,600	174,400	178,700	179,900	168,800	176,500
C	178,000	181,600	195,600	192,700	167,100	183,000
Colorimetric ($SbCl_3$)[d], U. S. P. units per gm.						
A	194,000	214,000	214,000	207,500	207,500	207,400
B	198,500	209,300	213,200	213,100	201,300	207,100
C	195,500	181,000	200,100	205,200	194,500	195,300
Average of Morton-Stubbs and $SbCl_3$ values, U. S. P. units per gm.						
A	190,100	203,000	204,600	198,000	196,200	198,400
B	189,500	191,900	195,900	196,500	185,000	191,800
C	186,800	181,300	197,800	199,000	180,800	189,100
Ratio of $SbCl_3$ to Morton-Stubbs values						
A	1.04	1.11	1.10	1.10	1.10	1.09
B	1.10	1.20	1.19	1.18	1.19	1.17
C	1.10	1.00	1.03	1.07	1.16	1.07
Multiple level bio-assay, U. S. P. units per gm.						
D	258,000	238,000	209,000	232,000	203,000	228,000
E	221,000	220,000	183,000	186,000	197,000	201,000
F	189,000	174,000	197,000	Not tested	171,000	183,000

[a] Capital letters in the line headings refer to laboratories participating in the collaborative study.
[b] $E^{1\%}_{1cm.}$ 328 mμ value of isopropanol solution × 1894.
[c] $E^{1\%}_{1cm.}$ 325 mμ value of isopropanol solution of the unsaponfiable extract × 1894, after correction for irrelevant light absorption.
[d] Test conducted on an aliquot of the same unsaponifiable extract used in the Morton-Stubbs spectrophotometric analysis; the unsaponifiable extract of the U.S.P. reference oil in chloroform solution was used to establish the reference curve for the colorimetric evaluation.
[e] Samples 1 and 5 were two different blends of food fish liver oils of high potency. Samples 2, 3 and 4 were three different preparations of distilled vitamin A esters.

TABLE II

Comparison of Values Obtained by Different Assay Procedures in Determining the Vitamin A Content of Concentrates Used in Fortifying Margarine[a]

Sample		Non-biological assay methods			Average of Morton-Stubbs and $SbCl_3$ values	Ratio of $SbCl_3$ to Morton-Stubbs values	Multiple level biological assays
		Spectrophotometric		Colorimetric[d] $SbCl_3$			
No.	Identity	Whole oil[b]	Morton-Stubbs[c]				
		U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram		U.S.P. units per gram
1	Blend of food fish liver oils of high potency	201,700	181,600	196,000	188,800	1.08	223,000
2	Distilled vitamin A esters	202,500	182,700	201,400	192,100	1.10	211,000
3	Distilled vitamin A esters	204,600	189,800	209,100	199,400	1.10	196,000
4	Distilled vitamin A esters	205,300	187,100	208,600	197,800	1.11	209,000
5	Blend of food fish liver oils of high potency	199,400	175,100	201,100	187,300	1.15	190,000
Average for all samples		202,700	183,300	203,200	193,100	1.11	205,800

[a] Average values obtained by the collaborating laboratories are listed.
[b, c, d] Same footnotes as in Table I.

Results and Discussion

Two types of vitamin A concentrates were used in this study, namely, a blend of food fish liver oils of high potency and distilled vitamin A esters, all with potencies in the order of 200,000 U.S.P. units per gram. Table I shows a detailed comparison of values found on the five samples by six participating laboratories. These data encompass a spectrophotometric evaluation of the whole oil, the U.S.P. XIV procedure (Morton-Stubbs correction of the spectrophotometric assay and the colorimetric assay), and multiple-level bio-assays conducted in accordance with the U.S.P. XIV assay method. The values from all laboratories are summarized in Table II.

It is apparent from the data presented that satisfactory interlaboratory results can be obtained by the physico-chemical methods of assay. The biological assays on this series of vitamin A oils are regarded as estimates of the true vitamin A unitage. The agreement between laboratories is as good as one can expect, using the bio-assay even with multiple feeding levels of both test and reference oils.

