Estimation of Vitamin A in Margarine. IV. Assay Procedures for Yellow Margarine Made With and Without Carotene

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MARGARINE, as produced in the United States, is colored by any one of three materials, namely carotene concentrates, extracts of annatto seeds, or the certified food colors F. D. & C. Yellows No. 3 and No. 4. Carotene, in addition to imparting a yellow color to margarine, acts as a provitamin in that it is readily converted to vitamin A in the animal body. Indeed, the major portion of the supply of vitamin A to the adult organism is carotene derived from fruits and vegetables. The carotenoids in annatto extracts are devoid of vitamin A activity, but their use also constitutes coloring of margarine with naturally-occurring materials. F. D. & C. Yellows No. 3 and No. 4 are harmless materials permitted by the U. S. Food and Drug Administration for the coloring of foods, including margarines and winter butters.

The objects of the present investigation were to determine what influence these coloring agents have on the physico-chemical methods used for the estimation of vitamin A in margarine. Full credit must be given to the vitamin A contributed by the carotene, while steps must be taken to prevent erroneously high estimates being made in assaying margarines colored with the other materials. Assay methods are presented for the determination of vitamin A in colored margarines, and the limitations of these methods are discussed.

Quality Tests on the Margarine Coloring Agents

The certified food colors F. D. & C. Yellows No. 3 and No. 4 are used predominantly to color margarines manufactured in this country, carotene is used by some manufacturers, and annatto extracts only occasionally. The latter material imparts an undesirable reddish cast to the margarine and hence has not enjoyed much favor. Carotene cannot be used for various reasons for the coloring of white margarine in the home. Carotene is expensive, costing almost twice as much as preformed vitamin A on an equivalent vitamin A unitage. Hence the manufacturer must of necessity take full advantage of its potential vitamin A contribution. In those states where only white margarine is sold, the product prior to the addition of the carotene by the housewife would contain much less than the declared 15,000 USP units of vitamin A per pound.[1] Furthermore, were the carotene to be supplied separately as a coloring agent, the housewife might use variable amounts of the carotene concentrate in coloring the product, and this in turn would contribute to a variable vitamin A content. For economic reasons therefore the color wafer or capsule used in home coloring of white margarine must contain F. D. & C. Yellows No. 3 and No. 4 in a suitable carrier.

F. D. & C. Yellows No. 3 and No. 4 are blended usually in equal amounts for coloring margarine. Both colors in cyclohexane solution have an absorption maximum at 435 mμ; the $E^{1\%}_{1cm.}$ for F. D. & C. Yellow No. 3 is 580, while that for F. D. & C. Yellow No. 4 is 590. The absorption curves are almost identical from 300 to 500 mμ. These materials must comply with the specification for F. D. & C. Yellows No. 3 and No. 4 as listed in the regulations promulgated under the authority of the Federal Food, Drug, and Cosmetic Act (1). Of the possible coloring agents for margarine, only these materials react with strong hydrochloric acid to produce an intense red color; the test is easily carried out on the colors, even in the oil separated from margarine.

Chemical methods for the detection of the carotenoids in extracts of annatto seeds have been published (2). A special test applicable to the oil separated from margarine is given for the qualitative identification of these carotenoids. A positive reaction is the appearance of a purple color when stannous chloride is allowed to react with the dried aqueous extract of the annatto carotenoids separated from margarine oil.

Currently, there is only one manufacturer making a quality carotene concentrate suitable for vitamin fortification and coloring of margarine.[2] The carotene is derived from carrots. The carotene-protein complex, washed out from finely comminuted carrots, is concentrated by heat-coagulation and salting-out. The carotene is separated from the protein and attendant salt by solvent extraction. After heat-volatilization of the solvent, the crystalline carotene material is suspended in cottonseed oil and finally reduced in the ball-mill to particles of about 10 microns in diameter. The commercial product consists of 30 parts of carotene material and 70 parts of cottonseed oil and furnishes about 400,000 USP units of vitamin A per gram.

Harry J. Deuel Jr., at the recent hearing before the Federal Security Administrator on amending the Standard of Identity of Oleomargarine, recommended that the above product be used to supply part of the vitamin A in margarine, bearing in mind its chromogenic value in margarine. He also broadened his recommendation (6) as follows: "A provitamin A preparation suitable for vitamin A fortification of margarine is one which contains not less than 50% of natural carotenoids, which in turn consist of not less than 80% beta-carotene. The irrelevant material in the carotene preparation is the natural edible material 'carrying through' in the course of isolating the carotene fraction." Actually in carrots the carotene present is about two-thirds beta-carotene and about one-third alpha-carotene (7, 8). In the carotene preparation described above, the beta-carotene is slightly more than 80%. Apparently in the preparation of the concentrate there is a preferential isolation of beta-carotene. Indeed, in the earlier preparations (9), when the total yield of isolated carotene was only one-half that currently attained, the ratio of beta to alpha carotene was as 90:10. Chromatographic resolution of the commercial micro-crystalline carotene suspension

[1] In manufacture of yellow margarine the carotene is added in such amounts as to furnish from 6,000 to 7,000 USP units of vitamin A per pound.

[2] Barnett's microcrystalline carotene, obtained from the Barnett Laboratories, Long Beach, Calif. (3-5).

TABLE I

Analytical Constants Relating to the Spectrophotometric Findings Plotted in Figure 1[a]

Solution	Description	$E^{1\%}_{1cm.}$ 455 mμ	$E^{1\%}_{1cm.}$ 340mμ	Ratio E 362 mμ/E 340 mμ
A	Pure beta-carotene in cyclohexane	2,470	.....	.....
B	As A, but in hexane	2,470	140	1.38
C	As A, but heat isomerized in hexane	2,340	237	0.80
D	As A, but iodine isomerized in hexane	1,950	335	0.74
E	Commercial carotene preparation in cyclohexane	616	.....	.....
F	As E, but in hexane	616	40.0	1.00
G	As E, but heat isomerized in hexane	570	68.3	0.72
H	As E, but iodine isomerized in hexane	490	75.2	0.71
I	Commercial carotene in margarine oil, cyclohexane as solvent, versus uncolored control oil in cyclohexane	0.0243	.....	.....
J	As I, for reading at 455 mμ, versus uncolored control oil alone at 340 and 364 mμ	0.0243	0.0017	0.99
K	As I, but separated from fresh margarine. Readings as in J	0.0243	0.0023	0.90
L	As I, but separated from the margarine held 7 weeks at 75°F. Readings as in J	0.0213	0.0037	0.88

[a] Readings of light absorption in the ultra-violet on the systems exhibiting stereoisomerization are those at the respective maxima and minima, i.e. at 340 ± 2 mμ and at 362 ± 2 mμ.

in oil, using a calcium hydroxide-celite (3:1) column with petroleum ether (b.p. 60-70°C.) as the developing solvent, has demonstrated that the preparation consists largely of beta-carotene and is practically free from stereoisomers (10).

