Studies in Vitamin A

4. SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN A IN LIVER OILS. CORRECTION FOR IRRELEVANT ABSORPTION

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The ultraviolet absorption spectrum of vitamin A with its maximum near 328 mμ. was described nearly 20 years ago (Morton & Heilbron, 1928*a*, *b*). The intensity of absorption shown at that wave length by a fish-liver oil or concentrate was conveniently expressed in terms of $E^{1\%}_{1\,cm.}$ 328 mμ. (Gillam & Morton, 1929), and this magnitude was early seen to afford a guide to potency. If a number of fish-liver oils covering a fair range of potency was examined in three different ways, namely, (*a*) by the method of biological assay, (*b*) by the antimony trichloride colour test, and (*c*) by the spectrophotometric method, the results showed fair agreement. From a strictly quantitative standpoint the bio-assays were primitive, the colour test was handicapped by a highly variable degree of inhibition and the ultraviolet absorption by superimposed irrelevant absorption.

Experience gained by a very large number of workers has since shown that bio-assays cannot compete with the spectrophotometric method in terms of either convenience or accuracy. In fact, for a bio-assay to be worth doing to-day a very large number of animals needs to be used, and the data must be subjected to rigorous statistical study. This makes it all the more necessary to dispose of difficulties and complications in the spectroscopic procedure.

The broader aspects of the problem of vitamin A activity raise acute difficulties (Morton, 1942; Hickman, 1943; Gridgeman, 1944), and it is proposed at this stage to confine attention mainly to the optimal use of spectrophotometry. We now enjoy advantages which were previously lacking. Vitamin A and its acetate are now available as pure, crystalline substances, and photoelectric spectrophotometry is capable of a higher degree of accuracy than photographic methods. Irrelevant absorption can be allowed for (Morton & Stubbs, 1946) so that the true intensity of absorption attributable to the vitamin A can be determined. By the use of such a correction procedure one major difficulty has been removed. This difficulty concerned the relationship between $E^{1\%}_{1\,cm.}$ 328 mμ. and potency determined by large-scale co-operative feeding trials with (*a*) halibut-liver oil and (*b*) crystalline vitamin A β-naphthoate. The conversion factors differed significantly, but the discrepancy disappeared when the gross intensity of absorption shown by the halibut-liver oil was suitably corrected for irrelevant absorption (Morton & Stubbs, 1947).

The purely analytical problem of determining the vitamin A content of an oil does not, however, of necessity require any reference to conversion factors or to the international unit of vitamin A activity (0·6 μg. of β-carotene). From many points of view further studies on the wider problem would be made easier if 'potencies' could be replaced by 'vitamin content' expressed in mg./g.

Spectrophotometric characteristics of vitamin A (free alcohol) and vitamin A acetate

Any attempt to determine vitamin A in an oil (where it normally occurs mainly as esters), or in an unsaponifiable fraction, requires at least accurate values for the molecular extinction coefficient (ε) or $E^{1\%}_{1\,cm.}$ at the maximum for the free or esterified vitamin dissolved in a specified solvent. For the extended use of spectrophotometry the whole absorption curve needs to be known somewhat accurately so that irrelevant absorption can be estimated.

Crystalline vitamin A acetate dissolved in *cyclo*hexane shows a maximum at 328 mμ. The intensities of absorption at different wave lengths are shown in Table 1 and are expressed as fractions of the peak value. The two wave lengths, one on each side of the maximum, at which the intensity of absorption is exactly 6/7 of the peak value have been determined as carefully as possible as 313 and 338·5 mμ. The same wave lengths apply to the purest natural ester preparations (Morton & Stubbs, 1946). It is to be noted that these figures apply to *cyclo*hexane solutions, because the absorption spectra of vitamin A and its esters vary a little from solvent to solvent. The absorption curve for the free vitamin differs a little from that of the esterified vitamin. Small though the difference is, it must be taken into account in applying correction procedures. Table 1 contains enough information for most purposes. The only significance of the ratio 6/7 is that it is empirically appropriate in relation to the wave length range covered and to the performance of the spectrophotometer.

Table 1. *Intensities of absorption of vitamin A and vitamin A acetate in different solvents, expressed as a fraction of $E_{max.}$ for wave lengths between 220 and 450 mμ.*

