The physical constants of fraction 3 from the refractionation were found to be:

Specific gravity at 25° C.	0.9328
Angle of rotation 100 mm. 20° C.	+24.6°
Index of refraction at 25° C.	1.4837

Inasmuch as the above constants correspond closely to those of pulegone it is obvious that pulegone is the principal constituent of the oil.

A COLLABORATIVE INVESTIGATION OF THE SPECTROPHOTOMETRIC METHOD FOR ASSAY OF VITAMIN A.*

BY C. L. BARTHEN, F. F. BERG, E. B. CARTER, D. M. COPLEY, R. J. FOSBINDER, T. LEWIS AND F. O. TAYLOR.[1]

At the combined A. D. M. A. and A. P. M. A. Contact Committee Meeting (held at the Washington Hotel, Washington, D. C., March 28, 1938) the Chairman of the Sub-Committee on Physical Tests read a report of a preliminary investigation showing to what extent, in six (6) different laboratories, the biological vitamin A assays of various fish liver oils parallel the spectrophotometric determination. The general discussion which followed the reading of the report was terminated by the acceptance of a proposal that the members of the Sub-Committee on Physical Tests and the members of the A. D. M. A. Vitamin Assay Committee (who were present) meet and outline a program for further study of this subject.

At the meeting of the combined committees, the Chairman of the Sub-Committee on Physical Tests was instructed to prepare a bulletin covering the suggested tentative program and submit copies of this bulletin to the members of both committees for their comments, corrections, etc.

In view of the report of the Vitamin Assay Committee (1) and the paper by Barthen and Leonard (2) and in accordance with the suggestions made in the replies to the bulletin, it was decided that six (6) samples be submitted for optical readings with various types of instruments, in as many laboratories as were willing and in a position to do so.

Inventors of physical instruments which are specially designed for the estimation of vitamin A (such as the photoelectric photometer, photoelectric colorimeter, monochrometer, spectrophotometer and vitameter) claim to achieve results by means of these instruments that are comparable with biological assays. Since the laboratories employing these various instruments expressed a willingness to collaborate, it seemed apparent that this study afforded an excellent opportunity to demonstrate whether or not, in the determination of vitamin A potency, the results obtained by physical instruments are as reliable as those obtained by biological assay.

The committee takes this occasion to express its sincere appreciation and gratitude to:

Atlantic Coast Fisheries Corp.	Mead Johnson & Co.
Distillation Products, Inc.	National Oil Products Co.

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* Read before the Scientific Section at the Twenty-eighth annual meeting of the American Drug Manufacturers' Association, The Homestead, Hot Springs, Va., May 1, 1939.

[1] Sub-Committee on Physical Tests.

Duke University
Electrical Testing Laboratories
The Fleischmann Laboratories
The Maltbie Chemical Co.
The Norwich Pharmacal Co.
Parke, Davis & Co.
The E. L. Patch Company
United Drug Co
White Laboratories, Inc.

for their gracious coöperation in this particular piece of research.

Procedure.—The following information, concerning the specimens, instructions, etc., were submitted to each laboratory coöperating in this investigation:

"It is our desire that the vitamin A potency of the specimens submitted be determined by any physical instrument suitable for the purpose. These specimens represent different kinds of fish liver oils or blends, covering a wide range of potency and having been biologically assayed at recent dates in different laboratories.

"The potencies given below are listed merely for the convenience of the operator and economy of material and labor:

No. 1. Less than 10,000 U. S. P. vitamin A units per Gm.
No. 2. Less than 10,000 U. S. P. vitamin A units per Gm.
No. 3. Between 25,000 and 100,000 U. S. P. vitamin A units per Gm.
No. 4. Between 25,000 and 100,000 U. S. P. vitamin A units per Gm.
No. 5. Between 150,000 and 400,000 U. S. P. vitamin A units per Gm.
No. 6. Between 150,000 and 400,000 U. S. P. vitamin A units per Gm.

"It is suggested that two samples of each specimen be weighed out and subjected to the optical determinations. In the event the readings of the two samples do not check within ±5 per cent, a third sample of the specimen should be submitted for reading. The average of the two readings that check each other within the limit set, and the conversion factor established for the particular instrument employed should be used in calculating the equivalent in U. S. P. units.

"It is requested that isopropanol (99 per cent) be used in making dilutions of the specimens submitted.

"The purpose of this collaborative investigation is either to prove conclusively that, when the proper conversion factors are employed, there is definite agreement in the results obtained in different laboratories with various instruments; or, in the event there are differences in the results, to ascertain whether these differences are significant when compared to the acknowledged variations in biological assays."

COMMENTS ON SPECIMENS.

The six samples sent out for these coöperative tests were identified by number only, with the statement in the accompanying letter as to the probable range of potency for each specimen. The actual identity and potencies as determined by bio-assays were as follows:

TABLE I.