Theoretically a preparation consisting of 80% beta-carotene and 20% alpha-carotene should have a biological potency as vitamin A of about 90% of that of pure beta-carotene since the alpha-isomer is only about one-half as potent as beta-carotene (11). The light absorption curves of alpha- and beta-carotene are so similar (12) that it is not possible by spectrophotometric analyses to resolve the composition of mixtures. Only by chromatographic techniques (7, 8, 10) can resolution be effected.

Isomerization of carotene is another potential difficulty in the regular use of the provitamin in vitamin A fortification of margarine. It is well known (13-17) that the introduction of one or two *cis* double bonds into a naturally-occurring, all-trans carotenoid most often brings about a sharp reduction in the provitamin A potency. This tendency for isomerization and a reduction in biological potency, not reflected by a change in the shape of the visible light absorption curve, has been responsible for difficulties in the use of the former International Vitamin A Standard. The latter consisting initially of pure *all-trans* beta-carotene in coconut oil lost 25-30% of its potency over a period of six months. About 10% of the loss was due to oxidation, and the remainder was due to the spontaneous appearance of *cis* forms in the oil solution (18).

In the top row of Figure 1 are presented light absorption curves for pure beta-carotene,[3] the commercial carotene concentrate, and the latter in margarine oil. The pure beta-carotene and the commercial carotene preparation in organic solvents were read in the Beckman Spectrophotometer versus the corresponding solvent. The margarine oil solution of the commercial product was read versus a blank uncolored margarine oil, both of the same concentration in cyclohexane when obtaining the absorption curve in the visible region, and without solvent when readings were taken in the ultra-violet. The high dilution of the carotene in the margarine oil made this necessary since even then absorbancy values at 340 mμ were rather small, varying from 0.160 to 0.370. The curves on these systems from 325 mμ to higher wave-lengths blended well with the curves obtained with dilutions of the oils in cyclohexane. Below 325 mμ discrepancies were noted, due undoubtedly to the necessity of taking readings on undiluted oils. Rather than plot extinction coefficients, the spectrophotometric findings have been plotted in terms of extinction ratios, that is, the ratio of the extinction coefficient (or absorbancy) at a given wave-length to that at an absorption maximum. By this method of plotting, ready comparison can be made between the absorption curve of any

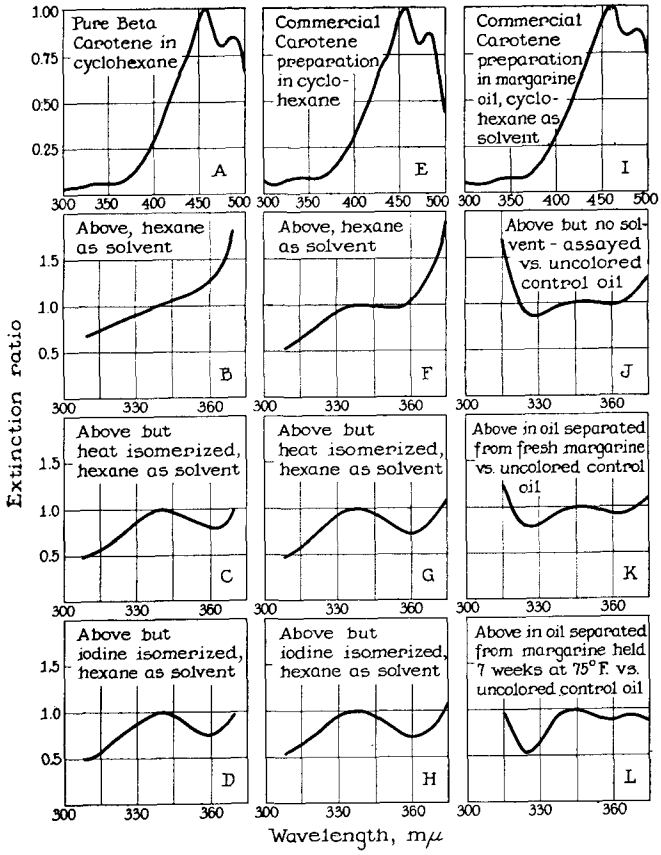

FIG. 1. Visible and ultra-violet light absorption curves of pure beta-carotene, of a commercial carotene preparation, and of the latter preparation in margarine oil.

[3] Kindly furnished by L. Zechmeister, California Institute of Technology, Pasadena, and used in the present study to establish light absorption curves for reference purposes.

sample, regardless of dilution of the test material, and that of a product of known purity, for example pure beta-carotene. It will be noted that the shapes of the visible-light absorption curves for the three test systems are practically identical. Analytical data relating to the spectrophotometric findings plotted in Figure 1 are listed in Table I.

Zechmeister and Polgar (19), in a study of stereoisomerization of the carotenoids, have pointed out that the general shape of the visible-light absorption curves is very little affected by *trans-cis* rearrangements. The presence of the usual absorption maxima in the visible region is evidently connected with the presence of the long conjugated system (20). In contrast, stereochemical alterations are reflected by changes in the ultra-violet light absorption curve between 320 and 380 mμ. In this region each of the C_{40}-carotenoids develops on stereoisomerization a moderate but marked maximum; in the case of beta-carotene a maximum develops at 340 mμ (± 2 mμ) with a minimum at about 362 mμ (± 2 mμ). This phenomenon has been termed by Zechmeister and Polgar (19) as the "*cis*-peak" effect.

In the second row of Figure 1 of this report are plotted the absorption curves for the same test materials shown in the first row, but with emphasis on the shape of the absorption curves in the ultra-violet. It will be noted that there is no evidence of a "*cis*-peak" in the curve obtained with pure beta-carotene. There is a suggestion of slight stereoisomerization having taken place in the commercial preparation of the carotene concentrate, and this is confirmed in the spectrophotometric examination of the material dissolved in margarine oil.[4]

Using the heat and iodine isomerization techniques described by Zechmeister and Polgar (19), it was possible to produce the "*cis*-peak" effect in spectrophotometric studies on both pure beta-carotene and on the commercial carotene preparation. The change in the shapes of the ultra-violet absorption curves and the extent of the variations in the $E^{1\%}_{1\,cm.}$ values at 455 and 340 mμ are confirmatory of those previously reported (19). It will be noted with pure beta-carotene (see Table I) that for a given percentage decrease in the $E^{1\%}_{1\,cm.}$ 455 mμ value there is a 7 to 14-fold percentage increase in the $E^{1\%}_{1\,cm.}$ 340 mμ value. The ratio of change in the $E^{1\%}_{1\,cm.}$ 455 mμ to the 340 mμ values for the commercial carotene concentrate following isomerization is on first examination not quite as large (4.5 to 10-fold) as that obtained with pure beta-carotene. This is attributed to some stereoisomers present in the commercial concentrate as received. Calculation based upon a ratio of the E 364/E 340 mμ value of 1.39 rather than the 1.00 obtained has shown that here there is actually an 8 to 15-fold percentage increase in the $E^{1\%}_{1\,cm.}$ 340 mμ value for a given percentage decrease in the $E^{1\%}_{1\,cm.}$ 455 mμ value.