λ (mμ.)	Crystalline vitamin A alcohol		Crystalline vitamin A acetate		
	*cyclo*Hexane	Ethanol	*cyclo*Hexane	Ethanol	75% (v/v) aqueous ethanol
220	0·143	0·089	—	0·106	—
230	0·127	0·093	—	0·106	—
240	0·128	0·102	—	0·113	—
245	—	0·107	—	—	—
250	0·125	0·112	0·135	0·123	0·123
255	0·126	0·112	0·137	—	—
260	0·123	0·109	0·136	0·122	0·133
265	0·127	0·115	0·138	—	—
270	0·150	0·139	0·158	0·150	—
275	0·187	0·175	0·193	—	—
280	0·236	0·224	0·238	0·254	0·265
285	0·300	0·313	0·300	0·337	—
290	0·393	0·414	0·373	0·410	0·405
295	0·515	0·518	0·469	0·514	—
300	0·611	0·609	0·571	0·604	0·592
305	0·718	0·720	0·668	0·716	—
310	0·841	0·844	0·795	0·826	0·814
311	0·861	0·857	—	0·845	—
311·5	—	—	—	0·854	0·840
312	0·877	—	0·838	0·864	0·844
312·5	—	—	—	—	0·858
313	0·888	—	0·858	0·878	0·863
315	0·906	0·898	0·889	0·897	0·888
317·5	0·923	0·922	0·915	0·927	—
320	0·950	0·950	0·934	0·953	0·946
322·5	0·978	0·983	0·961	—	—
325	0·998	1·000	0·988	0·999	0·991
326	1·000	1·000	—	1·000	0·999
326·5	—	—	—	—	1·000
327	0·998	0·990	—	1·000	0·999
328	0·994	—	1·000	0·990	0·991
330	0·972	0·957	0·995	0·976	0·982
335	0·880	0·869	0·925	0·905	0·914
335·5	—	0·861	—	—	—
336	0·861	0·849	—	0·886	—
337	—	—	—	0·868	—
337·5	—	—	—	0·860	0·872
338	—	—	—	0·852	—
338·5	0·810	—	0·857	—	0·855
340	0·776	0·772	0·828	0·812	0·827
345	0·646	0·638	0·707	0·689	—
350	0·513	0·509	0·572	0·557	0·580
355	—	0·397	0·440	0·437	—
360	0·293	0·278	0·331	0·317	0·359
365	0·207	0·172	0·239	—	—
370	0·129	0·108	0·151	0·132	0·175
375	—	0·074	—	—	—
380	0·046	0·048	—	0·043	—
390	0·025	0·024	—	0·013	—
400	0·012	0·013	—	0·004	—
450	0·0003	0·0018	—	0·0002	—
	$\lambda_{max.}$ 326 mμ.	$\lambda_{max.}$ 325 mμ.	$\lambda_{max.}$ 328 mμ.	$\lambda_{max.}$ 326·5 mμ.	$\lambda_{max.}$ 326·5 mμ.
	$\epsilon_{max.}$ 48,310	$\epsilon_{max.}$ 51,100	$\epsilon_{max.}$ 48,460	$\epsilon_{max.}$ 50,020	$\epsilon_{max.}$ 50,510
	$E_{311}=E_{336}$	$E_{311}=E_{335\cdot5}$	$E_{313}=E_{338\cdot5}$	$E_{311\cdot5}=E_{337\cdot5}$	$E_{312\cdot5}=E_{338\cdot5}$
	$\frac{E_{326}}{E_{311}}=7/6$	$\frac{E_{325}}{E_{311}}=7/6$	$\frac{E_{328}}{E_{313}}=7/6$	$\frac{E_{326\cdot5}}{E_{311\cdot5}}=7/6$	$\frac{E_{326\cdot5}}{E_{312\cdot5}}=7/6$

The need for correction of data on liver oils

For many years it has been the custom to prepare the unsaponifiable fractions of low-potency oils prior to making spectrophotometric readings. It is not necessary to weigh the unsaponifiable fraction, as the intensity of absorption is more conveniently expressed in terms of the $E^{1\%}_{1\,cm.}$ value calculated on the weight of oil used. As compared with readings made on unsaponified oils, $E_{max.}$ is significantly reduced in low-potency oils, and the whole absorption curve approximates in shape to that shown by high-potency oils and rich concentrates. It was not unreasonable to assume that substances giving rise to irrelevant absorption and rendered water soluble by saponification would occur to roughly the same extent in all fish-liver oils. If that were the case such irrelevant absorption would constitute a negligible fraction of the total absorption at 328 mμ. in rich oils. Support for the assumption was provided by the observation that practically all the absorbing material of rich oils remained in the unsaponifiable fraction.

The advantage of the data in Table 1 is that the presence of irrelevant absorption becomes easy to detect. If the sample under test, e.g. a fish-liver oil, is free from irrelevant absorption the line joining the 313 and 338·5 mμ. points on its absorption curve will be horizontal at a level 6/7 of the intensity at 328 mμ. (esterified vitamin A in *cyclo*hexane). This condition is rarely satisfied, and some correction is needed even with high-potency oils. A good example is provided by the halibut-liver oils assayed carefully in 1936 and retested spectrophotometrically in 1946 (Morton & Stubbs, 1947).

The reason why irrelevant absorption occurs so frequently in high-potency oils is that vitamin A decomposition products are formed either before or during the process of extracting livers. There may also be deterioration during transit or storage. Whatever the exact reason for their occurrence, the fact of contamination by such artifacts is undeniable. Most of the decomposition products remain in the unsaponifiable fraction.

Correction procedure

The simple correction procedure described by Morton & Stubbs (1946) rests upon the assumption that the irrelevant absorption 'curve' is linear over the approximate range 310–340 mμ., i.e. that no impurity or artefact shows a maximum very close to that of vitamin A. A good deal is now known concerning the spectra of vitamin A_2 and oxidation products of vitamin A, and it can be said that over the range 310–340 mμ. their absorption curves are at least approximately linear. Nevertheless, slight departures from linearity could give rise to appreciable errors in the final result, and the final verdict on the validity of the assumption must rest on the kind of experience recorded below.

