SOME OBSERVATIONS ON THE DETERMINATION OF VITAMIN A IN COD-LIVER OIL

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IT has long been known that naturally occurring vitamin A esters which are present in fish liver oils exhibit selective absorption of ultra-violet radiation of wavelength of about 328 mμ. For many years the value obtained by multiplying $E_{328\ m\mu}$* obtained on the unsaponifiable matter by the factor declared by the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations was regarded as indicating the potency of cod-liver oil. This was the procedure from 1936 until 1948, and a factor of 1600 was used. The B.P. 1948 laid down that the factor 1600 should be used to convert spectrophotometric data into biological units per g. The Addendum 1951 to the B.P. 1948 stipulates that cod liver oil shall be saponified, the unsaponifiable matter dissolved in *cyclo*hexane and optical density values determined at 3 stated wavelengths. A formula is then applied to the results and a corrected $E_{326\cdot5\ m\mu}$ obtained, which is then multiplied by a factor of 1900 to indicate the potency. This departure from the procedure indicated in the B.P. 1948, arose largely as a result of the work of Morton and his colleagues[1] who developed a geometrical formula which would allow for the contribution of irrelevant material to the absorption at the wavelength of maximum absorption. It is a prerequisite of the application of this formula that the irrelevant absorption should be linear at the specified wavelengths. It is also assumed that no impurity or artefact has a maximum very close to that of vitamin A. The development of this method was made possible by the development of the photoelectric spectrophotometer, whereby optical densities may be taken with ease and speed at different wavelengths, and by the production of synthetic all-*trans* vitamin A alcohol and vitamin A acetate. Morton *et al*[2] have published data giving the ratio of optical densities at $E_{max.}$ to that at other stated wavelengths in specified solvents, and have also given correctional formulæ for the determination of vitamin A in these solvents. Corrected values ($E_{max.}$ (corr.)) may be multiplied directly by the factor 1900. It should be mentioned that a factor of 1900 is employed to convert to biological units $E_{max.}$ values determined on pure all-*trans* vitamin A alcohol and pure all-*trans* vitamin A acetate when dissolved in *cyclo*hexane. The U.S.P. up to the 14th revision specified that vitamin A assays should be carried out biologically, and no physical method of assay was given. The U.S.P. XIV specifies that vitamin A shall be determined on the unsaponifiable matter followed by the application of a formula which corrects for irrelevant absorption. Materials other than vitamin A which occur in cod-liver oils and which may tend to vitiate the results

* Throughout this paper $Ex_{m\mu}$ is used to indicate $E_{1\ cm.}^{1\ per\ cent.}$ at wavelength x mμ.

obtained by a correctional formula are anhydro vitamin A, vitamin A_2, neo-vitamin A, kitol, oxidation products and polyene acids.

Vitamin A_2 shows selective absorption at 351 mμ ($E = 1460$) with a subsidiary peak at 287 mμ ($E = 820$), and has a biological potency of about 40 per cent. of that of all-*trans* vitamin A. Vitamin A_2 reacts with a solution of antimony trichloride in chloroform giving a blue colour with maximum absorption at 693 mμ. Vitamin A gives a similar colour with maximum absorption at 620 mμ.[3] From readings taken at 620 mμ and at 693 mμ of the colour developed with antimony trichloride by the unsaponifiable matter of a cod-liver oil, the absorption at 693 mμ due to vitamin A_2 may be calculated. The contribution of the vitamin A_2 absorption at 328 mμ may then be estimated using data given by Morton for vitamin A and by Shantz for vitamin A_2.[3]

Neo-vitamin A has an absorption curve which is similar to that of all-*trans* vitamin A, but the wavelength of maximum absorption is moved 3 mμ towards the visible range.[4] Its absorption at the wavelength of maximum absorption is 10 per cent. lower than that of all-*trans* vitamin A at its wavelength of maximum absorption. In the region 280 to 330 mμ differences between the absorption spectrum of neo-vitamin A and all-*trans* vitamin A are small. From 330 to 390 mμ the neo-form shows higher absorption. It is stated by Morton that neo-vitamin A esters are more difficult to saponify than all-*trans* vitamin A esters.