The ultra-violet absorption curves in Figure 1 and the data in Table I, bearing on the fate of carotene in margarine oil, shows that the oil separated from the freshly prepared product contains a measurable concentration of stereoisomers. Holding the margarine under abusive conditions of storage, seven weeks at 75°F., is responsible for some additional increase in stereoisomerization. How much of the apparent isomerization noted is due to the heating required to separate the margarine oil is not known. Nevertheless it is to be noted that the extent of stereoisomerization of the carotene in the oils separated from the margarines is very much less, on the basis of the ratio of E 362/E 340 mμ values, than that noted following heat or iodine isomerization of either pure beta-carotene or the commercial concentrate.

Based on theoretical considerations Zechmeister (21) has pointed out that a quasi-equilibrium mixture of carotene and its stereoisomers obtained by refluxing beta-carotene in petroleum ether suffers only little loss in biological potency. Such a mixture is composed of 86 parts unchanged all-trans, 4 parts neo U, 8 parts neo B beta-carotene, and 2 parts of labile isomers. This mixture is calculated to be 92% as effective as pure beta-carotene as a source of vitamin A. For the iodine-catalyzed stereoisomeric beta-carotene mixture the corresponding figure is about 87%.

From the above it may be postulated that the loss in vitamin A due to the stereoisomerization noted in spectrophotometric assays of the commercial carotene concentrate, before and after use in margarine manufacture, is negligible; the loss would appear to be no greater than that reflected by the decrease in the $E^{1\%}_{1\,cm.}$ 455 mμ values. This conclusion, based upon Zechmeister's postulation, was confirmed by multiple-level biological assays of pertinent test systems; the physico-chemical estimates of vitamin A content were based upon the spectrophotometric method ($E^{1\%}_{1\,cm.}$ 455 mμ values) subsequently described in this paper. The results of the assays are given in Table II.

TABLE II

Biological and Non-Biological Assays of the Commercial Carotene Concentrate Before and After Use in Margarine Manufacture[a]

Sample	Vitamin A Content	
	Spectrophotometric Assay	Multiple Level Bioassay
	USP Units	USP Units
	per gm.	*per gm.*
Carotene concentrate	405,000	377,000
	per lb.	*per lb.*
Carotene fortified margarine	6,100	7,500[b]
Above stored 7 weeks at 75°F	5,350 (12% loss)	6,200 (17% loss)

[a] The test samples were the same as those covered in Figure 1 and Table I of this paper.

[b] Average of two values, 7,100 and 7,900 USP units per pound of margarine, obtained by two independent multiple-level biological assays conducted in two different laboratories.

It will be noted that the bio-assay value for the concentrate was 93% of the spectrophotometric value. In the spectrophotometric estimate no correction was made for the presence of alpha-carotene in the concentrate. If this were done, the bio-assay value would be 98% of the spectrophotometric estimate. In the case of the margarines the bio-assay estimates are 120% of the spectrophotometric values (125% when the latter is corrected for the alpha-carotene present). Thus, in margarine, loss of vitamin A potency attributable to stereoisomerization is not apparent. This is due in large measure to the fact that in a margarine vehicle there is an augmentation of the provitamin A

[4] To yield a special margarine with carotene as the sole source of vitamin A and fortified to a level of approximately 6,000 USP units of vitamin A per pound.

TABLE III

Reproducibility and Quality of the Commercial Carotene Concentrate Currently Available[a, b]

Batch	E λ/E 455 mμ at					E λ/E 340 mμ at			
	442 mμ	455 mμ	473 mμ	483 mμ	487 mμ	308 mμ	340 mμ	362 mμ	455 mμ
1	0.78	1.00	0.81	0.87	0.84	0.60	1.00	1.31	18.1
2	0.80	1.00	0.81	0.88	0.84	0.59	1.00	1.30	18.3
3	0.80	1.00	0.81	0.87	0.83	0.61	1.00	1.31	18.7
4	0.83	1.00	0.83	0.87	0.80	0.59	1.00	1.39	19.2
5	0.83	1.00	0.82	0.87	0.79	0.60	1.00	1.38	19.3
6	0.84	1.00	0.83	0.87	0.80	0.60	1.00	1.35	18.5
7	0.83	1.00	0.81	0.86	0.79	0.61	1.00	1.35	18.7
8	0.81	1.00	0.81	0.85	0.77	0.59	1.00	1.30	17.2
9	0.78	1.00	0.81	0.87	0.84	0.61	1.00	1.34	18.6
10	0.84	1.00	0.83	0.87	0.80	0.60	1.00	1.34	18.6
11	0.83	1.00	0.83	0.87	0.80	0.61	1.00	1.32	18.4
12	0.83	1.00	0.81	0.86	0.79	0.61	1.00	1.36	19.4
13	0.80	1.00	0.81	0.88	0.84	0.62	1.00	1.35	19.3
14	0.80	1.00	0.81	0.87	0.83	0.61	1.00	1.34	19.1
15	0.83	1.00	0.82	0.87	0.80	0.64	1.00	1.39	19.7
Mean	0.81	1.00	0.82	0.87	0.81	0.61	1.00	1.34	18.7
Standard deviation	0.021	0.00	0.010	0.007	0.026	0.026	0.00	0.065	0.96
Pure beta-carotene	0.80	1.00	0.80	0.85	0.85	0.68	1.00	1.38	17.7

[a] Cyclohexane of the quality previously described (24) used as the solvent for readings in the visible range of the spectrum while purified hexane (B.P. 65°C.) used as the solvent for readings in the ultra-violet. The latter solvent is purified as follows: to 4 liters of the hexane are added 50 gm. of activated vegetable charcoal (West Virginia Pulp and Paper Co., Carbon C-115-N). The filtered hexane is tested versus distilled water in the Beckmann Spectrophotometer using the hydrogen discharge lamp and quartz cells. The transmission readings should be not less than 98.5% at 300 mμ, 99% at 310 mμ, and 100% at 320 to 500 mμ.

[b] The mean $E^{1\%}_{1cm.}$ value at 455 mμ for the concentrates was 586, S.D. ± 21.2; and at 340 mμ it was 31.3, S.D. ± 1.3.

potency of carotene (22). It should be noted however that the loss in vitamin A as a result of abusive storage of the test margarine is of the same order of magnitude based on the spectrophotometric or biological assay, bearing in mind the lower precision of the latter method. Thus the data in Tables I and II justify the conclusion that stereoisomerization of carotene is no problem when a quality carotene concentrate is employed to color margarine and at the same time contribute substantially to the vitamin A content.