The three chosen wave lengths, $\lambda_{max.}$ for vitamin A alcohol or ester in a specified solvent and the two at which the E value is 6/7 of $E_{max.}$, may be called for convenience 'fixation points'. With a known concentration of oil or concentrate (in the specified solvent), such that $E_{max.}$ for a 1 cm. cell occurs in the region of optimal performance for the photoelectric spectrophotometer, E values are determined at the three fixation points. Normally the values on either side of the maximum are different and both exceed 6/7 of the peak value. The correction is made in two steps as in the example given below:

Cod-liver oil, 1 % solution in *cyclo*hexane:

mμ.	E	Δ mμ.	
313	0·640	15	25·5
328	0·712	10·5	
338·5	0·620		

(i) $E_{313\,m\mu.} - E_{338\cdot5\,m\mu.} = 0\cdot020$:

By similar triangles the correction for slope is

$$\frac{0\cdot020 \times 10\cdot5}{25\cdot5} = 0\cdot008,$$

$$0\cdot712 - 0\cdot008 = 0\cdot704.$$

(ii) Correction for the height of the general absorption:

$$\frac{0\cdot704 - x}{0\cdot620 - x} = \frac{7}{6}, \quad x = 0\cdot116,$$

$$0\cdot704 - 0\cdot116 = 0\cdot588,$$

$E^{1\%}_{1\,cm.}$ 328 mμ. (0·712 gross) = 0·588 (corrected).

Given this one point, i.e. E 328 mμ., corrected, the whole corrected vitamin A absorption curve can be constructed from the data in Table 1. The difference between this curve and the uncorrected curve will represent the spectrum of substances other than vitamin A, provided that the assumption of linearity over the fixation range is valid.

It will be convenient first to consider tests carried out on liver oils from the tunny (*Thunnus thynnus*). Portuguese workers (Gouveia, Coelho, Gouveia & Paz, 1943), in confirmation of other experience, have recorded high figures for tunny-liver oils. In some fishes, livers weighing more than 1 kg. yielded 10 % of oil, some 40 % of which seemed to consist of vitamin A esters. The absorption curves of commercial tunny-liver oils now being imported into this country appear at first sight 'normal' enough to need little correction, but when judged by the criteria of Table 1 substantial correction is seen to be essential. Granted that the uncorrected curve fails to satisfy the fixation criteria it is desirable to

provide independent checks on the correction procedure, and so indirectly to test the validity of the assumption that irrelevant absorption is linear over the fixation range.

If the correction procedure is found to withstand successfully the most searching tests which can be devised, it will then be of interest to ascertain the magnitude of the correction appropriate to some of the principal types of fish-liver oils and concentrates.

The next step will be to subject the correction procedure to the severe test of indicating correctly the proportion of vitamin A_2 in liver oils which contain both vitamin A and vitamin A_2.

The most severe test of all will be to apply the procedure to whale-liver oils, first those of high quality, i.e. refined oils in which the main absorbing constituent other than vitamin A is kitol, and secondly to crude oils in which the gross absorption is several times greater than that properly attributable to vitamin A. If the method can bear this weight of interpretation the purely analytical problem will have been solved and the way cleared for studies concerned with utilization of vitamin A in the body.

RESULTS

The Beckman ultraviolet photoelectric spectrophotometer has been used throughout.

Spectroscopic data on high-potency tunny-liver oils

Sample A

(i) Solution in *cyclo*hexane, 0·001 %; $\lambda_{max.}$ occurred at 328 mμ., $E^{1\%}_{1\,cm.}$ 328 mμ., 482 (gross); inflexions near 370 and 392 mμ., indicative of 'cyclized' or anhydro-vitamin A, were observed. $E^{1\%}_{1\,cm.}$ 328 mμ., 400 (corrected on the basis of esterified vitamin A). The subtraction curve was almost horizontal at 310–340 mμ., but irrelevant absorption was considerable, because of the presence both of 'cyclized' vitamin A and oxidation products (Fig. 1).

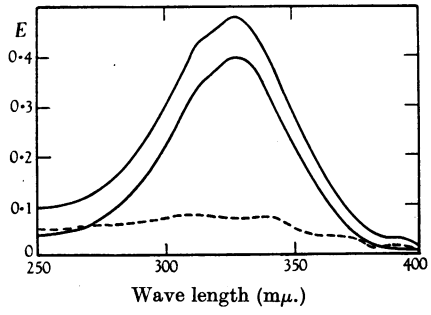

Fig. 1. Absorption curve for high-potency tunny-liver oil; upper curve observed on 0·001 % solution in *cyclo*hexane; lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(ii) A portion of oil (0·5 g.) was saponified; yield of unsaponifiable fraction 56 %, $E^{1\%}_{1\,cm.}$ 326 mμ., 802; $SbCl_3$ colour test: $E^{1\%}_{1\,cm.}$ 693 mμ., 168; 618 mμ., 2300; 582 mμ., 1105; some vitamin A_2 was present because the 693 mμ. maximum was seen clearly. $E^{1\%}_{1\,cm.}$ 326 mμ. (corrected on basis of free vitamin A) 637; recalculated on weight of oil taken, 357.