The high quality of these concentrates used for vitamin A fortification of margarine is indicated by the ratio of the colorimetric assay to the spectrophotometric values corrected for irrelevant absorption by the Morton-Stubbs procedure. These ratios are well under the 1.30 maximum specified in the U.S.P. XIV.

From the data presented in Table II it is evident that the spectrophotometric figures obtained on the whole oils and the colorimetric values on the unsaponifiable extracts are in good agreement with the estimates of potency derived from the biological assays. The fact that the values obtained by the Morton-Stubbs procedure are much less than the estimates based upon spectrophotometric assays on the whole oils, the colorimetric tests conducted on the unsaponifiable extracts, or the bio-assays is attributed to over-correction. This view is supported by the results of the U.S.P. collaborative study.

In Table III is a comparison of the results obtained in the current investigation with those reported by the collaborators in the U.S.P. study. It will be apparent that even in the latter investigation, which included oils of low biological potency, the spectrophotometric estimates of vitamin A unitage following the U.S.P. (Morton-Stubbs) procedure were less than the values obtained by collaborative bio-assays. Indeed, not one oil in either series gave a corrected spectrophotometric value greater than the bio-assay estimates. The magnitude of discrepancy between the estimates by the two assay methods in the U.S.P. study was only one-half that noted in the current

TABLE III

Comparison of Average Values Reported in Present and in U.S.P. Collaborative Studies of Vitamin A Assay Procedures[a]

Assay Procedure	Present study	U.S.P. study
	Per cent of bio-assay values	
Spectrophotometric-whole oil[b]	98.4	116.7
Morton-Stubbs method[c]	89.1	94.1
Colorimetric ($SbCl_3$) method[d]	98.7	106.9
Average of Morton-Stubbs and $SbCl_3$ values	93.8	100.5
Ratio of $SbCl_3$ to Morton-Stubbs values	1.11	1.14

[a] Test samples in the present study were five quality vitamin A concentrates of high vitamin A potency, in the neighborhood of 200,000 U.S.P. units per gram. In the U.S.P. study the test samples were five fish liver oils varying in potency from 1,300 to 130,000 U.S.P. units per gram.
[b, c, d] Same footnotes as in Table I.

investigation. However it must be pointed out (and this is evident from the data in Table III) that oils of poorer quality were used in the U.S.P. study—oils containing more irrelevant light-absorbing materials and more irrelevant chromogenic products when subjected to the antimony trichloride test.

Over-correction of the spectrophotometric data by the Morton-Stubbs procedure is attributed to two factors: a) the presence of neovitamin A in most fish liver oils and b) the presence of vitamin A_2 in some fish liver oils. Neovitamin A is a geometrical isomer of vitamin A; it is believed that the former has a *cis* configuration about the double bond nearest the hydroxyl group (3). Vitamin A in the U.S.P. Reference Standard has the all-*trans* structure ($E^{1\%}_{1cm.}$ 325 mμ = 1750). Neovitamin A has an ultraviolet absorption curve shifted about 3 mμ toward the longer wavelengths ($E^{1\%}_{1cm.}$ 328 mμ = 1645). Thus it suffers a correction under the new U.S.P. XIV spectrophotometric procedure. On the other hand, neovitamin A is chromogenically somewhat more active than all-*trans* vitamin A. Thus the presence of this vitamin A isomer in fish liver oils to the extent of one-third the total vitamin A (3) and its absence from the U.S.P. Reference Standard is largely responsible for a ratio of colorimetric to spectrophotometric (corrected) estimate above 1.00, even in tests of high quality oils such as those used in the present study.[2] Whereas it was originally reported (3) that the biological potency of neovitamin A was identical to that of vitamin A, a subsequent report (5) sets its potency at 85% of that of vitamin A. This latter finding is surprising inasmuch as the rat is able to convert all-*trans* to neovitamin A and vice versa (3, 5). Accepting the potency value most recently reported, we would expect fish liver oils with 1 part

[2] Pure neovitamin A has been found (4) to have a ratio of 1.43 for the values obtained by the colorimetic and U.S.P. XIV spectrophotometric tests.

neovitamin A and 2 parts of all-*trans* vitamin A to have a factor for converting the $E^{1\%}_{1cm.}$ 325 mμ value (test on unsaponifiable extract) about 5% lower than that of a similar preparation of all-*trans* vitamin A. In our studies the discrepancy of the Morton-Stubbs or U.S.P. XIV value from the bio-assay estimate was actually 10.9%.