No.	Description of Specimens.	U. S. P. Vitamin A Units per Gm.
1.	U. S. P. reference oil	3,000
2.	50 per cent dilution of U. S. P. reference oil (No. 1) with cottonseed oil	1,500
3.	Halibut liver oil	70,000
4.	50 per cent dilution of halibut liver oil (No. 3) with cod liver oil (destearinated)	35,000
5.	Distilled vitamin A ester product	300,000
6.	50 per cent dilution of distilled vitamin A ester product (No. 5) with cod liver oil (destearinated)	150,000

The unit values by bio-assay shown in this table were assigned on the basis of the following data:

1. The U. S. P. reference oil, used here as an unknown sample, is generally accepted as of 3000 unit value per Gm.

2. The U. S. P. reference oil diluted to half stregnth must therefore be 1500 units per Gm.

3. This halibut liver oil had been assayed three times in two laboratories and accepted as 70,000 units per Gm.

4. By calculation this sample, half the strength of No. 3, must be 35,000 units per Gm.

5. This sample was supplied by Dr. Hickman as of 300,000 units per Gm.

6. By dilution and calculation this sample must therefore be 150,000 units per Gm.

As a check on the dilutions and to ascertain whether the potencies assigned to the various specimens were correct, a sample of each specimen was again subjected to biological assay. Adequate quantities of each specimen were prepared to insure all investigators receiving identical samples. Data concerning these assays are given in Table II.

TABLE II.

Specimen No.	Description.	Unit Level Per Gm.	Amount Fed Daily, Mgm.	No. of Rats Wt. Gain 12–60 Gm.	Average Wt. Gain 12–60 Gm.	Date of Assay Report.
	U. S. P. reference oil (control)	3,000	1.2	9 of 10	41.3	8/12/38
1.	U. S. P. reference oil	3,000	1.2	8 of 10	34.6	8/12/38
2.	50 per cent dilution of U. S. P. reference oil with cottonseed oil	1,500	2.4	10 of 10	38.0	8/12/38
3.	Halibut liver oil, RM 1005	70,000	0.051429	10 of 10	37.0	8/12/38
4.	50 per cent dilution of halibut liver oil, RM 1005, with cod liqer oil (destearinated)	35,000	0.102857	8 of 10	35.1	8/12/38
5.	Distilled vitamin A ester product	300,000	0.012000	9 of 10	35.0	8/12/38
6.	50 per cent dilution of distilled vitamin A ester product with cod liver oil (destearinated)	150,000	0.024000	9 of 10	34.3	8/12/38

These final check biological assays were conducted in accordance with the U. S. P. method for vitamin A.

Although the specimens, recorded in Table II, were assayed in the same laboratory at the same time, it will be noted that the group of rats fed the U. S. P. reference oil as the positive control shows an average gain in weight of 41.3 Gm., whereas the group fed the U. S. P. reference oil submitted as specimen No. 1 shows an average gain in weight of 34.6 Gm. Apparently there is no accounting for this phenomenon other than the variation in biological response. Examination of data on U. S. P. reference oil accumulated in the laboratory that conducted the above-mentioned assays, shows the average gain in weight of 53 groups (each of 10 animals) run in the past 20 months is 37.1 Gm. with a standard deviation of 3.51 Gm. It is evident that the spread between these two groups may well occur.

The determination of vitamin A potency by biological assay is subject to many variable factors which may cause deviations in the results. From the many opinions expressed in the literature, a conservative estimate of the error in biological assays which may occur in any individual laboratory is approximately ±20 per cent or a spread of 40 per cent. However, the results of a series of assays, of the same material, conducted in six or more different laboratories, are likely to show a deviation of approximately ±30 per cent or a spread of 60 per cent. E. M. Hume (6) recently reported a collaborative study by 10 laboratories in which the results of the biological assays of the U. S. P. reference cod liver oil ranged from 1334 to 3270 I. U. of vitamin A per Gm., with a weighted mean of 2619 I. U. per Gm. This represents a deviation of −49.1 per cent and +24.9 per cent, or a spread of 74.0 per cent.

The preceding data justify the conclusion that the average response obtained with the U. S. P. reference oil, submitted as specimen No. 1, might properly be employed as the standard for comparison with the average response obtained with the other specimens.

In Table II it will be further noted that the responses of the groups fed specimens Nos. 2, 3, 4, 5 and 6, when compared with the response of specimen No. 1, are all within what may be considered the permissible limits of biological variation. Consequently, we are of the opinion that the vitamin A potencies assigned to the various specimens are sufficiently accurate for the purposes of this study.