(iii) A portion of oil (0·5 g.) was saponified in the presence of a little quinol as anti-oxidant; yield of unsaponifiable matter 57·2 %; $\lambda_{max.}$ occurred at 326 mμ. (*cyclo*hexane); $E^{1\%}_{1\,cm.}$ 326 mμ., 791, or 452·5 calculated on oil; $SbCl_3$ test $E^{1\%}_{1\,cm.}$ 693 mμ., 160; 618 mμ., 2226; 582 mμ., 1094. $E^{1\%}_{1\,cm.}$ 326 mμ., 661 (corrected), or 378 calculated on oil (Fig. 2).

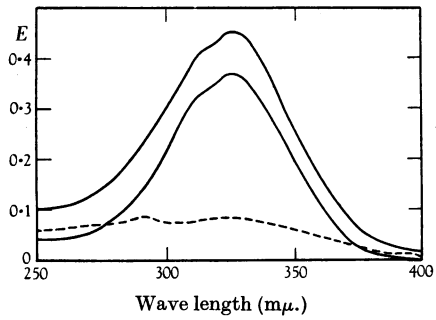

Fig. 2. Absorption curve for unsaponifiable fraction of the tunny-liver oil of Fig. 1, 0·001 %, calculated on weight of oil used; upper curve observed, lower curve corrected vitamin A absorption; broken curve, irrelevant absorption. 1 cm. cell.

(iv) Unsaponifiable matter (0·2655 g.) was partitioned between 83 % (v/v) aqueous ethanol and light petroleum: (*a*) ethanolic fraction, $E^{1\%}_{1\,cm.}$ 326 mμ., 599 (gross), 434 (corrected), calculated on fraction (0·2086 g.); (*b*) light petroleum fraction (0·057 g.), $E^{1\%}_{1\,cm.}$ 295 mμ., 47; 350 mμ., 22·2; 370 mμ. 16·5; 390 mμ., 8·5. The *E* values are here expressed on the weight of crude unsaponifiable matter; the absorption curve is uncorrectable. The fraction (*b*) seemed to be rich in 'cyclized' vitamin A but free from the vitamin itself. Appreciable losses of vitamin A occurred in this experiment.

(v) After standing for some weeks the small remaining amount of sample A had deteriorated appreciably: $E^{1\%}_{1\,cm.}$ 328 mμ., 403 (gross), 318 (corrected). A portion, 0·1 g., was saponified and the unsaponifiable fraction extracted with 250 ml. of 50 % (v/v) aqueous ethanol by warming on a water bath with frequent shaking. The extract was filtered through kieselguhr, and the combined extract and washings made up to 500 ml. with absolute ethanol. $E^{1\%}_{1\,cm.}$ 325 mμ., 369 (gross); 315 (corrected). The 50 % ethanol thus dissolves all the vitamin A but only a small part of the material contributing irrelevant absorption.

Sample B

(i) Solution in *cyclo*hexane (0·001 %), $E^{1\%}_{1\,cm.}$ 328 mμ., 484 (gross); 397 corrected on the basis of esterified vitamin A as the predominant form.

(ii) A portion of oil (0·05 g.) in 10 ml. peroxide-free ether was poured on to a column of silver sand (20 × 1·5 cm.). The

solution dispersed the oil at the top quarter of the column and the solvent was removed by means of an upward current of nitrogen. The column was then washed twice with 90 ml. portions of 70% (v/v) ethanol-water. (*a*) The first washing was diluted 1 in 5 with absolute ethanol, after first making up to 100 ml. ($c = 0{\cdot}001\%$ calculated on the weight of oil used initially.) $E^{1\%}_{1\,cm.}$ 326·5 mμ., 49·3 (gross), 34·3 (corrected). Maxima at 370 and 392 mμ. due to anhydro-vitamin A were shown clearly. (*b*) The second washing, made up to 100 ml. was diluted 1 in 5 (absolute ethanol). $E^{1\%}_{1\,cm.}$ 326·5 mμ., 13·8 (gross), 9·8 (corrected). Bands at 370 and 392 mμ., were again shown. The column was then washed with five successive portions of 80% (v/v) ethanol-water. Each was made up to 100 ml. and appropriately diluted for spectrophotometric tests. The following results all refer to concentrations expressed in terms of the initial weight of oil:

	$E^{1\%}_{1\,cm.}$ 326·5 mμ.	
	Gross	Corrected
(*c*)	54·2	49·0
(*d*)	51·3	45·5
(*e*)	34·5	31·5
(*f*)	28·6	25·2
(*g*)	20·5	17·5

The column was then washed with absolute ethanol (90 ml.) and the washings made up to 200 ml. A portion was diluted twentyfold. $E^{1\%}_{1\,cm.}$ 326·5 mμ., 200·8 (gross), 156·8 (corrected). By adding together the E values for all the fractions, the total recovery was $E^{1\%}_{1\,cm.}$ 326·5 mμ., 453 (gross), 370 (corrected). A repetition of the experiment gave a total recovery of $E^{1\%}_{1\,cm.}$ 326·5 mμ., 451 (gross).