The Morton-Stubbs correction method is based upon two assumptions: a) irrelevant light absorbing substances other than vitamin A have a linear absorption between the two fixation points on either side of the maximum, and b) these substances have no vitamin A activity of their own. Neither of these assumptions is true in the case of neovitamin A; hence, the over-correction.

Many of the same points noted above apply also in a discussion of the applicability of the Morton-Stubbs correction to fish liver oils containing vitamin A_2. Pure vitamin A_2 alcohol has an absorption maximum at 351 mμ ($E^{1\%}_{1cm.} = 1460$) and because the absorption curve is rather broad (6) absorbs at 325 mμ to the extent of 80% of its peak value. Shantz and Brinkman (7) have shown that this vitamin A isomer has about 40% of the biological activity of vitamin A and that the portion of the 325 mμ absorption contributed by vitamin A_2 is credited with only one-half of this biological activity in the Morton-Stubbs spectrophotometric assay. Thus, in blends of food fish liver oil of high vitamin A potency containing as much as 10% of vitamin A in the form of vitamin A_2 (2), the Morton-Stubbs or the U.S.P. XIV spectrophotometric procedure would over-correct the estimate of potency by about 2% due to the presence of this isomer of vitamin A. Indeed, colorimetric ($SbCl_3$) tests conducted on the blends of fish liver oils covered in this report with readings taken at 620 and 690 mμ as suggested by Jensen and associates (8), have shown that these samples contained from 10 to 20% of the total vitamin A in the form of vitamin A_2.

We would conclude from the results reported in the present and the U.S.P. collaborative study that a conservative estimate of the biological potency of a vitamin A concentrate derived from fish liver oils is the average of the values obtained by the U.S.P. XIV spectrophotometric[3] and the colorimetric ($SbCl_3$) tests. The ratio of the colorimetric to the spectrophotometric value should be no greater than 1.20. That the recommended procedure can yield values satisfactory in purchasing concentrates is apparent from the data obtained independently in the laboratories of the buyer and seller and presented in Table IV. In only one case out of 10 was it necessary to call for the services of an outside referee laboratory, as indicated in the table. The precision of the colorimetric method is definitely superior to that of the spectrophotometric procedure when correction is made for irrelevant light-absorbing materials. Small errors in the spectrophotometric settings or in the readings taken at the 310 and 334 mμ fixation points (these are on the slopes of the vitamin A absorption curve) become magnified in calculating the corrected vitamin A potency. Within our own laboratory, using two different analysts and two different Beckmann Spectrophotometers, much better agreement between values is regularly obtained; deviations between the average of triplicate values obtained by the two analysts on the oil listed in Table IV averaged 1.25% ± 1.07 S. D. Thus subtle unsuspected differences in techniques in preparing the unsaponifiable extract, over and above mechanical losses, play a major role in causing interlaboratory variations. The inclusion of the colorimetric estimate in calculating the invoiceable vitamin A potency of the concentrate is responsible for better agreement between the values reported by the laboratories of buyer and seller. Indeed, there is no difficulty in the two laboratories obtaining values which deviate by no more than 5%. The data in Table I add further support to this statement. The deviations between laboratories in the ratios of colorimetric to spectrophotometric (Morton-Stubbs) estimates can obviously be large since the errors inherent in the two assay methods may at times vary in opposite directions (viz. last sample listed in Table IV). Nevertheless it can be safely concluded that all the concentrates listed in Table IV have ratios no greater than 1.20.

The averaging of values obtained by two unrelated assay procedures is not permitted in the U. S. Pharmacopoeia; for legal purposes only one assay method can be official. Conservatism led to the selection of the spectrophotometric method with correction made for irrelevant light absorbing materials. This was equivalent to making the U.S.P. unit based on spectrophotometric assay about 10% larger than the U.S.P. biological unit in assays of quality vitamin A concentrates, and this was reflected in a prompt price increase by suppliers of such concentrates.[4] However the margarine manufacturer is held responsible for the biological and not spectrophotometric units in the end-time, the margarine. Thus there is no restriction on the margarine manufacturer preventing him from averaging the results obtained by the spectrophotometric and colorimetric methods in assigning a potency to a reproducible quality concentrate used to fortify his product.