The problem of arriving at a satisfactory estimate of potency is therefore one of considerable difficulty. Unsaturated acids exhibiting absorption maxima at 270 mμ, 315 mμ and 320 mμ, may be removed by a saponification process, but saponification cannot remove either vitamin A_2 or kitol. In the present investigation we are considering only fresh cod-liver oils, and therefore, expect to find only a very small amount of oxidation products. Further, by a chromatographic method, details of which will be given later, the presence of only very small amounts of anhydro-vitamin A could be demonstrated.

A number of cod-liver oils when assayed by the B.P. Addendum 1951 method in this laboratory gave considerably lower results for the value $E_{326{\cdot}5\ m\mu}$ (corr.) $\times$ 1900 than for the uncorrected value $E_{326{\cdot}5\ m\mu}$ (gross) $\times$ 1600. It was decided to investigate the reason for this discrepancy. The Addendum specifies that vitamin A in cod-liver oils shall be determined on the unsaponifiable matter obtained by the method given by the B.P. 1948. The instructions are as follows: "Boil 1 g. of the cod-liver oil with 10 ml. of freshly prepared *N/2 alcoholic solution of potassium hydroxide* for five minutes or until clear. . . ."

The U.S.P. XIV specifies a half-hour saponification period. In our experience the solution of cod-liver oil in ethanolic potassium hydroxide solution becomes clear in a much shorter time than 5 minutes. The solution is apparently clear as soon as sufficient heat has been applied to the flask to effect solution of the oil. This occurs within 1 minute of placing the flask on the hot plate. We have saponified several cod-liver oils for periods of 5 minutes and longer. The saponification time has been accurately observed and has been taken to start from the condensation

of the first drop of ethanol from the end of the condenser, the flask being placed on a previously heated hot plate. The flask was removed from the hot plate at the end of the 5-minute period. The following figures illustrate the change in shape of the absorption curve of sample A with increase in the time of saponification.

TABLE I

ABSORPTION SPECTRUM OF THE UNSAPONIFIABLE MATTER OF COD-LIVER OIL, A, AFTER 5 MINUTES, AND AFTER 30 MINUTES, SAPONIFICATION

mμ	5 Minutes saponification	30 Minutes saponification
	$E^{1\text{ per cent.}}_{1\text{ cm.}}$	$E^{1\text{ per cent.}}_{1\text{ cm.}}$
300	0·333	0·289
305	0·359	—
310	0·389	0·357
312	0·412	0·371
312·5	0·416	0·372
313	0·423	—
314	0·426	0·379
315	0·427	—
316	0·424	0·387
317	0·423	—
318	—	0·390
320	0·416	0·398
322	0·416	0·404
326·5	0·423	0·414
328	0·422	0·414
329	0·418	—
330	0·415	0·409
335	0·384	0·382
336·7	0·372	0·372
340	0·346	0·346
Ratio $\frac{E_{312\cdot5\text{ mμ}}}{E_{326\cdot5\text{ mμ}}}$	0·985	0·899
Ratio $\frac{E_{336\cdot7\text{ mμ}}}{E_{326\cdot5\text{ mμ}}}$	0·879	0·899

The absorption spectrum of the extracted material after 5 minutes saponification exhibits two maxima, one at 315 mμ and another at 326 mμ. The absorption maximum at 315 mμ which is due to unsaturated acids is removed by a 30-minutes saponification period. A 30-minutes saponification period removes additional amounts of irrelevant absorbing material over that removed by a 5 minutes saponification period, as shown in Table I by the reduction of the ratio $\frac{E_{312\cdot5\text{ mμ}}}{E_{326\cdot5\text{ mμ}}}$ from 0·985 to 0·899.

The results shown in Table II were obtained on the oil and on the unsaponifiable matter.