Subsequent to the studies on stereoisomerization, it has become routine in our laboratory to take both visible and ultra-violet light absorption readings on each batch of carotene purchased for coloring margarine. The readings in the visible region of the spectrum (455 mμ) are used to establish the potency of the material as a source of vitamin A while those in the ultra-violet are used to confirm the absence of stereoisomeric forms of carotene and thereby justify the spectrophotometric estimates of vitamin A potency. The illustrative data given in Table III clearly demonstrate that the commercial carotene concentrate is a reproducible product of high quality, currently even superior to that employed in the earlier studies on stereoisomerization. The manufacturer in increasing his yield of carotene during the past few years has now eliminated completely the stereoisomers from the concentrate.

From the data presented in both Tables I and III it has been concluded that the following requirements[5] must be satisfied by a beta-carotene concentrate to be used in margarine manufacture:

a) In cyclohexane solvent the preparation should have absorption maxima at 455 ± 1 mμ and 483 ± 1 mμ with the ratio of extinction coefficients at 483 mμ to 455 mμ between 0.83-0.89;

b) The shape of the visible absorption curve in other details should correspond to that for pure beta-carotene;

c) The ratio E 455 mμ/E 340 mμ should not be less than 15.0 with hexane as the solvent for the readings in the ultra-violet, and

d) The ratio E 362 mμ/E 340 mμ should not be less than 1.00.

It is unnecessary to plot for each batch the full absorption curves in the ultra-violet and visible; readings at the more important wave-lengths as shown in Table III suffice.

Comparative Light Absorption Curves for Margarine Oils Containing Each of the Coloring Agents in Current Use and the Influence of These Supplements on the Spectrophotometric Assay for Preformed Vitamin A

It will be noted from the data plotted in Figure 2 that the shape of the visible light absorption curve (420-510 mμ) of the annatto extract is somewhat simi-

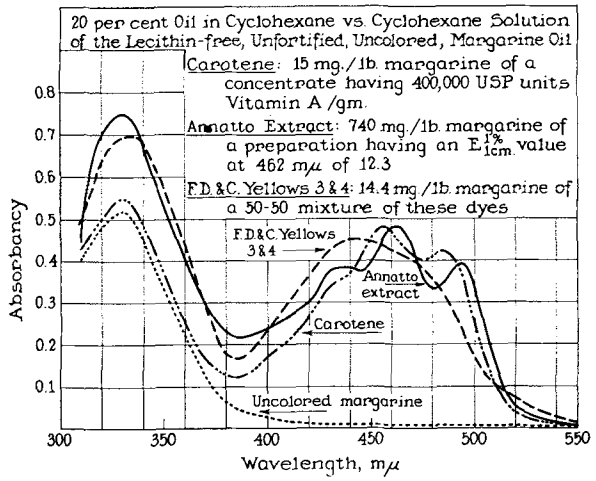

FIG. 2. Visible and ultra-violet light absorption curves of margarine oils separated from margarine, fortified to a level of 18,000 USP units with preformed vitamin A per pound, before and after supplementation with each of the three materials currently used to color margarine.

[5] That is over and above other requirements not presented here since they are not pertinent to the vitamin A picture.

TABLE V

Antimony Trichloride Reaction With Vitamin A and With the Materials Used to Color Margarine

Material[a]	Absorbancy at 620 Millimicrons					Comment
	4 seconds	1.0 minute	30 minutes	2 hours	3 hours	
10 USP units preformed vitamin A	0.220	0.161	0.009	0.000	0.000	Typical vitamin A reaction, blue fades to water white.
60 mcg. of beta-carotene	0.186	0.161	0.195	0.245	0.271	Typical carotene reaction, blue increases in intensity and develops a reddish cast.
12.3 mg. of annatto extract, $E^{1\%}_{1\,cm.}$ 462 mμ = 12.3	0.744	1.31[b]	1.72[b]	1.51[b]	1.61[b]	Greenish blue color and turbidity develops.
0.24 mg. F. D. & C. Yellows No. 3 and No. 4 (50-50 mixture)	0.308	0.308	0.308	0.298	0.298	Bright carmine red.

[a] In 1.0 cc. chloroform + 9.0 cc. of a 20.0% solution of antimony trichloride in chloroform.
[b] Obtained following a 1-2 dilution of the chloroform solution of annatto extract.

lar to that of the carotene preparation. The annatto extract shows three absorption maxima, 494 mμ, 462 mμ, and 436 mμ, while the carotene preparation exhibits maxima at 483 mμ and 455 mμ, and an inflection at 430 mμ. Earlier in this report a color test (2) was cited to which the carotenoids in the annatto extract respond; this can be used effectively to differentiate a margarine colored with annatto extract from one colored with carotene. However when mixtures of annatto extract and carotene are used to color margarine, both the color test and the spectrophotometric absorption curves are difficult to interpet. Fortunately the carotenoids in the annatto extracts are found to be completely water-soluble following saponification,[6] while carotene remains unchanged and entirely in the unsaponifiable fraction. This clean-cut separation of the two coloring agents permits ready identification.

The colors, F. D. & C. Yellows No. 3 and No. 4, behave like carotene in the saponification step, "carrying through" into the unsaponifiable extract. However the shapes of the light absorption curves are sufficiently dissimilar (see Figure 2) to permit differentiation between these two coloring materials. Furthermore these F. D. & C. colors react with strong acid to produce a red color (1) while carotene does not.

[6] In the current study little loss of annatto carotenoids occurred in preparing the unsaponifiable extract of margarine according to the method of Rice and associates (23). Fully 98% of the pigment was recovered, but all of this was found in the alkaline aqueous phase. The visible light absorption curve of the annatto carotenoids in the aqueous extract was practically the same as that of the same carotenoids dissolved in margarine oil, when plotted as extinction ratios to compensate for the concentration differences.

In a preceding paper in this series (24) a rapid precise and accurate spectrophotometric method was presented for the control of vitamin A fortification of uncolored margarine made in the plant. Also in Figure 2 are plotted the ultra-violet light absorption curves of margarine oils separated from a margarine fortified to a level of 18,000 USP units with preformed vitamin A per pound, before and after supplementation with each of the three materials currently used to color margarine.

It is apparent that the lecithin-free (25) unfortified margarine oil blank must contain the added coloring for reliable estimates of the preformed vitamin A content of yellow margarine. The values in Table IV, based on the data plotted in Figure 2, indicate the extent of the error resulting from the use of an uncolored unfortified oil blank in the spectrophotometric test. When these same test samples were read versus

TABLE IV

Extent of the Over-Estimates of the Preformed Vitamin A Content of Colored Margarine When the Lecithin-Free Unfortified Margarine Oil Blank Does Not Contain the Added Coloring

Margarine Sample	$E^{20\%}_{1\,cm.}$ 328 mμ Readings[a]	Preformed Vitamin A Content	
		Apparent	Over-estimate
		USP units per pound	*USP units per pound*
Uncolored	0.52	17,900	0
Colored with carotene	0.55	18,900	1,000
Colored with F. D. & C. Yellows No. 3 and No. 4	0.69	23,700	5,800
Colored with annatto extract	0.75	25,800	7,900

[a] Cyclohexane as the solvent.

