CXLIX. DETERMINATION OF VITAMIN A WITH THE HILGER VITAMETER EQUIPPED WITH A DEVICE FOR PHOTOGRAPHIC RECORDING.

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(*Received March 23rd, 1935.*)

The ultraviolet absorption at 328 $m\mu$ has now been generally accepted to be, with certain limitations, a measure of the content of vitamin A [League of Nations Commission, 1934]. The spectrographic determination of this, however, requires elaborate and expensive apparatus and a considerable amount of work by expert hands, which makes it unsuitable for use as a general method of analysis. For the Official Norwegian Cod Liver Oil Control Laboratories under technical leadership of the Fisheries Research Station, it was very desirable to be able to carry out the determination by means of some simpler apparatus, and as early as 1932 enquiries were made with the leading makers of optical instruments whether it was possible to manufacture a simple apparatus suitable only for measuring $E_{328\,m\mu}$. At that time only the usual spectrophotometers were available, but early in 1933 the firm A. Hilger, London, sent out their vitameter, which was exactly the instrument we were looking for, and the first one manufactured was delivered to our laboratories.

A description of the vitameter has been published by Adam Hilger, Ltd. The measurement, as far as the observer is concerned, consists of the matching of intensity of two fluorescent lines. We soon found that the matching of the intensity of the two fluorescent lines was difficult, determinations carried out at different times and by different observers giving discrepancies of up to $\pm$ 10–15 %. In order to avoid this I tried to record the intensity of the lines photographically, and it was found that they had sufficient photographic activity to give prints within a few seconds on the most vigorous gaslight printing paper available. This material has the great advantage of being independent of a dark-room, which makes developing *etc.*, a very simple matter.

I therefore equipped our vitameter with a simple device for photographing the lines on such paper, and this has been in constant use during the last year and a half and has enabled us to obtain exact and objective observations quickly and easily.

The construction of the device will be seen in Fig. 1. Immediately in front of the fluorescent screen there have been made two narrow slits to allow the insertion of a holder for the paper. This runs in rails that are fixed to the support for the screen, both holder and rails being made so as to get the paper as close as possible to the screen. The paper is held in place by the turned down edges of the holder and is pushed in till it meets a stop, to ensure that it is always held in the same place. On the back of the holder there are marks to show where to begin and how far it is to be moved for each exposure. We have found a size of paper 9 × 4 cm. suitable.

To carry out the determination a suitable solution is first prepared, which should have an extinction coefficient of 0·6–1·0. The absorption is first determined approximately by eye, a shutter is placed in front of the lens and the paper brought into position in the holder. It should be marked to show which

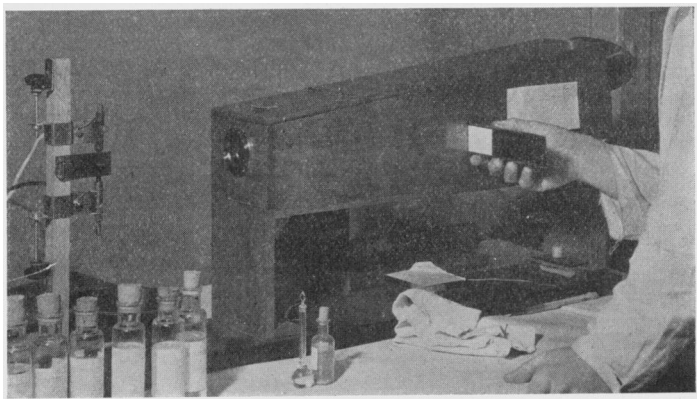

Fig. 1.

side is up. Exposures are then made at suitable intervals of E, the holder being moved about 5 mm. between each exposure. The time necessary increases as the aperture is reduced; we have found a suitable exposure to be ten times as many seconds as the aperture reading ($E = \log I_0/I$) running the copper arc at 45–50 volts, 0·45 ampere, and with a gap of about 1 mm. The paper is then developed for one minute in an ordinary, vigorous paper developer, and fixed. This can be done in electric or subdued daylight if the paper is protected from direct rays. Even greater freedom can be obtained by using a desensitising bath first.

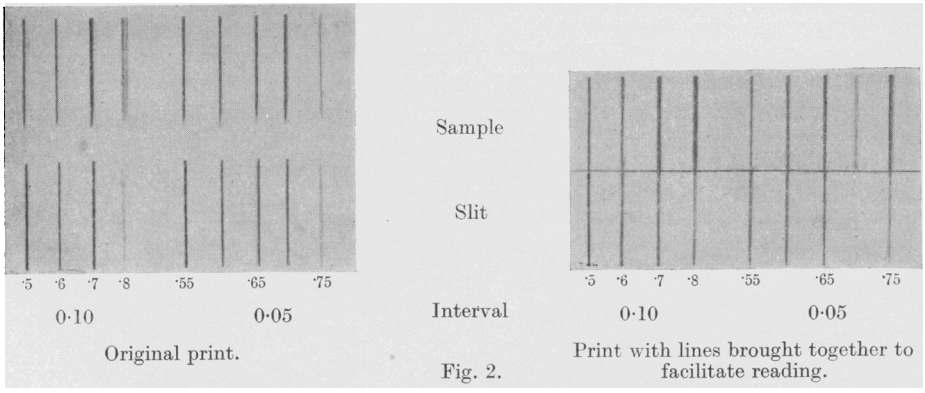

Fig. 2.