The above figures indicate that a period of saponification longer than 5 minutes has had little effect on the E (gross), but quite a large effect on the value of E (corr.). The application of the correctional formula to the unsaponifiable matter obtained by a 5-minute period of saponification

TABLE II

	$E_{max.}$ (gross)	$E_{max.}$ (gross) ×1600	E (corr.)	E (corr.) ×1900
On the whole oil	0·456	730	0·385	730
After 5 minutes saponification ..	0·422	675	0·224	425
After 30 minutes saponification ..	0·415	665	0·301	570

has in the above case led to erroneously low results. Tests carried out on further samples indicate the inadequacy of the current instructions for the determination of vitamin A.

TABLE III

ABSORPTION SPECTRUM OF THE UNSAPONIFIABLE MATTER OF COD-LIVER OIL, B, AFTER 5 MINUTES AND 30 MINUTES SAPONIFICATION

mμ	5 Minutes	30 Minutes
	$E_{1\text{ cm.}}^{1\text{ per cent.}}$	$E_{1\text{ cm.}}^{1\text{ per cent.}}$
310	0·980	0·905
312	1·040	0·939
313	1·066	—
314	1·072	0·965
315	1·082	0·974
316	1·080	0·983
317	1·075	0·990
318	—	0·994
320	1·072	1·010
326·5	1·113	1·048
Ratio $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·940	0·896
Ratio $\frac{E_{326\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·890	0·896

Sample B	$E_{max.}$ (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
On the whole oil	1·212	1940	1·008	1910
After 5 minutes saponification	1·113	1780	0·686	1300
After 30 minutes saponification	1·048	1680	0·777	1480

Table III gives the values on sample B obtained by taking the $E_{max.}$ (gross) and $E_{max.}$ (corr.) and multiplying by the factors 1600 and 1900 respectively. Figures are also given for the whole oil.

Table IV gives similar results for samples C and D.

TABLE IV

Sample C	E (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
After 5 minutes saponification	0·658	1050	0·497	945
After 5 minutes saponification	0·682	1090	0·399	760
After 5 minutes saponification	0·651	1040	0·539	1020
After 30 minutes saponification	0·661	1060	0·511	970
	$\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ E\mu}}$		$\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	
After 5 minutes saponification	0·872		0·910	
After 5 minutes saponification*	0·946		0·893	
After 5 minutes saponification	0·870		0·891	
After 30 minutes saponification	0·870		0·904	

Sample D	$E_{max.}$ (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
After 5 minutes saponification	0·474	760	0·385	730
After 5 minutes saponification	0·486	780	0·350	665
After 30 minutes saponification	0·475	760	0·364	690
	$\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$		$\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	
After 5 minutes saponification	0·876		0·890	
After 5 minutes saponification	0·893		0·906	
After 30 minutes saponification	0·883		0·893	

* The absorption spectrum of this extract showed a subsidiary maximum at 315 mμ.

Discussion of results obtained on the foregoing samples

Examination of the above samples indicates that a 5 minute saponification period is not sufficient to remove the non-linear irrelevant material. In general, improvement in the shape of the absorption curve is obtained by increasing the saponification time from 5 minutes to 30 minutes. The ratios $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$ and $\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$ are of value in indicating the efficiency of a given saponification. The variable nature of the results obtained after 5 minutes saponification is not due to faulty technique since only a few oils exhibit this variation. The variation may be attributed to the presence of matter which is more difficult to saponify than that which is generally found in medicinal cod-liver oils.

Table V indicates the repeatability of $E_{max.}$ (gross) and $E_{max.}$ (corr.) as determined on the unsaponifiable matter of several samples after a 30-minutes saponification period. It will be seen that the values of $E_{max.}$ (gross) are reproducible to within about 2 per cent. and that E (corr.) is reproducible to within about 5 per cent.