Indeed, the most realistic estimate of the vitamin A content of quality oils, such as those covered in the present report, is the $E^{1\%}_{1cm.}$ 328 mμ value on the whole oil times the 1894 conversion factor. Before the latter method can be adopted however, it is necessary for the margarine manufacturer to establish by assays of several batches of the same type of oil furnished by a given supplier that the simple spectrophotometric estimate is a true reflection of biological potency.

Even the conservative method suggested above (averaging of the U.S.P. XIV spectrophotometric and colorimetric values) for estimating the vitamin A content of concentrates cannot be used indiscriminately. In the recent hearings (1951) to amend the Standard of Identity of Oleomargarine, it was proposed that whale liver oil, solutions of synthetic vitamin A esters containing irrelevant reaction products, and pure synthetic vitamin A esters be permitted as optional ingredients for fortifying margarine. The presence of biologically inactive materials (kitol, anhydrovitamin A, and vitamin A oxidation products) in whale liver oil gives rise to serious difficulties when ultraviolet absorption or the antimony trichloride test is used to de-

[3] The conversion factor employed in this study for translating the E value of an oil into biological potency has been 1894 rather than the 1900 factor called for in the U.S.P. XIV. Since the 1894 factor was routinely employed when the biological assay was the basic reference method and since the biological assay remains the basic method for estimating the vitamin A content of margarine, justiification for retaining the 1894 factor exists.

[4] Commonly referred to in bulletins from vitamin A suppliers in early 1950 to users of their concentrates.

TABLE IV
Reproducibility of Estimates of Vitamin A Potency of Concentrates Employed in Fortifying Margarine[a]

Identity of concentrate	Laboratory	Estimate of vitamin A content: Spectrophotometric Morton-Stubbs[c] U.S.P. units per gram		Colorimetric[d] $SbCl_3$ U.S.P. units per gram		Avg. of Morton-Stubbs and $SbCl_3$ values U.S.P. units per gram		Ratio of $SbCl_3$ to Morton-Stubbs value	
Six different batches of blends of fish liver oils of high potency	A	180,500	(4.9%)	209,300	(2.4%)	194,900	(3.6%)	1.16	(2.7%)
	C	189,400		214,400		201,900		1.13	
	A	177,200	(7.0%)	204,700	(2.3%)	191,000	(4.5%)	1.15	(4.6%)
	C	189,600		209,300		199,500		1.10	
	A	185,600	(6.5%)	224,300	(1.7%)	205,000	(2.0%)	1.21	(8.4%)
	C	197,600		220,600		209,100		1.12	
	A	174,100	(12.9%)	227,300	(1.8%)	200,700	(4.5%)	1.31	(14.9%)
	C	196,500		223,400		210,000		1.14	
	D[b]	189,000		218,250		203,600		1.15	
	A	179,400	(9.1%)	218,200	(0.9%)	198,800	(4.6%)	1.22	(8.0%)
	C	195,800		220,250		208,000		1.13	
	A	182,000	(6.5%)	224,300	(2.1%)	203,150	(1.7%)	1.23	(8.9%)
	C	193,700		219,600		206,650		1.13	
Four different batches of distilled vitamin A esters.	A	188,900	(1.0%)	209,300	(1.7%)	199,100	(0.4%)	1.11	(2.7%)
	B	187,000		212,800		199,900		1.14	
	A	184,100	(3.1%)	218,100	(2.5%)	201,100	(2.8%)	1.17	(0.9%)
	B	189,800		223,500		206,650		1.18	
	A	182,200	(4.7%)	213,900	(1.0%)	198,100	(2.7%)	1.17	(3.5%)
	B	190,700		216,100		203,400		1.13	
	A	194,900	(10.1%)	235,100	(2.3%)	215,000	(3.3%)	1.21	(13.1%)
	B	214,500		229,800		222,200		1.07	
Average deviation between laboratories		6.58% (±3.46 S.D.)		1.87% (±0.55 S.D.)		3.01% (±1.49 S.D.)		6.77% (±1.45 S.D.)	