(iii) A quantity of oil (0·1323 g.) was saponified. The unsaponifiable fraction was extracted with 250 ml. of 50% (v/v) aqueous ethanol by warming on a water bath with frequent shaking for 0·5 hr. The solution was filtered through kieselguhr and the latter was washed with 100 ml. of 50% ethanol. The combined filtrate and washings were made up to 500 ml. with absolute ethanol. The material retained by the kieselguhr was washed by means of redistilled ether into the flask containing the matter insoluble in 50% ethanol. The solvent was removed and the residue dissolved in *cyclo*hexane:

(*a*) Fraction soluble in 50% ethanol. $E^{1\%}_{1\,cm.}$ 325 mμ., 427 (gross), 392 corrected (Fig. 3).

(*b*) Remainder of unsaponifiable matter, $\lambda_{max.}$ 305 mμ., inflexions at 370 and 392 mμ. indicating the presence of 'cyclized' or anhydro-vitamin A; $E^{1\%}_{1\,cm.}$ 326 mμ., 52 (gross), 15 (corrected).

Sample C

(i) $E^{1\%}_{1\,cm.}$ 328 mμ., 395 (gross) in *cyclo*hexane; 323 (corrected) on the basis of esterified vitamin A.

(ii) A quantity of oil (0·1696 g.) was saponified. The unsaponifiable matter was extracted as before with 50% (v/v) aqueous ethanol. The soluble portion was appropriately diluted with absolute ethanol for spectrophotometric examination: $E^{1\%}_{1\,cm.}$ 325 mμ., 357 (gross), 320 (corrected).

Sample D

Four samples representative of another batch of tunny-liver oils were examined in *cyclo*hexane solution and corrected on the basis of esterified vitamin. The results were very close: $E^{1\%}_{1\,cm.}$ 328 mμ., 402 (gross), 334 (corrected).

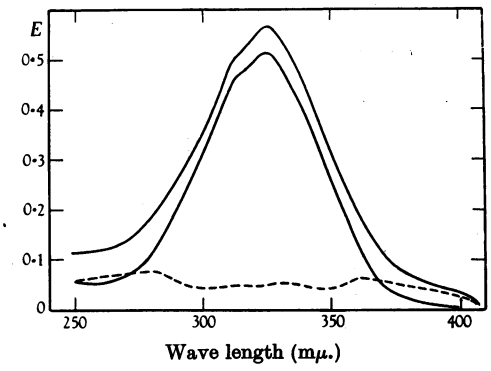

Fig. 3. Absorption spectrum of fraction soluble in 50% ethanol of the unsaponifiable fraction of tunny-liver oil (0·001323% calculated on oil used); upper curve, observed; lower curve, corrected; broken curve, irrelevant absorption. 1 cm. cell.

Discussion of the experiments on tunny-liver oils

The tunny-liver oils examined show exceptionally high values of $E^{1\%}_{1\,cm.}$ 328 mμ., indicating that they contain 40–50% of vitamin A esters. The gross values of the absorption at 328 mμ. determined directly on a *cyclo*hexane solution lead, however, to a considerable over estimate of the vitamin A content because of the presence of material contributing irrelevant absorption. When the spectra are corrected using the gross readings at the fixation points (313, 328 and 338·5 mμ.) appropriate to vitamin A esters in *cyclo*hexane, the vitamin A contribution is seen to be 82–83% of the gross absorption at 328 mμ. The validity of the correction, as already explained, rests upon the assumption that irrelevant absorption is linear over the fixation range.

In order to test the assumption various methods of fractionation have been studied. Partition of the total unsaponifiable material between 83% (v/v) aqueous ethanol and light petroleum led to appreciable losses of vitamin as shown by the results on sample A. In all cases where the unsaponifiable fractions of these oils were freed from the last traces of solvent by means of steam heating in a current of nitrogen, marked losses of vitamin have occurred. It would seem that the free vitamin A as it occurs in the unsaponifiable matter of these very potent oils is more liable to oxidation than the vitamin in the corresponding fractions derived from oils of lower potency.

The method of fractionation suggested by Chevallier, Manuel & Faubert (1941) has only recently become known to us through the courtesy of M. Faubert. It consists of dispersing an oil upon silver sand as an inert support and eluting various fractions by different mixtures of ethanol and water. In trying out the method on sample B, three main fractions were obtained:

(*a*)–(*b*) fraction soluble in 70 % ethanol.
(*c*)–(*g*) fraction soluble in 80 % ethanol.
(*h*) fraction soluble in absolute ethanol.

The possibilities of the method have not yet been fully explored, but for our present purposes the use of sand is not essential.

It has been found that almost all the free vitamin A in the unsaponifiable fraction is readily soluble in 50 % aqueous ethanol. Whereas the corrected *E* value for the oil is 82 % of the gross value it is 92 % of the gross value for the soluble part of the unsaponifiable fraction (see sample B (iii)). Moreover, for samples B and C, the simple correction procedure applied to *cyclo*hexane solutions of the oils, gives the same final result, within very narrow limits, as the fuller fractionation procedure.