TABLE V

REPRODUCIBILITY OF $E_{max.}$ (GROSS) AND $E_{max.}$ (CORR.) AFTER 30 MINUTES SAPONIFICATION

Sample	$E_{max.}$ (gross)	$E_{max.}$ (corr.) Formula			E_{max} (gross) $\times 1600$	$E_{max.}$ (corr.) $\times 1900$ Formula		
		(*a*)	(*b*)	(*c*)		(*a*)	(*b*)	(*c*)
6	0·525	0·385	0·376	0·390	840	730	715	740
6	0·535	0·399	0·402	0·399	855	760	765	760
6	0·535	0·399	—	—	855	760	—	—
7	1·000	0·805	—	—	1600	1530	—	—
7	1·000	0·784	0·757	0·799	1600	1490	1440	1520
8	1·135	0·924	—	—	1820	1750	—	—
8	1·143	0·875	0·852	0·905	1830	1660	1620	1720
10	1·050	0·826	0·826	0·872	1680	1570	1570	1660
10	1·067	0·847	—	—	1710	1610	—	—
12	0·477	0·343	0·334	0·350	765	650	635	665
12	0·475	0·336	—	—	760	640	—	—

(*a*) (*b*) and (*c*) are formulæ given by Morton[2] which correct the E (gross) for irrelevant linear absorption.

It should be noted that good agreement is obtained between E (gross) values as carried out in duplicate, and also good agreement between E (corr.) results when determined using *one* given formula (*a*), (*b*) or (c). However, inter-agreement is not always good between results obtained using (*a*), (*b*) and (*c*). Poor inter-agreement tends to indicate that the irrelevant absorption is non-linear.

It was next decided to obtain a comparison of E (max.) values, both gross and corrected on the whole oil, on the unsaponifiable matter and by chromatography on alumina, containing 5 per cent. of added water. These values are given in Table VI.

All E values given in Table VI, which have not been determined in

*cyclo*hexane, have been calculated to those which would have been obtained in *cyclo*hexane.

Table VI illustrates good agreement between potencies obtained by fully correcting chromatographic and unsaponifiable matter *E* (gross) values for the presence of 10 per cent. of vitamin A_2 and the presence of 25 per cent. of neo-vitamin A,* and those obtained by multiplying the gross absorption of the unsaponifiable fraction by the factor 1600. These results are marked by an asterisk. Results obtained by applying the correctional formula given in the B.P. Addendum 1951, and multiplying the corrected *E* value by a factor of 1900 are given in the column marked with two asterisks and on the whole, give low results for the vitamin A potency.

The details of the chromatographic procedure are as follows, and are, in the main, similar to those of Gridgeman for the determination of vitamin A in whale liver oil.[5] The alumina used for chromatography was that of Peter Spence Grade O "activated alumina for chromatography." The alumina was heated for 2 hours before use in a furnace at 800° C. and then allowed to cool to room temperature. The chromatographic column consisted of a glass tube about 45 cm. long, and 7 mm. internal diameter. It was plugged with cotton wool at the lower end. A wider tube, forming a reservoir, 50 cm. long and of 12 mm. internal diameter was sealed to the top end of the narrower tube. 10 ml. of activated alumina, as measured in a 25 ml. cylinder of diameter $\frac{3}{4}$ inch were covered with light petroleum (b.pt. 40° to 60° C.) in a mortar, 0·5 ml. of water was added in drops from a pipette with a fine orifice, the pipette being moved about above the surface of the light petroleum during the addition. The moistened alumina was then ground and mixed under the surface of the light petroleum. The column was then set vertically in a stand, the lower end of the column was closed by means of a cork, and the narrow part of the column half filled with light petroleum. The moistened alumina from the mortar was then washed into the wide reservoir and allowed to settle, the tube being rotated during the period of settling. An air pressure of 40 cm. of mercury was applied to the top of the column, the pressure being released when the solvent surface had sunk to within 1 or 2 cm. above the surface of the material in the column. 1 g. of cod-liver oil, dissolved in 20 ml. of light petroleum, was then washed into the reservoir, the air pressure re-applied, and the portion of the eluate which fluoresced yellowish-green when examined in ultra-violet light, was collected separately. It was found to be necessary to add 20 ml. of light petroleum to the reservoir, when practically all the cod-liver oil solution had left, in order to remove all traces of vitamin A from the column. The pressure was then re-applied and the yellowish-green fluorescent fraction added to that portion previously collected. The whole was then made up to volume with light petroleum and suitably diluted for spectrophotometry.

In the early experiments the course of the chromatography was followed by means of the antimony trichloride reagent, but observation of the fluorescence in the ultra-violet light proved to be less troublesome and

* Hereafter referred to as fully corrected chromatographic and fully corrected unsaponifiable *E* values.