TABLE VI

Implications of the Chromogenicity of the Margarine Coloring Agents, Following Reaction With Antimony Trichloride, on the Colorimetric Estimate of Vitamin A Content

Test Material	Quantity for $SbCl_3$ Reaction		Absorbancy at 620 mμ[a]		Calculated Apparent Vitamin A Content of Yellow Margarines of Equal Color Intensity[b]
	Absolute	Vitamin A Equivalent	Observed	Apparent Vitamin A Equivalent	
		USP units		*USP units*	*USP units/lb.*
Preformed vitamin A	3 mcg.	10	0.220	10.0	.....
Beta-carotene	*60 mcg.*	*100*	0.186	8.2	492
Annatto extract $E^{1\%}_{1\,cm.}$ 462 mμ = 12.3	12.3 mg.	0	0.744	37.0	2,220
F. D. & C. Yellows No. 3 and No. 4	0.24 mg.	0	0.308	14.6	875

[a] At the end of 4 seconds at which time the preformed vitamin A reaction product absorbs maximally (see Table V). Apparent vitamin A equivalent obtained by interpolating absorbancy values on vitamin A reference curve.
[b] Margarine free of preformed vitamin A and colored to the same degree of yellow with one of the coloring agents: 6,000 USP units of vitamin A as carotene, 740 mg. of annatto extract, or 14.4 mg. of F. D. & C. Yellows No. 3 and No. 4 per pound of margarine.

TABLE VII

Vitamin A ($SbCl_3$) Assays of Yellow Margarine Containing One of the Three Coloring Agents in Current Use[a]

Margarine	Vitamin A Fortification		$SbCl_3$ Assay of Separated Margarine Oil		Error in Vitamin A Estimate	
	Preformed	Provitamin	Whole Oil[b]	Unsap. Extract[c]	Whole Oil Assay	Unsap. Extract Assay
	USP units/lb.	*USP units/lb.*	*USP units/lb.*	*USP units/lb.*	*Percent*	*Percent*
Uncolored	18,500	0	18,500	18,400	0	0
Colored with carotene	12,500	6,000	13,500	13,000	−27	−30
Colored with annatto extract	18,500	0	20,200	18,600	+11	0
Colored with F. D. & C. Yellows No. 3 and No. 4	18,500	0	19,600	19,700	+ 6	+ 7

[a] For quantities of the coloring agents employed see footnote "b" of Table VI.
[b] Using the increment procedure (26, 23).
[c] Using the method previously described (24).

the appropriate colored margarine oil blanks, the discrepancy between apparent and true vitamin A content (preformed) disappeared.

Reactions of the Margarine Coloring Agents with Antimony Trichloride and Their Influence on the Colorimetric Test for Preformed Vitamin A

In Table V are shown the results of the antimony trichloride reaction with vitamin A, carotene, annatto carotenoids, and F. D. & C. Yellows No. 3 and No. 4. The quantities of the annatto extract and the F. D. & C. colors used are equivalent in yellow color to the amount of carotene employed in these tests. The quantity of beta-carotene used (60 micrograms) is such that it exhibits an absorbancy at 620 mμ comparable to that obtained with 10 USP units of vitamin A, following reaction with antimony trichloride (26). From the data in Table V it is to be noted that both the annatto carotenoids and F. D. & C. Yellows No. 3 and No. 4 react with antimony trichloride producing colors absorbing strongly at 620 mμ.

If one were to conduct colorimetric assays of unsaponified oils separated from margarines as suggested by some analysts (23), it would follow that erroneously high estimates would be obtained in assays of margarines colored with an annatto extract or F. D. & C. Yellows No. 3 and No. 4 and erroneously low estimates for the carotene-colored product. This is clearly shown by the data in Table VI. It is worth emphasizing that carotene on an equivalent vitamin A unitage basis is only about one-twelfth as chromogenic as preformed vitamin A in the colorimetric test.

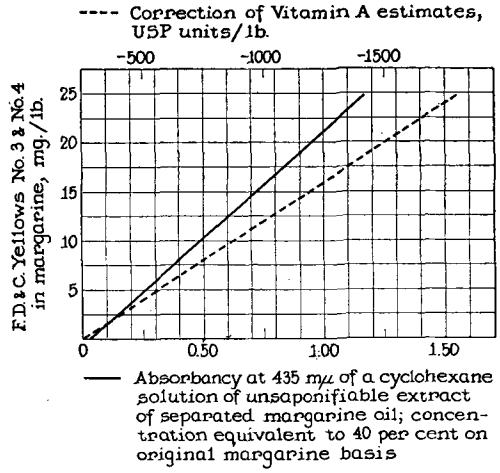

FIG. 3. Chart for correcting colorimetric ($SbCl_3$) estimates of the vitamin A content of margarine colored with F. D. & C. Yellows No. 3 and No. 4.

When the assays are conducted on the unsaponifiable extract of the margarine oils, the interference in the vitamin A test by one coloring agent, the annatto carotenoids, is eliminated. However with the F. D. & C. Yellows No. 3 and No. 4 erroneously high and with carotene erroneously low estimates of vitamin A content are still obtained (see Table VII).

It is possible to correct for the over-estimate of vitamin A content attributable to the reaction between the F. D. & C. colors and the antimony trichloride reagent. In Figure 3 is presented a chart illustrating how this correction is made. The $E^{1\%}_{1\,cm.}$ 435 mμ values of the unsaponifiable fractions of fortified margarines (or of separated margarine oils) colored with known increasing amounts of F. D. & C. Yellows No. 3 and No. 4 are plotted, absorbancy versus concentration of coloring material. The desirable concentration of the unsaponifiable fraction in cyclohexane solvent is 40% on the original margarine basis. Another aliquot of the unsaponifiable extract (23), now in chloroform solution and also in 40% concentration on the original margarine basis, is allowed to react with the antimony trichloride reagent and the apparent vitamin A content estimated (24). The increments in apparent vitamin A content over that obtained in assaying the uncolored but fortified margarine are plotted versus concentration of color in the margarine; these increments are the corrections to be applied to the $SbCl_3$ assays of margarine colored with F. D. & C. Yellows No. 3 and No. 4. That the margarine is actually colored with these materials can be demonstrated by the tests mentioned earlier.

F. D. & C. Yellow No. 3 is only 70% as chromogenic as F. D. & C. Yellow No. 4. For the most part the colors are used in a 50:50 ratio for coloring margarine. If one coloring agent were used exclusively, the correction would be either too low or too high by 15%. This is of little concern since at the usual level of color in margarine, about 14 mg. per pound, this amounts to only 140 USP units of apparent vitamin A per pound and in terms of the fortified margarine (usually about 18,000 USP units per pound) would introduce an error of only 0.8%. In so far as $E^{1\%}_{1\,cm.}$ 435 mμ readings of the cyclohexane solution of the unsaponifiable fraction are concerned, it makes no difference which of the colors may have been used.