Whether the potency as determined by a physical instrument represents the biological response as determined by a biological assay is a controversial subject, which has been discussed in many articles in the literature and at various scientific meetings. Furthermore, comparative studies of the spectrophotometric and biological assays for vitamin A have been conducted by a number of investigators: namely, Barthen and Leonard (2), Black, *et al.* (4), Pritchard, *et al.* (5), Hume (6). In addition there is a considerable amount of data on this particular subject, that is available although not yet published. In all of the studies mentioned, a great number of specimens, which represented a large variety of vitamin A bearing substances and a wide range of potencies, were employed. Therefore, for the purposes of this study, it was decided that a few representative specimens would be sufficient; but that as many types of instruments and as many laboratories as possible should be engaged in the project.

DATA CONCERNING THE SPECTROPHOTOMETRIC ASSAYS.

Laboratory No.	Instruments Employed in This Project.
1	Hilger Spectrophotometer.
2	Hilger Spectrophotometer.
3	Bausch & Lomb Spectrophotometer.
4	Hilger Vitameter.
5	Hilger Spectrophotometer.
6(s)	Hilger Spectrophotometer.
6(v)	Hilger Vitameter.
6(p)	Specially designed Photometer.
7	Specially designed Vitamin A Meter.
8(s)	Bausch & Lomb Spectrophotometer No. 748
8(v)	Hilger Vitameter (1938).
9	Evelyn Photoelectric Colorimeter.
10	Hilger Vitameter.
11	Hilger Spectrophotometer.
12	Hilger Vitameter with S-I Lamp.
13	Monochromator, Tungsten Light Source, Photocell Detector.

In the tabulations of data in Table III, the first seven lines are those obtained with spectrophotometers, the next four lines present data obtained with vitameters and the remaining lines cover data obtained by various specially designed instruments.

TABLE III.

		Equivalents in U. S. P. Units per Gm.					
Date.*	Lab. No.	Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.
6/12/38	1	3,060	1,776	62,274	32,528	289,328	151,298
6/15/38	2	3,128	1,754	65,900	34,200	302,500	156,600
8/22/38	5	2,875	1,692	54,517	29,999	279,316	135,475
8/12/38	6(s)	2,888	1,606	62,152	30,739	285,726	142,985
8/26/38	11	3,500	1,880	68,000	34,600	315,000	158,000
6/28/38	3	3,100	1,675	61,400	31,100	294,000	144,800
7/29/38	8(s)	3,310	1,726	60,736	29,536	271,144	147,264
6/20/38	4	3,186	1,572	59,558	32,525	277,728	157,978
6/14/38	6(v)	2,750	1,415	64,809	31,880	416,333	156,449
7/27/38	8(v)	3,099	1,674	61,984	30,680	272,792	139,360
8/23/38	10	2,943	1,659	59,428	32,271	278,414	139,314
6/15/38	6(p)	3,220	1,470	73,203	36,346	350,393	175,027
6/23/38	7	3,218	1,595	74,420	34,205	339,600	138,900
8/19/38	9	..	..	...	...	....	....
8/11/38	13	2,921	1,575	58,850	28,676	277,130	135,034
9/19/38	12	2,465	1,368	61,150	30,500	....	125,150
Totals		45,663	24,437	948,381	479,785	4,249,404	2,203,634

Averages	3,044	1,629	63,225	31,986	303,529	146,909
Bio-assays	3,000	1,500	70,000	35,000	300,000	150,000
Deviation of av. results from bio-assays	+44 +1.47%	+129 +8.6%	−6,775 −9.68%	−3.014 −8.61%	+3,529 +1.18%	−3,091 −2.06%
Greatest deviation from mean of results plus (+)	456 14.98%	251 15.41%	11,195 17.71%	4,360 13.63%	112,804 37.16%	28,118 19.14%
Greatest deviation from mean of results minus (−)	579 19.02%	261 16.02%	8,708 13.77%	3,310 10.35%	32,385 10.67%	21,759 14.81%
Spread	34.00%	31.43%	31.48%	23.98%	47.83%	33.95%

* Readings were made within a week before or after date given in table.

In Table III are shown the calculated equivalents in U. S. P. units, for the various specimens, as reported by the different laboratories. In compiling the data for this table no significance was given to any particular type of instrument employed in making the determinations; because, it was desired merely to reveal the extent of agreement in the results obtained with various instruments in different laboratories. Also to demonstrate to what extent the average potencies, as determined by physical instruments, deviate from the potencies determined by biological assay.

Laboratory No. 9 did not report any equivalents in U. S. P. units; consequently no results for this laboratory appear in this table.

Examination of Table III will reveal that the averages of the equivalents, obtained for the respective specimens, approximate the potencies indicated by the biological assays. However, the results obtained in some of the laboratories, in one or more instances, are so far out of line with the other results obtained with the same specimen, as to make the deviations from the mean and the spread of results with each specimen unduly great. Elimination of the highest and lowest equivalents from the calculations would reduce the percentage spread of results, given in Table III, considerably. For examples:

With Specimen No. 1: Elimination of the two extremes would reduce the percentage spread from 34.00 per cent to 18.34 per cent.