TABLE VI

COMPARISON OF $E_{max.}$ AND ($E_{max.}$ × FACTOR) FOR A NUMBER OF COD-LIVER OILS

Sample	E (gross)			E (corr.)			E (gross) × 1600			E (corr.) × 1900			(Fully corrected) ×1825		$\lambda_{max.}$ mμ oil	$\lambda_{max.}$ mμ chromatography
	Whole oil	Unsaponifiable matter	Chromatography on oil	Whole oil	Unsaponifiable matter	Chromatography on oil	Whole oil	Unsaponifiable matter*	Chromatography on oil	Whole oil	Unsaponifiable matter**	Chromatography on oil	Unsaponifiable matter*	Chromatography on oil*	Whole oil	Chromatography on oil
1	1·305	1·175	1·084	1·09	0·94	1·020	2080	1880	1730	2070	1780	1940	1940	1790	—	325
2	0·814	0·758	0·718	0·679	0·588	0·650	1300	1210	1150	1310	1120	1230	1260	1180	—	—
3	0·743	0·701	0·694	0·623	0·567	0·610	1190	1120	1110	1180	1080	1160	1150	1140	—	—
4	0·597	0·522	0·533	0·526	0·406	0·464	955	835	850	1000	770	880	860	875	—	319
5	0·865	0·822	0·827	0·728	0·665	0·710	1380	1310	1320	1380	1260	1360	1350	1360	—	325
6	0·576	0·535	0·560	0·476	0·399	0·451	920	855	895	905	760	855	880	920	323	322
7	1·013	1·000	0·990	0·858	0·805	0·902	1620	1600	1580	1630	1530	1710	1640	1640	327·5	325
8	1·217	1·135	1·182	1·050	0·924	1·063	1950	1820	1890	1995	1750	2020	1860	1940	327·5	325
9	1·277	1·204	1·260	1·068	0·994	1·141	2040	1930	2020	2030	1890	2170	1980	2070	327·5	325
10	1·147	1·067	1·100	0·980	0·847	0·935	1830	1710	1760	1860	1610	1780	1760	1810	324·5	320
11	0·540	0·467	0·476	0·434	0·357	0·424	860	745	760	825	680	805	770	780	321·5	320
12	0·547	0·475	0·480	0·439	0·335	0·431	875	760	770	835	640	820	780	790	321	320
13	0·450	0·415	0·425	0·363	0·301	0·378	720	665	680	690	570	720	680	700	323	322
14	1·212	1·093	1·070	1·008	0·651	0·990	1940	1750	1710	1920	1640	1880	1770	1760	—	325
15	0·716	0·661	0·660	0·632	0·511	0·597	1130	1060	1060	1200	970	1130	1085	1085	324	325
16	0·522	0·475	0·484	0·426	0·364	0·426	835	760	775	810	690	810	790	780	322·5	322
17	0·582	0·514	0·482	0·504	0·371	0·437	930	820	770	960	705	830	845	790	—	—
18	0·848	0·725	—	0·713	0·553	—	1335	1160	—	1355	1050	—	1190	—	—	—
19	0·456	0·415	0·425	0·385	0·301	0·378	730	665	680	730	570	715	680	695	—	320

much quicker. 4 chromatograms could be carried out in the course of 4 hours by this means. The equations of Morton, quoted below, correcting for irrelevant absorption, were applied to density readings obtained by chromatography.[2]

$$E \text{ (corr.)} = 7\ (E_{325}-0{\cdot}399\ E_{310}-0{\cdot}601\ E_{334{\cdot}9}) \quad .. \quad .. \quad (a)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}5\ (2E_{325}-E_{313}-E_{337}) \quad .. \quad .. \quad .. \quad .. \quad (b)$$