It will be noted that the plot of the absorbancy values in Figure 3 meets the abscissa at an 0.03 absorbancy value. This is the irrelevant absorbancy in the unsaponifiable extract derived from the margarine oil itself. Tests conducted on light and dark oils varying in Lovibond color values from 25Y-2.5R to 55Y-11.8R gave absorbancy values at 435 mμ of

TABLE VIII

Extent of Error in Colorimetric ($SbCl_3$) Assays of Margarines Containing F. D. & C. Yellows No. 3 and No. 4 and the Reliability of the Correction Procedure Presented[a]

Sample	$SbCl_3$ Values for Uncolored Margarines	F. D. & C. Yellows No. 3 and No. 4		Spectrophotometric Values for Margarines[b]	$SbCl_3$ Values for Colored Margarines		
		Added	Found		Observed	Correction[c]	Corrected
	USP units per lb.	*mg. per lb.*	*mg. per lb.*	*USP units per lb. of margarine*	*USP units per lb. of margarine*	*USP units per lb. of margarine*	*USP units per lb. of margarine*
1	18,600(+6.8%)	10	9.8	17,400	19,200(+10.4%)	−600	18,600(+6.8%)
2	18,400(+5.8%)	10	10.5	17,400	18,600(+6.9%)	−650	18,000(+3.5%)
3	18,400(+7.0%)	14	14.5	17,200	19,300(+12.2%)	−900	18,400(+7.0%)
4	18,600(+5.7%)	14	13.9	17,600	19,500(+10.8%)	−870	18,600(+5.7%)
5	19,000(+5.6%)	14	13.9	18,000	19,900(+10.6%)	−870	19,000(+5.6%)
6	18,600(+6.8%)	18	17.8	17,400	19,900(+14.4%)	−1,190	18,700(+7.5%)
7	18,400(+5.8%)	18	18.2	17,400	19,600(+12.6%)	−1,200	18,400(+5.8%)

[a] The values in parentheses are the percentage over-estimates in vitamin A content by the assay methods indicated.
[b] For either the uncolored or colored margarines, when the test solutions were read versus the proper unfortified oils (24, and see text of this report). These values are the true estimates of vitamin A content.
[c] Based upon procedure described in text and in Figure 3.

0.02 to 0.04 (unsaponifiable extracts in cyclohexane at a 40% concentration on the original margarine basis), with no correlation between Lovibond and photometric readings.

The correction chart in Figure 3 is presented for illustrative purposes. Each laboratory should establish its own chart since vagaries exist between laboratories in preparing unsaponifiable extracts and in conducting the $SbCl_3$ test. That the method presented is satisfactory in eliminating the interference of F. D. & C. Yellows No. 3 and No. 4 in the vitamin A assay is shown by the data in Table VIII.

The correction method however does not in any way compensate for the tendency of the $SbCl_3$ assay to over-estimate the vitamin A content of margarine (24). This is also illustrated by the data in Table VIII.

Assay of Carotene-Colored Margarine with Proper Allowance for the Vitamin A Contribution by the Provitamin

In a preceding section mention was made of the fact that, for routine plant control of the vitamin A fortification of margarine, the spectrophotometric method previously presented (24) could be used satisfactorily to determine preformed vitamin A content. It was pointed out that the blank unfortified oil must contain the coloring material in same concentration when used to set the spectrophotometer at 328 mμ at 100% transmission prior to readings of the test solution. Procedures have also been presented for eliminating the interference of the annatto carotenoids and of the F. D. & C. Yellows No. 3 and No. 4 in the colorimetric ($SbCl_3$) assay method. The present section presents methods for the determination of vitamin A in carotene-colored margarine with full allowance of the vitamin A contribution by the provitamin.

Plant control procedure. The test margarine oil in 40% cyclohexane solution is read spectrophotometrically versus the proper unfortified uncolored oil in similar concentration (24) at 455 mμ and the absorbancy multiplied by 6,300 to convert to USP units of vitamin A per pound of margarine; this gives the vitamin A contributed by the carotene. We have obtained an $E^{1\%}_{1\,cm.}$ 455 mμ value of 2,470 for pure beta-carotene,[3] using cyclohexane as the solvent. Others (28, 29) have reported values of 2,490 and 2,400 with the same solvent. We have used a value of 2,400 in calculating the conversion factor noted above. Furthermore no correction has been made for the 10 to 20% alpha-carotene present in the commercial carotene concentrate. Justification for the use of a somewhat liberal factor converting absorbancy to USP units of vitamin A per pound of margarine is the fact that carotene, when fed in a margarine vehicle, exhibits an augmented biological potency (22). The margarine manufacturer is held responsible for the biological, not spectrophotometric, unitage of the product marketed.

The 6,300 conversion factor employed in our laboratories is derived from the equation:

$$\text{Conversion Factor} = A \times 694 \times 454 \times 0.8/40.$$

Where A = Absorbancy of the carotene in the fortified oil at 455 mμ

694 = Factor for converting absorbancy to USP vitamin A units[7]

454 = Grams per pound

0.8 = Fraction of margarine as fat

40 = Grams of margarine oil in cyclohexane per 100 cc. overall volume.

For the assay of the carotene concentrate, the $E^{1\%}_{1\,cm.}$ 455 mμ value is multiplied by the 694 factor to obtain USP units of vitamin A per gram of product.

Since vitamin A fortification with preformed vitamin A and carotene in the plant is made at one and the same time, it is difficult to obtain an oil colored with carotene and free from preformed vitamin A. Accordingly in the assay for preformed vitamin A we have preferred to read the colored margarine oil versus the proper, unfortified, uncolored oil as described in an earlier paper (24), using the 40% solution of margarine oil in cyclohexane. Correction is then made for the relatively small absorption at 328 mμ by the carotene present. The absorbancy at 328 mμ of carotene in margarine oil, fortified to a level of 6,000 USP units of vitamin A per pound of margarine, is 0.06 ± 0.01 when read as the 40% solution of oil in cyclohexane (see Figure 2, where an increment of 0.03 in absorbancy at 328 mμ is obtained for a 20% oil solution). Hence the modified formula for calculating preformed vitamin A in carotene-colored margarine is:

$$[A - (0.01 \times P)] \times 1{,}894 \times 454 \times 0.80/40 = \text{USP units per lb. of margarine}$$

[7] On the basis that $E^{1\%}_{1\,cm.}$ 455 mμ for beta-carotene = 2,400 and that 0.6 mcg. of beta-carotene furnishes 1.0 USP unit of vitamin A, it follows that $\frac{1{,}000{,}000}{0.6 \times 2{,}400} = 694$.