[a] Data obtained in large scale purchases of vitamin A concentrates. Percentages in parentheses indicate the deviations in values, based on the lesser of the two figures reported by the two laboratories.
[b] Referee laboratory; the data from this source were not included in calculating the precision of the assay methods in actual practice.
[c, d] Same footnotes as in Table I.

termine vitamin A potency of whale liver oils (9,10). Most such oils absorb light maximally in the region of 310 to 320 mμ and exhibit colorimetric ($SbCl_3$) figures in excess of the true biological values; the ratio of colorimetric to corrected spectrophotometric values can easily exceed the maximal 1.30 ratio specified in the U.S.P. XIV test. A chromatographic spectrophotometric method has been proposed (10) for the determination of vitamin A in whale liver oil, but this is of limited value in the control of margarine manufacture due to the time required for an analysis, and it is dubious whether the method can be applied at all to the assay of the end-item.

Synthetic vitamin A preparations must be used with caution inasmuch as certain related but biologically inactive compounds (11) can be present which have absorption curves simulating that of vitamin A and react with antimony trichloride to yield colored products absorbing light at 620 mμ. It is true that the currently available commercial products satisfy the U.S.P. XIV test for vitamin A. Hence pharmaceutical manufacturers, whose products can be tested by the U.S.P. XIV method, can and do use the synthetic concentrates with assurance that legal requirements are satisfied. The margarine manufacturer responsible for the potency of his product determined by biological assay must either check by bio-assay the synthetic concentrate and/or the fortified margarine or rely upon the integrity of the vitamin A producer that the concentrate is made by a reproducible process time after time. Changes in the synthetic procedure cannot be approved, insofar as the margarine manufacturer is concerned, simply because the final concentrate satisfies the U.S.P. XIV non-biological test requirements. Fortunately, only companies of high repute are currently manufacturing synthetic vitamin A, and each time a change in synthesis is made, confirmation of biological activity is obtained by biological assay. Records of comparative physico-chemical and biological values are open to examination by the margarine manufacturer.

Pure synthetic vitamin A esters as such or in a vegetable oil solvent at a potency level of 200,000 U.S.P. units per gram or more may be employed in margarine manufacture, using the U.S.P. XIV non-biological assay, provided that a) the spectrophotometric value on the whole oil is the same as that on the unsaponifiable extract, b) the spectrophotometric assays show the material to be free from irrelevant light-absorbing materials so that no correction in the Morton-Stubbs procedure is required, and c) the ratio of colorimetric to spectrophotometric values is 1.00.

In order to check further on our recommendations of the proper non-biological assay procedure to be used for controlling the vitamin A fortification of margarine with quality concentrates, biological and physico-chemical assays were conducted on two batches of margarine prepared on a plant scale. One batch of margarine was fortified with a blend of food fish liver oils of high potency, the other with a concentrate of distilled vitamin A esters. The assay data on the concentrates are presented in Table V. The resulting margarines were subjected to a variety of non-biological and biological (multiple level) assays

TABLE V

Vitamin A Potency of Concentrates Used in Fortifying Margarines on a Plant Scale[a]

Identity of concentrate	Blend of food fish liver oils of high potency			Distilled vitamin A esters		
	A	C	Avg.	A	B	Avg.
Assay laboratory	U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram	U.S.P. units per gram
Vitamin A content of concentrate:						
Spectrophotometric-whole oil[b]	206,000	Not reported	(206,000)[e]	206,000	Not reported	(206,000)[e]
Morton-Stubbs method[c]	180,500	189,400	185,000	188,900	187,000	188,000
Colorimetric ($SbCl_3$) method[d]	209,300	214,400	211,900	209,300	212,800	211,100
Average of Morton-Stubbs and $SbCl_3$ values	194,900	201,900	198,400	199,100	199,900	199,500
Ratio of $SbCl_3$ to Morton-Stubbs values	1.16	1.13	1.15	1.11	1.14	1.13

[a, b, c, d] Same footnotes as in Table I.