With Specimen No. 2: Elimination of the two extremes would reduce the percentage spread from 31.43 per cent to 22.15 per cent.

With Specimen No. 3: Elimination of the two extremes would reduce the percentage spread from 31.48 per cent to 22.77 per cent.

With Specimen No. 4: Elimination of the two extremes would reduce the percentage spread from 23.98 per cent to 15.87 per cent.

With Specimen No. 5: Elimination of the two extremes would reduce the percentage spread from 47.83 per cent to 26.14 per cent.

With Specimen No. 6: Elimination of the two extremes would reduce the percentage spread from 33.95 per cent to 15.69 per cent.

The average spread calculated from Table III is 33.78 per cent. With the elimination of the extremes the average spread would be 20.16 per cent.

In Table IV are shown the average E. values, for the various specimens, as reported by the different laboratories. This table is divided into three parts so that comparisons of the results may readily be made as to variations between laboratories employing the Spectrophotometers, the Hilger Vitameter and the specially designed instruments.

Laboratories No. 6(p) and No. 12 did not report any E. values; consequently no results for these laboratories appear in the table.

The E. values reported by laboratory No. 7 are omitted from this table, because they were determined at the wave-length of 3303 Angstrom units, and it is the conclusion of this laboratory that this difference in wave-length of light used affects the results to so great an extent that they may not fairly be compared with the E. values reported by the other laboratories and determined at 3280 Angstrom units.

As specimen No. 1 represents the U. S. P. reference cod liver oil, it was believed to be expedient to determine to what extent the conversion factors, employed by the collaborating laboratories, differed from the conversion factors calculated from the E. values reported for this specimen.

In Table V are shown:

A. The conversion factors reported and employed in calculating the equivalent in U. S. P. units per Gm.

B. The corrected conversion factors calculated from the E. values reported for specimen No. 1.

%±. The deviation in percentage, of A from B.

It will be noted that the deviations extend from minus 4.19 per cent to plus 16.77 per cent. (Laboratory No. 4 reported 2 conversion factors. See remarks under General Discussion.)

TABLE IV.

Date.*	Lab. No.	Average E. Values: Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.
		Spectrophotometer.					
6/12/38	1	1.43	0.83	29.1	15.2	135.2	70.7
6/15/38	2	1.58	0.886	33.3	17.25	152.8	79.1
8/22/38	5	1.495	0.880	28.35	15.6	145.25	70.45
8/12/38	6(s)	1.51	0.909	29.29	14.565	134.09	67.18
8/26/38	11	1.66	0.89	32.2	16.40	149.0	74.9
6/28/38	3	1.55	0.838	30.7	15.55	147.0	72.4
7/29/38	8(s)	1.59	0.83	29.2	14.2	130.5	70.8
Average	(of 7)	1.545	0.866	30.31	15.54	141.98	72.22
		Hilger Vitameter.					
6/20/38	4	1.54	0.76	28.2	15.4	131.5	74.8
6/14/38	6(v)	1.593	0.901	34.646	16.731	(215.7)[1]	81.67
7/27/38	8(v)	1.49	0.805	29.8	14.75	131.15	67.0
8/23/38	10	1.375	0.775	27.77	15.08	130.1	65.1
Average	(of 4)	1.50	0.810	30.10	15.49	130.9	72.14
		Special Instruments.					
6/15/38	6(p)	..	..	..	..	..	..
8/19/38	9	..	..	29.5	14.6	147.0	72.6
8/11/38	13	1.365	0.736	27.5	13.4	129.5	63.1
6/23/38	7	..	..	..	..	..	..
9/22/38	12	..	..	..	..	..	..
Average	(of all)	1.515	0.837	29.97	15.29	138.59	71.52
Greatest deviation from mean of results plus (+)		0.145	0.72	4.676	1.96	14.21	10.15
		9.57%	8.60%	15.60%	12.82%	10.25%	14.19%
Greatest deviation from mean of results minus (−)		0.150	0.101	2.47	1.89	9.09	8.42
		9.90%	12.07%	8.24%	12.36%	6.56%	11.77%
Spread		19.47%	20.67%	23.84%	25.18%	16.81%	25.96%

The average spread calculated from Table IV is 21.99 per cent.

* Readings were made within a week before or after date given in table.

[1] Omitted from calculations.

Laboratories Nos. 6(p), 9 and 12 did not report any E. values or conversion factors for specimen No. 1; consequently no results of calculations for these laboratories appear in Table V. In the case of laboratory No. 6(v) the corrected conversion factor was calculated from the E. values reported.