The corrected results appearing in the "oil" columns of Table VII were obtained by using the equations,

$$E \text{ (corr.)} = 7\ (E_{327{\cdot}5}-0{\cdot}405\ E_{312{\cdot}5}-0{\cdot}595\ E_{337{\cdot}7}) \quad .. \quad .. \quad (a)$$
$$E \text{ (corr.)} = 6{\cdot}58\ (E_{328}-0{\cdot}412\ E_{313}-0{\cdot}588\ E_{338{\cdot}5}) \quad .. \quad .. \quad (b)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}52\ (E_{328}-E_{316}-E_{340}) \quad .. \quad .. \quad .. \quad .. \quad (c)$$

The equations:

$$E \text{ (corr.)} = 7\ (E_{326{\cdot}5}-0{\cdot}422\ E_{312{\cdot}5}-0{\cdot}578\ E_{336{\cdot}7}) \quad .. \quad .. \quad (a)$$
$$E \text{ (corr.)} = 6{\cdot}82\ (E_{326}-0{\cdot}4\ E_{311}-0{\cdot}6\ E_{336}) \quad .. \quad .. \quad .. \quad (b)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}36\ (2E_{327}-E_{315}-E_{339}) \quad .. \quad .. \quad .. \quad .. \quad (c)$$

were applied to results obtained on the unsaponifiable matter.

Agreement within 5 per cent. between the values obtained by the application of given sets of correctional formulæ indicates that the irrelevant absorption is linear. It will be seen from an examination of the above data, that, although in some cases the agreement indicates that the irrelevant absorption is linear, in other cases the indication suggests non-linearity. In cases where a single result is given, only equations (*a*) have been applied.

The E values in the chromatographic experiments were determined using light petroleum as a solvent, but the values given in Tables V and VI are calculated to those which would be obtained using *cyclo*hexane as solvent, in order that the E values may be comparable.

The chromatographic results are rather variable. In some cases E values were obtained which, when expressed as a ratio $E_{max.}$, agreed with the published data with $\pm$ 2 per cent. over the range 310 to 340 mμ. In cases where appreciable amounts of unsaturated acids were present, chromatography failed to remove them completely, and maxima were found at wavelengths less than 325 mμ.

The agreement between the wavelengths of maximum absorption of the chromatographic fraction and that as determined on the oil is very good. In the case of those oils which contain unsaturated acids, displacement of the wavelength of maximum absorption towards the 320 mμ region is noted, and a similar displacement is found in the case of the chromatographic fraction.

The results appearing in the fully corrected unsaponifiable matter column are results compensated for the presence of 25 per cent. of neo-vitamin A of 80 per cent. of the activity of the all-*trans* variety and also for the presence of a 10 per cent. contribution by vitamin A_2 to the $E_{max.}$ (gross) value. In the course of a number of determinations of vitamin A_2 a contribution to the $E_{max.}$ (gross) by vitamin A_2 of not more than 10 per cent. of the value of $E_{max.}$ (gross) has been found. The factor 1825 was

calculated using the values $E_{max.}$ for all-*trans* vitamin A alcohol in *cyclo*hexane = 1760 and $E_{max.}$ for neo-vitamin A alcohol in *cyclo*hexane = 1650.

TABLE VII

Comparison of potencies and $E_{max.}$ values obtained by the use of correctional formulæ

Sample	Oil E (corr.)			Unsaponifiable matter E (corr.)			Chromatographic E (corr.)		Oil E (corr.) × 1900			Unsaponifiable matter E (corr.) × 1900			Chromatographic E (corr.) × 1900	
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)
6	0·476	0·446	0·442	0·385	0·376	0·374	0·451	0·428	905	850	840	730	715	710	855	810
7	0·858	0·842	0·842	0·784	0·757	0·799	0·902	0·885	1630	1600	1600	1490	1440	1520	1710	1680
8	1·050	1·032	1·068	0·875	0·852	0·905	1·063	1·067	1995	1960	2030	1660	1620	1720	2020	2030
9	1·068	1·063	1·097	—	—	—	1·141	1·137	2030	2020	2080	—	—	—	2170	2160
10	0·979	0·947	0·942	0·826	0·826	0·872	0·935	0·965	1860	1800	1790	1570	1570	1660	1780	1830
11	0·434	0·416	0·384	—	—	—	0·424	0·394	825	790	730	—	—	—	805	750
12	0·439	0·395	0·345	0·343	0·334	0·350	0·431	0·405	835	750	655	650	635	665	820	770
13	0·363	0·347	0·347				0·378	0·345	690	660	660				720	655
15	0·632	0·658	0·638				0·597	0·568	1200	1250	1210				1130	1080
16	0·426	0·390	0·358				0·426	0·397	810	740	680				810	755
18	0·713	0·592	0·561				—	—	1355	1215	1065				—	—