The method of fractionation with 50 % aqueous ethanol offers an alternative correction procedure to those workers who lack an accurate photoelectric spectrophotometer. It provides a means for the quantitative recovery of the vitamin A while at the same time eliminating at least half of the irrelevant absorption. It also obviates much of the risk of losing vitamin A in the final stages of preparation of unsaponifiable extracts, since after the removal of ether, the 50 % ethanol can be added at once without trying to get rid of the last traces of ethanol and water.

Conclusions from the experiments on tunny-liver oil

The correction procedure, based on the three point fixation applied to *cyclo*hexane solutions of whole oils, is justified (*a*) by fractionations of the oil and (*b*) particularly by fractionation of the unsaponifiable fraction. If facilities are lacking for the application of the full correction, the best estimate of vitamin A potency is obtained by spectrographic measurements at 325 mμ. on the 50 % ethanol extract of the unsaponifiable fraction.

The results on sample B (ii) show that with both 70 and 80 % ethanol, the amount of vitamin extracted by successive portions of solvent decreases steadily. The oil probably contains a little free vitamin A (easily soluble in 70 % ethanol). A difference is suggested between the esterified vitamin A which is soluble in 80 % ethanol from that requiring absolute ethanol, since the former solvent is progressively less effective in successive elutions despite the fact that more than half the amount of vitamin used is still available. A possible explanation of the difference is that the two fractions differ in respect of the fatty acids with which the vitamin A is esterified. Although the corrected *E* values for the different fractions vary from 70 to 92 % of the gross values, the addition of gross and corrected values shows that 82 % of the absorption is due to the vitamin, a result agreeing with that observed on the whole oil.

Spectrophotometric data for a range of commercial fish-liver oils

The application of the correction procedure to the determination of vitamin A in cod-liver oils has been discussed elsewhere (Morton & Stubbs, 1946). Its bearing on the conversion factor problem has been studied using the halibut-liver oil samples employed in the large-scale co-operative work organized by the Vitamin A Sub-Committee of the Accessory Food Factors Committee (Hume, 1937, 1939; Irwin, 1944). For the fully corrected $E^{1\%}_{1\,cm.}$ value a conversion factor of 1800 is appropriate (Morton & Stubbs, 1947). It is proposed here to summarize experience gained in the use of the method applied to a wide variety of vitamin A-containing materials. Table 2 shows the results obtained for a number of commercial halibut-liver oils examined in *cyclo*hexane solution.

Table 2. *Intensities of absorption shown by commercial halibut-liver oils*

Oil no.	$E^{1\%}_{1\,cm.}$ 328 mμ. Gross	$E^{1\%}_{1\,cm.}$ 328 mμ. Corrected	Vitamin A absorption as percentage of gross absorption
1	28·8	24·2	84·2
2	54·9	47·6	86·8
3	63·4	54·0	85·2
4	39·9	34·3	86·0
5	25·6	21·1	82·4
6	29·1	24·5	84·1
7	14·6	14·0	95·9
8	98·4	95·8	97·4
9	38·0	35·7	94·0
10	26·2	23·4	89·4
11	52·2	46·2	88·6
12	58·2	49·0	84·2
13	37·5	32·1	85·6
14	29·9	28·0	93·6
15	15·0	12·6	84·0
16	25·8	24·3	94·2
			Mean 88·5

For many years the gross $E^{1\%}_{1\,cm.}$ 328 mμ. for halibut-liver oils has been multiplied by a conversion factor of 1600 in order to express the results in international units. When there is no irrelevant absorption the appropriate conversion factor is 1800. Thus only when the corrected *E* value is 88·9 % of the gross

Table 3. *The extent of correction needed in the spectrophotometric measurement of the vitamin A content of fish-liver oils*

Type	No. of samples	$E^{1\%}_{1\,cm.}$ 328 mμ. gross	$\frac{E \text{ corrected}}{E \text{ gross}}$	Average conversion factor for E gross
Very rich oils	12	*c.* 200	0·79–0·89	1500
Rich oils	20	*c.* 100	0·80–0·90	1550
Halibut-liver oils	16	*c.* 40	0·82–0·97	1600
Shark-liver oils	15	*c.* 25	0·77–0·90	1510
Dogfish-liver oils	3	*c.* 6	0·82–0·94	1560
Hake-liver oils	6	*c.* 5	0·85–0·93	1610
Other liver oils	20	*c.* 25	0·70–0·94	1540

value will $E^{1\%}_{1\,cm.}$ 328 mμ. (gross) × 1600 give the true potency. Since Table 2 shows that on the average the corrected E value for halibut-liver oil is 88·5 % of the gross value, 1600 remains the appropriate conversion factor if correction cannot for lack of the right equipment be undertaken. It is quite clear, however, that omission of correction may give rise to serious errors in some cases; the oils most contaminated with artefacts will be over valued and those least contaminated may be very significantly under valued (cf. oils 5, 7 and 8 in Table 2).