In view of the preceding data, it was deemed advisable to ascertain whether the results

(equivalents in U. S. P. units) obtained by employing the corrected conversion factors, would be in closer agreement than the results obtained by employing the conversion factors established in and reported by the different laboratories. Therefore, in Table VI are shown the equivalents in U. S. P. units, obtained by multiplying the reported E. values, for the various specimens, by the corrected conversion factors (see B in Table V).

TABLE V.

Laboratory No.	Conversion Factors. A.[1]	B.[2]	% ±.[3]
1	2140	2098	+ 2.00
2	1980	1899	+ 4.27
5	1923	2007	− 4.19
6(s)	1920	1987	− 3.37
11	2110	1807	+16.77
3	2000	1936	+ 3.31
8(s)	2080	1887	+10.23
4	2069	1948	+ 6.21
4	2112	1948	+ 8.42
6(v)	..	1883	...
8(v)	2080	2014	+ 3.28
10	2140	2182	− 1.93
6(p)	..	..	...
7	2780	2591	+ 7.29
9	..	..	...
13	2140	2198	− 2.64
12	..	..	...

[1] The reported conversion factors.

[2] The corrected conversion factors.

[3] The deviation, in percentage, of A from B.

Laboratories Nos. 6(p), 9 and 12 were omitted from this table for the same reason as mentioned under Table V.

TABLE VI.

Laboratory No.	Equivalents in U. S. P. Units Per Gm. Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.
1	3,000	1,741	61,052	31,890	283,650	148,329
2	3,000	1,683	63,237	32,758	290,167	150,211
5	3,000	1,766	56,898	31,309	291,517	141,393
6(s)	3,000	1,806	58,199	28,941	266,437	133,487
11	3,000	1,608	58,185	29,635	269,243	135,344
3	3,000	1,622	59,435	30,105	284,592	140,166
8(s)	3,000	1,566	55,100	26,795	246,254	133,600
4	3,000	1,480	54,934	29,999	256,162	145,710
6(v)	3,000	1,697	65,246	31,503	406,163	153,785
8(v)	3,000	1,621	60,017	29,707	264,136	134,938
10	3,000	1,691	60,594	32,905	283,660	142,048
6(p)	..	..	...	...	....	....
7	3,000	1,487	69,361	31,947	316,517	129,472
9	..	..	...	...	....	....
13	3,000	1,618	60,445	29,453	284,641	138,694
12	..	..	...	...	....	....
Total	39,000	21,386	782,703	396,947	3,743,139	1,827,177

Table VI.—(*Continued from page 667.*)

Averages	3,000	1,645	60,208	30,534	287,934	140,552
Bio-assays	3,000	1,500	70,000	35,000	300,000	150,000
Deviation of av. results from bio-assays	..	+145	−9,792	−4,466	−12,066	−9,448
	..	+8.81%	−16.26%	−14.63%	−4.19%	−6.72%
Greatest deviation from mean of results plus (+)	..	161	9,153	2,371	118,229	13,233
	..	9.79%	15.20%	7.77%	41.06%	9.42%
Greatest deviation from mean of results minus (−)	..	165	5,274	3,739	41,680	11,080
	..	10.03%	8.76%	12.25%	14.48%	7.88%
Spread	..	19.82%	23.96%	20.02%	55.54%	17.30%

The average spread calculated from Table VI is 27.33 per cent.

Table VI illustrates to what degree the corrected conversion factors affect the calculated potencies of the different specimens in the individual laboratories; also demonstrates to what extent the corrected factors influence the statistical calculations. It will be noted in this table that the averages of the equivalents, for the respective specimens, do not approximate the potencies indicated by the biological assays as well as those in Table III.

A comparison of Tables III and VI will reveal that, with the exception of specimen No. 5, the calculated equivalents in Table VI are in closer agreement than those in Table III. Furthermore, with the elimination of the extreme results obtained with specimen No. 5 from both tables, the spread for this specimen would then be less in Table VI than in Table III.

Upon further examination of Table III, it is evident that some of the laboratories obtained results which show a much greater spread of deviation from the averages than other laboratories whose results are more in line with the averages or otherwise more consistently high or low.

Table VII shows to what extent, in per cent, each laboratory deviates from the average equivalent given in Table III for each specimen; also shows the spread of deviations in each laboratory.

In this table it will be noted that seven laboratories, namely Nos. 5, 8(s), 4, 6(v), 6(p), 7 and 12 show a spread greater than 15 per cent, which would indicate unreliability due to the technique or instrument employed in making the determinations. It therefore seemed that it would be interesting and informative to determine another set of averages, on the basis of the equivalents as shown in Table III, from the eight remaining laboratories only.

TABLE VII.

Per Cent Deviation from Average.