[e] Although only the value from one laboratory is listed here, it can be assumed that the other laboratory would have obtained practically the same figure. This is apparent from the excellent reproducibility between laboratories of the spectrophotometric values on the whole oil; see data in Table I.

TABLE VI

Vitamin A Potency of Margarines Manufactured on a Plant Scale

Identity of the vitamin A concentrate used	Blend of food fish liver oils of high potency	Distilled vitamin A esters
	U.S.P. units per lb. of margarine	
Theoretical vitamin A content of the margarine based upon potency of concentrate		
Routine spectrophotometric-whole oil	17,700	17,600
Morton-Stubbs spectrophotometric unsap. ext.	15,900	16,000
Colorimetric ($SbCl_3$)-unsap. ext.	18,100	18,000
Average of Morton-Stubbs and $SbCl_3$ value	17,000	17,000
Estimate of vitamin A content based on routine spectrophotometric assays of the fortified oils prior to churning	17,400	18,300
Estimate of vitamin A content based upon assays of the margarine		
Routine spectrophotometric-whole oil	17,000	18,200
Colorimetric ($SbCl_3$)-unsap. ext.	17,600	19,000
Multiple level biological assay—		
Collaborator D	19,300	18,500
Collaborator F	17,300	15,400
Average	18,300	17,000

See Table V for potency estimates of the concentrates by the various assay methods employed; in the above table, the calculations of the theoretical potencies of the margarines were based upon the average values reported by the two collaborating laboratories.

to determine their vitamin A content; the findings are summarized in Table VI. The non-biological methods employed and their reliability for the assay of vitamin A in margarine are discussed in a subsequent paper (12).

Comparisons of the results of the physico-chemical assays of the margarines with those of the biological assays again show good agreement between the theoretical vitamin A potency based on the spectrophotometric assay of the whole oil used in fortifying the margarine and the values obtained by multiple-level biological assays. Bearing in mind the relatively poor precision of the biological assay, it must be concluded that the figures based upon the average of the colorimetric and Morton-Stubbs values are conservative estimates of the vitamin A content. The fact that the Morton-Stubbs procedure gives erroneously low estimates of the vitamin A potency of the quality concentrates employed in this study is again demonstrated.

Summary

A series of 5 vitamin A concentrates of 2 different types were assayed by physico-chemical methods and by multiple level biological assays. Three independent laboratories collaborated on the physico-chemical assays and three other laboratories conducted the biological assays. The test oils contained about 200,000 U.S.P. units of vitamin A per gram and were either blends of food fish liver oils of high potency or were distilled vitamin A esters in a vegetable oil solvent. Margarines fortified with each type of concentrate were made on a plant scale and also assayed for vitamin A, using both the physico-chemical and biological assay techniques.

The data show that the type of vitamin A bearing oils for margarine fortification used in the present collaborative study are of high quality and contain considerably less extraneous (non-vitamin A) materials than the oils used in a comparable U.S.P. collaborative study. A valid and precise estimate of the vitamin A potency of such oils can be obtained by spectrophotometric assay of the whole oil or by colorimetric ($SbCl_3$) assay of the unsaponifiable extract. A conservative estimate of the vitamin A potency is obtained by averaging the colorimetric and the U.S.P. XIV (or Morton-Stubbs) spectrophotometric values derived from assays of the unsaponifiable extract. It is evident that the Morton-Stubbs procedure over-corrects the spectrophotometric estimates of the potency of such vitamin A concentrates. Largely responsible for the over-correction are the presence of neovitamin A and vitamin A_2 in the natural concentrates and their absence from the U.S.P. Reference Standard. The use of other sources of preformed vitamin A to fortify margarine, such as a) fish liver oils or concentrates of poorer quality than those evaluated in the present study (more irrelevant light-absorbing materials at 328 mμ and more irrelevant chromogenic materials in the $SbCl_3$ test), b) whale liver oil and/or c) synthetic vitamin A preparations, will introduce complications if non-biological assay methods should be used exclusively by the margarine manufacturer in controlling the vitamin A potency of his product. The same applies to federal and state control laboratories using non-biological assays as a screening device prior to scheduling biological assays.

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