Similar results were obtained on a large number of fish-liver oils. In Table 3 many of these oils have been grouped at different potency levels rather than according to species because many commercial oils are obtained from mixed livers.

Throughout Table 3 an average empirical conversion factor between 1500 and 1600 would be justified for use with the uncorrected $E^{1\%}_{1\,cm.}$ 328 mμ. value. The range of variation in the magnitude of the correction again shows, however, that over a period some oils would be over valued and others under valued, but on the average the conversion factor of 1600 has given results surprisingly near the truth.

Spectrophotometric data on commercial molecular distillates (ester concentrates)

As might have been expected, molecular distillates are easily distinguishable from ordinary commercial liver oils. The correction for irrelevant absorption is much smaller and the spectra of some samples examined are within experimental error identical in form with the standard spectrum of crystalline vitamin A acetate dissolved in *cyclo*hexane. It is significant, however, that none of the distillates so far examined require zero or negative correction as would be expected if the observed corrections were all within the experimental error of the procedure (Table 4).

Except for one sample the corrected values are over 92 % of the gross value, and in general the use of a conversion factor of 1700 is justifiable. It is not unlikely that the freshly prepared molecular distillates may have needed little or no correction. Certainly samples 7, 12 and 15 must have been representative of remarkably stable products.

Table 4. *Intensities of absorption of commercial molecular distillates in* cyclo*hexane*

Distillate no.	$E^{1\%}_{1\,cm.}$ 328 mμ. Gross	$E^{1\%}_{1\,cm.}$ 328 mμ. Corrected	Vitamin A absorption as percentage of gross absorption
1	100·7	92·8	92·2
2	102·2	96·6	94·5
3	102·4	95·2	93·0
4	85·8	74·2	86·5
5	85·8	82·6	96·3
6	86·8	84·0	96·8
7	87·6	86·8	99·1
8	87·8	81·9	93·3
9	86·6	80·5	93·0
10	88·2	81·2	92·1
11	86·4	81·2	94·0
12	86·8	85·4	98·4
13	87·2	81·2	93·1
14	86·0	81·2	94·4
15	85·8	84·0	97·9
			Mean 94·3

Spectrophotometric data on ling cod-liver oils

The liver oil of the ling cod (*Ophiodon elongatus*, Girard) and also the commercial oils known as 'red' and 'black' cod-liver oils are interesting in several ways. For marine fish-liver oils they are exceptionally rich in vitamin A_2 as shown by the antimony trichloride colour test. The main selective absorption in the colour test for vitamin A occurs at 617–620 mμ. whereas that characteristic of vitamin A_2 occurs at 693 mμ. In ling cod-liver oils the relative intensities of the two bands are 7–8 to 1, the latter band being the weaker. For many other liver oils from salt-water fish the corresponding ratio is 15–20 to 1. Since the potency of a commercial ling cod-liver oil may be of the order 300,000 i.u./g. the absolute amount of vitamin A_2 present is considerably higher than that normally obtainable from fresh-water fish-liver oils. After saponification and desterolation of the unsaponifiable fraction, a concentrate containing 10 % of vitamin A_2 is obtained.

From the analytical standpoint a study of such oils illustrates the possibility of further extensions of the correction for irrelevant absorption. The ultra-violet absorption curve for a ling cod-liver oil is superficially a normal vitamin A curve (Fig. 4). The criterion that in *cyclo*hexane solution

$$E_{313\ m\mu.} = E_{338{\cdot}5\ m\mu.} = 6/7\ E_{328\ m\mu.}$$

is not, however, satisfied and there must therefore be irrelevant absorption. $E_{338{\cdot}5\ m\mu}$ exceeds $E_{313\ m\mu.}$ in most ling cod-liver oils indicating that the slope

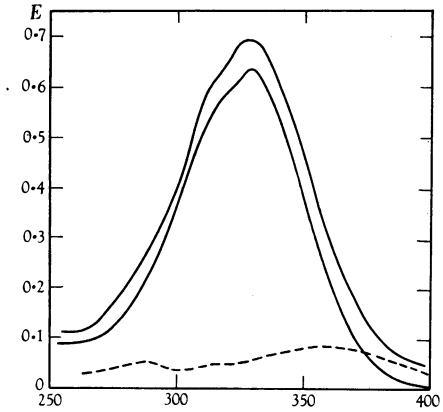

Fig. 4. Absorption spectrum of ling cod-liver oil, 0·0176% solution in *cyclo*hexane. Upper curve, observed; lower curve, corrected; broken curve, subtraction curve showing vitamin A_2 maximum. 1 cm. cell.