Laboratory No.	Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.	Spread of Deviations.
1	+ 0.5	+ 9.0	− 1.5	+ 1.7	−4.7	+ 3.0	13.7
2	+ 2.8	+ 7.7	+ 4.2	+ 6.9	− 0.3	+ 6.6	8.0
5	− 5.6	+ 3.9	−13.8	− 6.2	− 8.0	− 7.8	17.7
6(s)	− 5.1	− 1.4	− 1.7	− 3.9	− 5.9	− 2.7	4.5
11	+15.0	+15.4	+ 7.6	+ 8.2	+ 3.8	+ 7.5	11.6
3	+ 1.8	+ 2.8	− 2.9	− 2.8	− 3.1	− 1.4	5.9
8(s)	+ 8.7	+ 6.0	− 3.9	− 7.7	−10.7	+ 0.2	19.4
4	+ 4.7	− 3.5	− 5.8	+ 1.7	− 8.5	+ 7.5	16.0
6(v)	− 9.7	−13.1	+ 2.5	− 0.3	+37.2	+ 6.5	50.3
8(v)	+ 1.8	+ 2.8	− 2.0	− 4.1	−10.1	− 5.1	12.9
10	− 3.3	+ 1.8	− 6.0	+ 0.9	− 8.3	− 5.2	10.1
6(p)	+ 5.8	− 9.8	+15.8	+13.6	+15.4	+19.1	28.9
7	+ 5.7	− 2.1	+17.7	+ 6.9	+11.9	− 5.5	23.2
13	− 4.0	− 3.3	− 6.9	−10.3	− 8.7	− 8.1	7.0
12	−19.0	−16.0	− 3.3	− 4.6	..	−14.8	15.7

Table VIII shows the average equivalent in U. S. P. units of the eight selected laboratories, for each specimen; this table also gives, for each laboratory, the per cent deviation from the mean for each specimen, the average deviation and the new spread.

TABLE VIII.

	Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.	Average Per Cent Deviation.	Per Cent Spread.
Averages	3,080	1,700	62,499	31,849	289,361	145,924	...	...
Lab. No. 1	− 0.6	+ 4.5	−0.4	+ 2.1	0	+3.7	+1.6	5.1
Lab. No. 2	+ 1.6	+ 3.2	+5.4	+ 7.4	+4.5	+7.3	+4.9	5.8
Lab. No. 6(s)	− 6.2	− 5.5	−0.6	− 3.5	−1.3	−2.0	−3.2	5.6
Lab. No. 11	+13.6	+10.6	+8.8	+ 8.6	+8.9	+8.3	+9.8	5.3
Lab. No. 3	+ 0.6	− 1.5	−1.8	− 2.4	+1.6	−0.8	−0.7	4.0
Lab. No. 8(v)	+ 0.6	− 1.5	−0.8	− 3.7	−5.7	−4.5	−2.6	6.3
Lab. No. 10	− 4.4	− 2.4	−4.9	+ 1.3	−3.8	−4.5	−3.1	6.2
Lab. No. 13	− 5.2	− 7.4	−5.8	−10.0	−4.2	−7.5	−6.7	5.8

It was demonstrated that the results of the calculations given in Tables V and VI did not accomplish the hoped for explanation or correction of the wide discrepancies in the results obtained in some of the laboratories. Consequently, further analysis of the preceding data was indicated. The next step was to make a correction, by means of the average deviations in Table VIII, of the conversion factors used and reported by the eight laboratories under consideration.

In Table IX are shown:

A. The conversion factors reported and employed in calculating the equivalent in U. S. P. units per Gm.

B. The corrected conversion factors calculated from the E. values reported for specimen No. 1.

C. The corrected conversion factors obtained by adding to or subtracting from the reported factors, a figure representing the average per cent deviation given for each laboratory in Table VIII.

TABLE IX.

	Conversion Factors.		
Laboratory No.	A.[1]	B.[2]	C.[3]
1	2140	2098	2106
2	1980	1899	1883
6(s)	1920	1987	1981
11	2110	1807	1903
3	2000	1936	2014
8(v)	2080	2014	2134
10	2140	2182	2206
13	2140	2198	2283

[1] The reported conversion factors.

[2] The corrected conversion factors calculated from E. values for specimen No. 1.

[3] The conversion factors corrected by means of the average deviations.

TABLE X.