The potency of all-*trans* vitamin A alcohol is taken as $3{\cdot}33 \times 10^6$ I.U./g., and it has been stated[6] that the potency of neo-vitamin A is about 80 per cent. of the all-*trans* variety. On this basis the potency of neo-vitamin A is $2{\cdot}664 \times 10^6$ I.U./g. Hence the $E_{max.}$ (gross) values when corrected for vitamin A_2 may be multiplied by the factor 1825 to convert to biological units.

The percentage increase of E fully corrected unsaponifiable matter over the value of E (gross) unsaponifiable matter × 1600 is given in Table VIII.

From a consideration of Tables VI, VII and VIII the following facts emerge:

(1) There is fair agreement between the $E_{max.}$ (gross) as determined on the unsaponifiable matter and by chromatography on the whole oil. However, the chromatographic values corrected by Morton's formula tend to be greater than the unsaponifiable matter values, and are not considered to be reliable unless the shape of the absorption curve in the region 310 to 325 mμ indicates the absence of subsidiary maxima or a tendency to maxima. The absorption curves of samples 1, 5, 7, 8, 9, 14 and 15 do not indicate the presence of unsaturated acids.

(2) Chromatography of samples 1, 5, 7, 8, 9, 14 and 15 yields fractions agreeing with ± 2 per cent. with the published data for vitamin A acetate within the range 310 to 335 mμ. Outside this range, deviation occurs due to vitamin A_2. The column E (fully corrected) × 1825 chromatography must, therefore, represent a fairly true estimate of the potency of these samples. With the exception of sample 1, good agreement is obtained between these potencies and those of E (gross) × 1600 as obtained on the unsaponifiable matter.

TABLE VIII

THE INCREASE OF FULLY CORRECTED RESULTS OVER THOSE CALCULATED FROM E (GROSS) $\times$ 1600

UNSAPONIFIABLE MATTER

Sample	E (fully corrected) $\times$ 1825 I.U./g.	E (gross) $\times$ 1600 I.U./g.	Difference	Percentage
1	1940	1880	60	3·1
2	1260	1210	50	3·9
3	1150	1120	30	2·6
4	860	835	25	2·9
5	1350	1310	40	2·9
6	880	850	30	2·7
7	1640	1600	40	2·6
8	1860	1820	40	2·1
9	1980	1930	50	2·5
10	1760	1710	50	2·8
11	770	745	25	3·2
12	780	760	20	2·6
13	680	665	15	2·2
15	1085	1060	25	2·3
16	790	760	30	3·8
17	845	820	25	2·4
18	1190	1160	30	2·5
				Mean 2·7

(3) The unsaponifiable matter curves are more inclined to follow all-*trans* acetate curves above 330 mμ. This is supposedly due to the presence of, and the more difficult saponification of, neo-vitamin A.

(4) The E (gross) values as expressed on the whole oil are the greatest. This is due to the presence of irrelevant absorbing material.

(5) There is fair agreement between E (gross) $\times$ 1600 and E (corr.) $\times$ 1900 as determined on the whole oil.

(6) Good agreement may sometimes be obtained between E (corr.) values on the whole oil as calculated using formulæ (*a*) (*b*) and (*c*). Samples 7, 8, 13 and 15 show particularly good agreement between the sets of $E_{max.}$ values. This is strong indication that the irrelevant absorption is linear over the range 312·5 to 316 mμ, 327·5 to 328 mμ and 337·7 to 340 mμ. In the case of certain of the other samples the indication is that the irrelevant absorption is non-linear, and that the correctional formulæ should not be applied.

(7) There is good correlation between E (corr.) values as determined by the different formulæ on the whole oil, and on the chromatographic fraction.