TABLE IX

Comparison of Assay Procedures for Determining Vitamin A in Margarine Colored With Carotene

Sample	Spectrophotometric Assay of Separated Margarine Oil Using Unfortified Control Oil		Total	Colorimetric Assay of Unsap. Extract from Separated Margarine Oil	Spectrophotometric Assay of Unsap. Extract from Separated Margarine Oil	Total
	Vitamin A	Carotene		Vitamin A	Carotene	
	USP Units Vitamin A per lb. Margarine					
1	11,300	5,500	16,800	13,000	5,800	18,800
2	10,800	6,300	17,100	13,100	6,100	19,200
3	11,100	6,100	17,200	12,200	5,900	18,100
4	11,900	6,200	18,100	13,400	5,800	19,200
5	10,800	6,000	16,800	12,400	5,700	18,100
6	11,700	4,400	16,100	11,500	4,300	15,800
7	12,000	5,500	17,500	11,500	5,400	16,900
8	12,000	5,700	17,700	12,400	5,600	18,000
9	13,400	5,900	19,300	12,400	5,600	18,000
10	11,900	5,800	17,700	12,700	5,700	18,400
11	10,000	6,800	16,800	11,300	6,700	18,000
12	11,200	6,500	17,700	11,900	6,600	18,500
13	11,500	6,900	18,400	11,500	6,900	18,400
14	11,300	6,800	18,100	11,200	6,300	17,500
15	11,000	6,900	17,900	10,000	6,700	16,700
16	11,500	8,700	20,200	11,800	8,400	20,200
17	11,200	6,600	17,800	11,300	5,800	17,100
18	11,300	6,500	17,800	11,200	6,100	17,300
19	11,000	6,900	17,900	11,500	6,400	17,900
20	11,500	6,800	18,300	12,700	6,200	18,900
21	11,500	6,900	18,400	12,300	7,200	19,500
22	10,300	7,200	17,500	11,200	7,000	18,200
23	11,000	6,600	17,600	11,100	5,900	17,000
24	10,800	6,300	17,100	10,700	6,100	16,800
25	11,300	6,900	18,200	10,700	6,300	17,000
26	10,300	6,600	16,900	11,500	6,100	17,600
27	10,800	6,500	17,300	11,200	6,100	17,300
28	10,800	6,700	17,500	12,000	6,400	18,400
29	10,600	6,600	17,200	11,300	6,200	17,500
30	10,800	6,600	17,400	11,100	6,100	17,200
31	11,500	6,600	18,100	11,700	6,100	17,800
32	11,200	6,600	17,900	11,700	6,400	18,100
33	11,200	6,700	17,900	11,900	6,300	18,200
34	11,500	6,300	17,800	11,700	5,600	17,300
35	11,500	7,100	18,600	12,400	5,800	18,200
36	12,400	6,900	19,300	11,700	6,500	18,200
37	11,000	6,700	17,700	11,500	6,600	18,100
38	10,500	6,900	17,400	11,400	6,300	17,700
39	10,800	6,800	17,600	12,100	6,600	18,700
40	10,300	6,500	16,800	11,500	6,100	17,600
41	10,500	6,900	17,400	11,400	6,500	17,900
42	11,000	6,900	17,900	11,300	6,300	17,600
43	11,500	7,100	18,600	11,600	6,400	18,000
44	10,200	6,800	17,000	11,700	6,300	18,000
45	11,000	6,700	17,700	11,500	6,600	18,100
Mean	11,170	6,560	17,730	11,720	6,220	17,940
Standard Deviation	±632	±613	±792	±600	±576	±714

Where A = Absorbancy of the fortified oil read versus blank oil

P = Provitamin A activity of the carotene in 1,000 USP units per lb. of margarine

1,894 = Factor for converting absorbancy of preformed vitamin A to USP units (27)

454 = Grams per pound

0.80 = Fraction of margarine as fat

40 = Grams of margarine oil in cyclohexane per 100 cc. overall volume.

If fortification with carotene is at the level of 6,000 USP units per pound of margarine, absorbancy — 0.06 × 17,200 yields directly the preformed vitamin A unitage per pound. This figure plus that for the vitamin A contributed by the carotene is the total vitamin A content per pound of margarine.

Assay of open market samples. As discussed in a previous paper (24), the spectrophotometric method cannot be used to determine the vitamin A content of margarine without having available for test the blank unfortified margarine oils of the same history. Within certain limitations the colorimetric ($SbCl_3$) method can be used to determine preformed vitamin A content (24), and this value plus that obtained following spectrophotometric assay for the carotene present gives the overall vitamin A potency of the margarine.

In this type of assay 16.0 gm. of the separated margarine oil (equivalent to 20.0 gm. of margarine) are saponified (23) and brought to a volume of 50 cc. The ether solution is divided into two equal parts and both evaporated to dryness under nitrogen with gentle heating. One portion is then taken up with dry chloroform and brought to a 25-cc. volume; the other portion is brought also to a 25-cc. volume in cyclohexane. The identity of the coloring agent is established by plotting the visible-light absorption curve versus the cyclohexane solvent. Absorption maxima at 455 mμ and at 483 mμ should exist with an inflection at 430 mμ. Indeed the absorption curve on the unsaponifiable extract will simulate very closely that shown in Figure 2 for the whole margarine oil colored with carotene and read versus the blank unfortified oil. Confirmation that the coloring agent is carotene may be obtained by negative results in the qualitative chemical tests previously indicated for the annatto carotenoids and for the F. D. & C. Yellows No. 3 and No. 4. The absorbancy of the cyclohexane solution of the unsaponifiable extract in 40% concentration (on the whole margarine basis) minus 0.03 times the 6,300 conversion factor gives the vitamin A unitage per pound of margarine contributed by

the carotene.[8] Absorbancy measurements conducted on the unsaponifiable extracts of uncolored light and dark margarine oils in the same test concentration have indicated that at 455 mμ irrelevant absorption is 0.03 ± 0.01.

Colorimetric ($SbCl_3$) assays are conducted (24) on the chloroform solution of the unsaponifiable extract mentioned above. Because of the reaction between carotene and the $SbCl_3$ reagent, discussed earlier in this paper, the colorimetric estimate of preformed vitamin A content is corrected by a figure representing one-twelfth of the vitamin A value attributable to the carotene.[9] The values for vitamin A, as preformed vitamin and provitamin, are added to obtain the total vitamin A unitage per pound of margarine.