	Equivalents in U. S. P. Units per Gm.					
Laboratory No.	Specimen No. 1.	Specimen No. 2.	Specimen No. 3.	Specimen No. 4.	Specimen No. 5.	Specimen No. 6.
1	3,012	1,748	61,285	32,012	284,731	148,894
2	2,975	1,668	62,704	32,482	287,722	148,945
6(s)	2,991	1,801	58,023	28,853	265,632	133,084
11	3,159	1,694	61,277	31,209	283,547	142,535
3	3,122	1,688	61,830	31,318	296,058	145,814
8(v)	3,180	1,718	63,593	31,477	279,874	142,978
10	3,033	1,710	61,261	33,266	287,001	143,611
13	3,116	1,680	62,783	30,592	295,649	144,057
Totals	24,588	13,707	492,756	251,209	2,280,214	1,149,918

TABLE X.—(*Continued from page 669.*)

Averages	3,074	1,713	61,595	31,401	285,027	143,740
Bio-assays	3,000	1,500	70,000	35,000	300,000	150,000
Deviation of av. results from bio-assays	74 +2.47%	213 +14.20%	8,405 −12.01%	3,599 −10.28%	14,973 −4.99%	6,260 −4.17%
Greatest deviation from mean of results plus (+)	106 3.45%	88 5.14%	1,998 3.24%	1,865 5.94%	11,031 3.87%	5,205 3.62%
Greatest deviation from mean of results minus (−)	99 3.22%	45 2.63%	3,572 5.80%	2,548 8.11%	19,395 6.80%	10,656 7.41%
Spread	6.67%	7.77%	9.04%	14.05%	10.67%	11.03%

The average spread calculated from Table X is 9.87 per cent.

In Table X are shown the equivalents in U. S. P. units obtained by multiplying the E. values, reported by the eight selected laboratories for the various specimens, by the corrected conversion factors under C in Table IX.

It will be noted that in this table the calculated equivalents are in close agreement.

An examination of the tables will reveal that in Table X the deviations from the mean and the spread of results for each specimen are very much less than those in Tables III, IV and VI. For convenience in making a quick comparison, the average spreads, in per cent, are:

	Per Cent.		Per Cent.
Table III	33.78	Table VI	27.33
Table IV	21.99	Table X	9.87

GENERAL DISCUSSION.

Remarks submitted in the reports of some of the collaborators and the comments on them may afford a better interpretation of the results given in the various tables.

Laboratory No. 4 states: "In this Laboratory the average conversion factor for oils of a potency greater than 10,000 A per Gm., or for the unsaponifiable fraction of oils of a lower potency has been found to be 2112. The average conversion factor for the direct determination of oils lower than 10,000 A per Gm has been found to be 2069. In these calculations, both factors have been reported, as we could not tell in specimens 1 and 2 which was whole oil and which a solution of a non-saponifiable portion.

"Specimen No. 4. Run at two different temperatures to see what effect this would have."

As specimens Nos. 1 and 2 represent whole oils of potencies less than 10,000 units, the conversion factor of 2069 was employed in calculating the equivalents for these specimens, and the conversion factor 2112 was employed in calculating the equivalents of the other specimens given in Table III.

Laboratory No. 4 made determinations of specimen No. 4 at temperatures of 24° C. and 32° C.; the results obtained at these temperatures were the same. The temperatures at which the different laboratories conducted their determinations varied from 22.5° C. to 32° C. (72.5° F. to 90° F.). The temperatures may have influenced the results in some of the laboratories, yet there is no direct evidence to prove that the discrepancies in the results were due to temperatures at which the readings were made.

Laboratory No. 5 states: "Sample No. 2 did not give a good vitamin A absorption curve, absorbing quite strongly below 3000 A. Undoubtedly this increases the absorption at the vitamin A maximum 3280 A. A biological test would probably show less vitamin A potency than the optical test."

Evidence submitted by Haines and Drummond (3) led to the belief that the presence of a vegetable oil in combination with a vitamin A bearing oil may influence the shape of the absorption curve and be responsible for a false evaluation of the blend. Therefore, a product of this type was intentionally included in the group of specimens for this study.

It will be noted that 12 of the 15 equivalents in Table III and 11 of the 13 equivalents given in Table VI and all of the equivalents in Table X for specimen No. 2 show a greater potency than indicated by biological assay. However, when determinations were made on the unsaponifiable

fraction of this specimen, in two different laboratories, the equivalents in U. S. P. units were 1548 and 1530. These results check the biological assay.

Laboratory No. 6(s) states: " 'Uncorrected' values are simply the product of E. value × 1920; 'corrected' values are this product corrected for irrelevant absorption by our abritrary formula. This formula was worked out for the vitameter when used under certain conditions, but I am not at all sure it is applicable here.

"We consider 1600 the proper factor for International Units, 1920 the factor for the present issue of U. S. P. Reference Oil, the true potency of which is nearer 2500 I. U. than 3000."

Only the average of the corrected values are given in Table III; furthermore, the corrected values are in closer agreement with the mean of all the equivalents in this table than the uncorrected values.

Laboratory No. 6(v) states: " 'Corrected' values were derived from 'uncorrected' by our formula, with which you are familiar. Values were determined on the oils directly, without saponification. We do not use the E. value factor method, but instead use a calibration graph of the vitameter plotted against a series of dilutions of U. S. P. Reference C. L. O. Thus we avoid the controversy over what is the correct factor."