(8) The absorption spectra of many of the whole oils show a displaced maximum towards the 320 mμ region, indicating the presence of unsaturated acids.

(9) Good agreement is obtained between the results which are thought to be the most accurate in the table, viz. the unsaponifiable matter E (fully corrected) $\times$ 1825 and the "unsaponifiable matter E (gross) $\times$ 1600."

(10) Corrected values on the unsaponifiable matter tend to be of the order of 10 per cent. low.

(11) Table VIII indicates from a comparison of "unsaponifiable matter E (fully corrected $\times$ 1825" and "unsaponifiable matter E (gross) $\times$ 1600" that the factor 1600 for conversion to biological potencies is not likely

to be greatly in error, assuming the presence of a 10 per cent. vitamin A_2 contribution to $E_{max.}$ and the presence of 25 per cent. of neo-vitamin A.

(12) A good agreement is shown between potencies in the column unsaponifiable matter E (fully corrected) $\times$ 1825 and chromatography E (fully corrected) $\times$ 1825. The absorption spectra of the unsaponifiable fractions of the oils do not indicate the presence of unsaturated acids, whilst the spectra of eluates of certain of the chromatographed oils indicate the presence of these acids. The good agreement between the results in the two columns indicates that the effect of the presence of conjugated acids on the E (gross) value of the chromatographic fraction would not appear to be very great.

General Conclusions

1. Corrected E values as determined on the unsaponifiable matter are lower than those which may be obtained by any other method and are considered to undervalue a given oil.

2. It is considered that a fair estimate of the potency may be obtained by saponifying the oil for a period of 30 minutes followed by a determination of the $E_{326\cdot5\ m\mu}$ (gross). Optical density readings should also be taken over the range 310 to 360 mμ, those from 310 to 320 mμ being at intervals of 1 mμ, the others at 5 mμ intervals. The plotted optical densities between 310 and 360 mμ should present a smooth curve indicating the absence of oxidation products and of unsaturated acids with absorption maxima at 315 and 320 mμ. If maxima are found indicating the presence of unsaturated acids, the oil shall be saponified for a longer period. The $E_{326\cdot5\ m\mu}$ (gross) value may then be multiplied by the factor 1600 to obtain the potency in units per g.

3. The factor 1600 to convert E (gross) of the unsaponifiable matter to biological units is fairly reliable.

4. It has been shown that a 30 minutes saponification period as specified by the U.S.P. XIV is desirable, since a 5 minutes saponification period is sometimes insufficient to remove unsaturated acids. A symptom of incomplete saponification is a tendency to emulsification during separations.

5. Chromatography, using alumina containing 5 per cent. of added water, in some cases gives results in accordance with those obtained on the unsaponifiable matter but in other cases the results are unreliable. It is possible that use of alumina containing a smaller amount of added water might result in improved removal of unsaturated acids. Care would have to be taken to prevent de-esterification of the vitamin A esters, and/or subsequent loss of vitamin A on the column. Use of stoppered cells should be of value in preventing the evaporation of the light petroleum solvent. Chromatography possess the advantage over the saponification method in that there can be no doubt as to whether the natural esters present in a cod-liver oil are hydrolysed or not.

6. It is not justifiable to determine vitamin A on the whole oil by application of Morton's three point correctional formulæ when the wavelength of maximum absorption is displaced from 327·5 mμ. When

displacement is shown, a preliminary treatment, such as saponification, is necessary.

This investigation was carried out in part on a Beckman Quartz Spectrophotometer and in part on a Unicam Spectrophotometer, both instruments being calibrated using potassium dichromate solution and pure synthetic all-*trans* vitamin A acetate. Ratios of the optical density at a given wavelength, to that of the optical density at the maximum obtained using synthetic all-*trans* vitamin A acetate dissolved in *cyclo*hexane agreed with those of Morton.[2]

The spectrophotomers were calibrated for wavelength using the mercury lines of wavelength 2967Å, 3022Å, 3126Å, 3341Å, 3650Å, 4047Å and 5461Å. Wavelength settings on both instruments were found to be correct or not to differ from the correct values by more than 2Å.

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