of the irrelevant absorption is in the direction opposite to that found for most fish-liver oils. The correction procedure is, however, applicable and a reduced E value for the vitamin A contribution can readily be calculated at 328 mμ. If from the standard curve for vitamin A acetate the whole corrected vitamin A absorption curve is plotted, subtraction from the complete curve (uncorrected) of the true vitamin A contribution will give the irrelevant absorption curve. For most ling cod-liver oils the subtraction curve shows a maximum near 350 mμ. characteristic of the vitamin A_2 present in the oil, and furthermore the ratio $E_{328\ m\mu.}$ (corrected)/$E_{350\ m\mu.}$ (by subtraction) is 7–8 to 1, in very reasonable agreement in most cases with the ratio obtained from the colour test. The agreement is not strictly quantitative because there is some overlapping of the absorptions of vitamins A and A_2 in the colour test. The $E_{350\ m\mu.}$ value is the difference between two comparatively large values and as such is subject to appreciable error. There is no doubt, however, that if the position of $\lambda_{max.}$ for vitamin A_2 were not already known from data on preparations from fresh-water fish-liver oils, the subtraction procedure would have given it fairly correctly. This is of much wider significance than the present problem as it offers a means for arriving at the spectra of contaminants even though the latter may not be chemically separable from the main constituent.

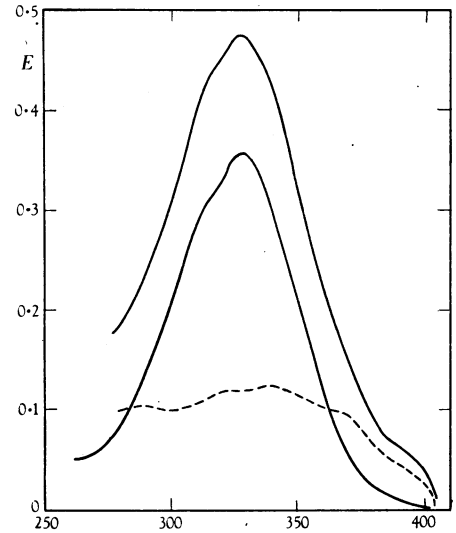

Fig. 5. Absorption spectrum of ling cod-liver oil, 0·015% solution in *cyclo*hexane. In this sample the subtraction curve shows the presence of decomposition products of vitamin A. 1 cm. cell.

Fig. 5 shows the results obtained with a ling cod-liver oil containing 'cyclized' or anhydro-vitamin A as well as vitamins A and A_2. In this case the maximum for vitamin A_2 in the subtraction curve is not so clearly shown owing to the superimposed absorption of the anhydro-product with its maxima at 350, 370 and 392 mμ. The method, however, reveals clearly the spectrum of the principal absorbing contaminant.

Data on whale-liver oils

The assay of whale-liver oils will be dealt with in a separate communication, but the problem may be referred to briefly here.

The spectra of whale-liver products vary rather widely, but in general $\lambda_{max.}$ is well on the short-wave side of 328 mμ. This is the result of very considerable irrelevant absorption due largely in refined oils to kitol with its maximum at 286 mμ. Crude oils are also contaminated with a variety of absorbing materials, especially oxidation products. The accurate assessment of vitamin A content is impossible on the uncorrected absorption, and as the correction

is very variable the use of an empirically based conversion factor leads to nothing better than a first approximation. Correction by our ordinary procedure gives results in surprisingly good agreement with very much more troublesome fractionation procedures. Given a corrected $E^{1\%}_{1\,cm.}$ 328 mμ. value on the oil, or corrected $E^{1\%}_{1\,cm.}$ 326·5 mμ. on the unsaponifiable fraction, the use of a conversion factor of 1800 gives results which are reasonably good.

SUMMARY

1. The absorption spectra of pure vitamin A and vitamin A esters have been determined in *cyclo*hexane, ethanol and 75% (v/v) aqueous ethanol. The results are expressed in terms of the fraction of $E_{max.}$ which corresponds to the absorption at different wave lengths between 250 and 400 mμ.

2. Fixation wave lengths, $\lambda_{max.}$ and two others at which E is 6/7 of $E_{max.}$, have been selected for vitamin A, free or esterified, in specified solvents. By their aid, irrelevant absorption in a liver oil or concentrate is readily detectable.

3. A correction procedure, previously found to be valid for cod-liver oils, has been carefully tested in relation to its validity for high-potency tunny-liver oils. The allowance for irrelevant absorption seems to be given with considerable accuracy.

4. Free vitamin A may be extracted from the total unsaponifiable fraction of fish-liver oils by 50% (v/v) aqueous ethanol. Much of the material giving rise to irrelevant absorption is left behind.

5. Experience gained in testing a wide range of fish-liver oils, including halibut, shark, hake, dogfish and many mixed liver oils shows that irrelevant absorption at 328 mμ. occurs in all, varying from 5 to 20% of the gross absorption. On the average the conversion factor of 1600 was fully justified if irrelevant absorption could not be allowed for, but the use of the factor of 1800 and of corrected E values permits the assay of individual oils with considerably improved precision.

6. Molecular distillates show from 10% to negligible irrelevant absorption. The average for the commercial samples studied is about 5%.

7. The correction procedure applied to the spectra of ling cod-liver oils enables the spectra of vitamin A_2 and anhydro-vitamin A to be obtained at least semi-quantitatively.

8. Crude and refined whale-liver oils are amenable to study by the method, and the results are an improvement upon those of any alternative procedure so far available.

Most of the fish-liver oils examined in the course of this work were obtained through the Ministry of Food.

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