Only the average of the corrected values is given in Table III; however, with four of the six specimens, the uncorrected values were in closer agreement with the mean of all the equivalents in this table than the corrected values for the same specimens.

Laboratory No. 6(p)—only the average of the corrected values is given in Table III. However, all of the uncorrected values are in closer agreement with the mean of all the equivalents in this table than the corrected values.

Laboratory No. 8(v) states: "The conversion factor is based on a large series of 'E.' determinations made with the U. S. P. reference cod liver oil, giving an average E. value of 1.44."

In Table IV it will be noted that this laboratory obtained with specimen No. 1 an E. value of 1.59 when it employed the spectrophotometer and an E. value of 1.49 when it employed the vitameter; for both instruments the same conversion factor was employed. Therefore, a difference in the calculated equivalents is to be expected. In the event the E. value of 1.59 is correct for specimen No. 1 the conversion factor should be 1887 (see Table V). The equivalents obtained in Laboratory No. 8 with the vitameter are in closer agreement with the mean of all the equivalents give in Table III than the equivalents obtained with the spectrophotometer. Employment of the corrected conversion factors (in Table V) does not bring the equivalents into closer agreement with the mean of results.

The figures given in this discussion will indicate that either the E. value for specimen No. 1 found by the spectrophotometer (in this laboratory) or the conversion factor reported, is incorrect.

Laboratory No. 10 states: "*Time Factor:* The assays were all made within 15 minutes after the solution was prepared.

"You will note that both the 'readings' and the 'E. values' for each sample checked within significantly less than ±5 per cent. In order that you may be able to check the 6 samples against the U. S. P. reference cod liver oil, which we procured from Professor Cook, we made an assay of it, using isopropanol, for vitamin A simultaneously with the assay of your samples. Our results are reported above."

This laboratory obtained an average E. value of 1.40 with the reference cod liver oil as a positive control and an average E. value of 1.375 with the reference cod liver oil as an unknown. The two results are in such close agreement that no further comment is necessary.

Laboratory No. 12 states: "The results reported were obtained with a vitameter equipped with an S-1 lamp in place of the copper arc, the readings being recorded photographically at 0.05 scale intervals. The instrument was standardized by plotting vitameter scale values against U. S. P. reference cod liver oil concentrations. The vitamin A content in U. S. P. units per Gm. in the unknown sample was calculated by application of the following formula:

$$\frac{\text{A units per ml. (obtained from the calibration curve)} \times 100}{\text{Concentration of sample in per cent}} = \text{vitamin A units per Gm.}"$$

In three of the five specimens, the equivalents obtained in this laboratory deviate considerably from the mean of all equivalents obtained in Table III for the same specimens. As no E.

values or conversion factors were reported by this laboratory no satisfactory comparisons can be made with the results obtained in the other laboratories.

It has been suggested that the U. S. P. adopt a spectrophotometric method for the determination of the vitamin A content of cod liver oil and other fish liver oils, as an alternate for the biological assay. However, the opinion persists that vitamin A-bearing oils can be adulterated with some substance which would so influence the optical absorption that the number of units, determined by means of a physical instrument, would be greater than the number of units determined by a biological assay.

In view of the acknowledged wide deviation in biological response, the nature of which prohibits accurate control, a supplier of a vitamin A-bearing oil should not be obliged to depend entirely upon the results of a biological assay. Nevertheless, to prevent deliberate or even unintentional fraud, it is suggested that a supplier be compelled to guarantee that a check biological assay of his product, whether it was standardized by means of a spectrophotometric or biological assay, will be a success at a level of not less than 75 per cent of the claimed potency. It is believed that the adoption of the above suggestion will practically obviate any controversy or litigation as to the accuracy, reliability or validity of a bio-assay.

SUMMARY.

In the determination of vitamin A potency by physical instruments, it is apparent that the conversion factor is of paramount importance.

To achieve the greatest degree of accuracy in results, the E. value, of the U. S. P. reference oil, its unsaponifiable fraction and/or any other suitable standard, should be determined every day and the conversion factor calculated to check the constancy of conditions necessary for accurate operation of the instrument. In this manner, errors due to conditions in the laboratory, the instrument or the human element, can be obviated or at least reduced to a minimum. However, it is obvious that expertness, as a consequence of experience in the technique and knowledge of the fundamental principles of the instruments, is an essential factor for the attainment of accurate results.

Under specified and well controlled conditions of operation of the physical instruments, capable of accuracy of measurement, results can and should be obtained which are in closer agreement than those obtainable by biological assay.

When there is any doubt or question as to the source of the oil under consideration, the optical determination should be made on the unsaponifiable fraction.

The suggestion that a spectrophotometric method be adopted as an alternate for the biological assay, is discussed.

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