Comparison of the results obtained by the two assay procedures for determining vitamin A in margarine colored with carotene. In Table IX are listed the results obtained by the two test methods in assaying 45 margarine samples. Mean values and the standard deviations of the means are also given. Attention is directed to the apparent better reproducibility of the figures for total vitamin A content as compared to that for either preformed vitamin A or provitamin A. This is due to the practice of fortifying margarine with carotene at either the 6,000 or 7,000 USP unit level per pound but keeping the total vitamin A level (18,000 USP units) the same in all distribution areas. The data in Table IX were subjected to further statistical analysis with the following results. For the difference between the mean values for preformed vitamin A by the two assay methods, "t" = 4.16; this is highly significant since the t value for p = 0.001 is 3.42. This is confirmatory of our findings in assays of uncolored margarine (24). The difference between the mean values for vitamin A as carotene by the two assay methods is small but statistically significant; t = 2.72 and the t value for p = 0.01 is 2.64. The difference between the mean values for total vitamin A content by the two assay methods is not statistically significant, t = 1.54. Apparently the small overestimate in preformed vitamin A content by the $SbCl_3$ procedure is neutralized by the small underestimate in provitamin A content in the associated test, so that the assay method for carotene-colored margarine in the absence of the blank unfortified margarine oil is as reliable as the spectrophotometric method used in controlling production.

[8] The 0.03 absorbancy correction is small, amounting to only about 3% of the true vitamin A unitage attributable to the carotene at 6,000 USP units per lb. of margarine.

[9] This correction of the $SbCl_3$ value is small, amounting to only about 4% of the preformed vitamin A unitage, when 12,000 USP units of vitamin A as such are added per lb. of margarine.

TABLE X

Reliability of Non-Biological Assay Procedures in Estimating the Vitamin A Content of Margarine Colored With Carotene

Sample No.	Non-Biological Assay of Separated Oil		Biological Assay of Margarine[c]
	Spectrophotometric[a] (Whole Oil)	Colorimetric ($SbCl_3$)[b] and Spectrophotometric (Unsaponifiable Extract)	
	USP Units of Vitamin A per lb. of Margarine		
1	17,600	19,800	20,900
2	17,400	18,000	18,200
3	17,400	19,100	21,600
4	16,700	18,200	15,500
5	17,500	18,300	18,800
6	17,000	18,700	19,000
7	16,700	17,000	17,200
8	17,200	17,300	20,500
9	17,600	17,400	21,600
10[d]	17,800	18,100	20,100
Mean	17,300	18,200	19,300
Standard Deviation	±381	±854	±1,989

[a] Both vitamin A and carotene determined spectrophotometrically with the Beckmann Spectrophotometer set at 100% transmission at the required wavelength with the appropriate unfortified control oil (see text).

[b] Preformed vitamin A determined colorimetrically and the carotene spectrophotometrically with no unfortified margarine oil as the control (see text).

[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.

[d] This sample after abusive storage, 7 weeks at 75°F., gave values by the above methods of assay of 15,500, 17,600, and 19,000 USP units per pound respectively.

Reliability of the Results Obtained by the Two Non-Biological Assay Procedures for Determining Vitamin A in Margarine Colored with Carotene

In Table X are presented the results of biological and non-biological assays conducted on 10 margarine samples. With the exception of Sample No. 10 all the others were picked up on the open market.

The augmentation of the provitamin A potency of carotene when fed in a margarine vehicle is noted (22). The results were subjected to statistical analyses with the following results. For the difference between the mean values obtained by the two non-biological assay methods for total vitamin A content, t = 3.05; this is significant since the t value for p = 0.01 is 2.88. This finding of a significant difference, not noted in the evaluation of the results presented in Table IX, is attributed to the fact that in stored margarine exhibiting some vitamin A loss, there is a greater tendency for the $SbCl_3$ procedure to overestimate preformed vitamin A content (24). This is illustrated by the values obtained by the non-biologicay assays of Sample No. 10, following abusive storage (see footnote d to Table X). However because of the augmentation of the provitamin A activity of carotene when fed in margarine, estimates of vitamin A potency by the colorimetric ($SbCl_3$) and associated spectrophotometric test on the unsaponifiable extracts are still reliable in assays of stored margarine. The difference between the mean values obtained by the latter method and by biological assay is not statistically significant, t = 1.68. As would be expected, the difference between the mean values obtained by the spectrophotometric (whole oil) assay and by the biological assay is statistically significant, t = 3.20.

From the results presented above, it is concluded that either one of the two non-biological assay methods can be employed to yield a reliable estimate of the vitamin A potency of margarine containing preformed vitamin A and colored with carotene.

Summary

Chemical and spectrophotometric methods have been presented for the identification and evaluation of quality of the agents (annatto carotenoids, F. D. & C. Yellows No. 3 and No. 4, and carotene) used to color margarine. A procedure for determining the extent of stereoisomerization in carotene concentrates has been described. A negligible loss of vitamin A occurs due to stereoisomerization, before and after use of a quality carotene concentrate in margarine manufacture and during the shelf life of the margarine. A specification based on analytical constants

is given for a carotene concentration suitable for coloring margarine and at the same time contributing substantially to the overall vitamin A content.

The spectrophotometric method, previously described, for the plant control of vitamin A fortification of uncolored margarine, has been shown to be applicable to the assay of margarines colored with annatto carotenoids or with F. D. & C. Yellows No. 3 and No. 4, provided the blank unfortified margarine oil contains also the coloring in the same concentration. By supplementing the value obtained following the spectrophotometric readings at 328 mμ with that obtained at 455 mμ, reliable estimates of the total vitamin A content of carotene-colored margarine can be made. Formulas for converting spectrophotometric values into USP units of vitamin A per pound of margarine are given.

Erroneously high vitamin A estimates can be obtained in colorimetric ($SbCl_3$) assays of margarines colored with the annatto carotenoids or with the F. D. & C. Yellows No. 3 and No. 4 and erroneously low estimates for the carotene-colored product. Carotene on an equivalent vitamin A unitage basis is only about one-twelfth as chromogenic as preformed vitamin A in the $SbCl_3$ test.

When the $SbCl_3$ test is conducted on the unsaponifiable extract of the margarine oils, the interference in the vitamin A test by the annatto carotenoids is eliminated. Correction for the over-estimate of vitamin A content, attributable to the reaction between the F. D. & C. coloring agents and the $SbCl_3$ reagent, is possible through a determination of the amount of these materials in the margarine and a knowledge of the extent to which they interfere in the vitamin A assay. A chart simplifying this correction procedure has been presented.

Reliable estimates of the total vitamin A content of open market samples of margarine colored with carotene can be obtained by a method described, even in the absence of blank unfortified margarine oils. The assay method is based upon a) spectrophotometric (455 mμ) determinaiton of the carotene in the unsaponifiable extract, with a small correction factor for irrelevant light absorption and b) colorimetric ($SbCl_3$) determination of preformed vitamin A in another aliquot of the unsaponifiable extract, with a small correction for the participation of carotene in the $SbCl_3$ test.

By comparative assays on the same margarine samples, using the procedures recommended for plant control and for assay of open-market samples, it has been shown that the two methods give the same values for vitamin A content. Comparison with the results obtained by biological assay has demonstrated that the recommended non-biological assay methods give reliable figures for the vitamin A potency of carotene-colored margarines.

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