It will be necessary to expose from about 20 % below to 20 % above the approximate value, the intervals being chosen according to the accuracy desired. If the apertures have been correctly chosen it will be found that the print of the line that has passed through the solution is weaker than that from the aperture for the lowest values, the difference growing less until there is equality and then the case is reversed. A typical set of exposures is shown in Fig. 2.

The intervals may be chosen down to $0{\cdot}05\,E$ and it is possible to read with an accuracy of $\pm 0{\cdot}01$. Parallel determinations should not differ more than $\pm 0{\cdot}02$, that is ± 3 % if the observation is carried out at 0·6–0·7.

It will be seen that the accuracy is considerably greater than is obtainable by ocular readings and approaches the accuracy obtainable by usual spectrographic methods.

Corrections. The increased accuracy makes it necessary to take certain precautions. The glass cell must be perfectly clean and should be polished before each observation with a piece of clean wash-leather. The solutions must be free from any turbidity, and a correction must be introduced for the solvent used. This makes the choice of solvent unimportant as long as its absorption is low and it does not in any way harm the vitamin.

Table I gives some of the values found for various solvents in one of the cells that have been used. It should be noted that these cannot be regarded as absolute values of $E_{328\,m\mu}$. They may be expected to vary with different instruments and cells, as the latter and the compensating glass plates may differ slightly. No special precautions have been taken to purify the solvents, the chloroform is ordinary B.P. and the alcohol may be expected to contain aldehyde, as it has not been protected from air.

Table I. E *for solvents in vita-glass cell No. II.*

						Mean value
Chloroform	0·04	0·05	0·03	0·04	0·03	0·04
Alcohol, absolute	0·07	0·09	0·11	0·10	0·08	0·09
The same + 10 % chloroform	0·07	0·07	0·05	0·07	0·08	0·07
Toluene	0·07	0·07	0·08	—	—	0·07

Although it has a relatively great absorption, and dissolves only a limited quantity of oil, we have found that absolute alcohol is the most suitable solvent, as vitamin A is more stable in this than in other solvents, and it does not evaporate very quickly.

Setting of the vitameter. It is imperative that the arc should be placed exactly at the correct distance from the lens, as the changes in aperture are not symmetrical. The prints of the two lines will then appear exactly one above the other and be equally broad, and variations in density across the lines, if any, should correspond. If the arc is out of focus, the line from the aperture will be blackened unsymmetrically, and it is difficult to obtain correct readings. To set the vitameter it is best to use a solution with a density of 0·8–1·0. The arc is then set and the lines matched as nearly as possible by eye. Exposures are made with the arc at slightly different distances (2–4 mm.), it being then easy to see from the prints which is the right distance. The setting can be checked by determining the same substance in several concentrations. Table II gives some values of the same oil in two concentrations in different solvents.

Table II.

Solvent	Concentration g./l.	$E_{328\,m\mu}$	Correction for solvent	Corrected $E_{328\,m\mu}$	$E^{10\,g./l.}_{328\,m\mu}$
Chloroform	13·0	0·92	0·04	0·88	0·68
,,	6·4	0·47	0·04	0·43	0·67
Alcohol	13·0	0·96	0·09	0·87	0·67
,,	6·4	0·52	0·09	0·43	0·67
Toluene	13·0	0·90	0·07	0·83	0·64
,,	6·4	0·50	0·07	0·43	0·67

It will be seen that the various conditions give good agreement for $E^{10\,g./l.}_{328\,m\mu}$.

Vitameter values as compared with spectrographic determinations.

It is of course vital to the value of the readings in the vitameter that they agree with or stand in some constant relation to values determined spectrographically. We have carried out numerous determinations on cod-liver oils, halibut-liver oils and also various concentrates that give good agreement, and the values give the usual parallelism with the blue values obtained.

Table III shows results which have been found after the present technique had been worked out.

Table III.

Sample	Colour 2 cm. layer Y.V.	Colour 2 cm. layer R.V.	Free fatty acid g./100 g.	Kreis test R.V.	B.V. calculated for 0·04 g. oil	$E^{10\,g./l.}_{328\,m\mu}$ Spectrograph	$E^{10\,g./l.}_{328\,m\mu}$ Vitameter
Cod-liver oil 1	1·5	0·0	0·95	3·0	7·7	0·50	0·46
Concentrate of same	—	—	—	—	15·5	0·42	0·39
,, (quick method)	—	—	—	—	—	0·42	0·38
Cod-liver oil 2	4·4	0·4	1·40	6·0	17·0	0·89	0·90
Concentrate of same	—	—	—	—	29·0	—	0·77
,, (quick method)	—	—	—	—	—	—	0·77
Cod-liver oil 3	7·0	1·2	3·20	9·6	15·0	0·76	0·75
Concentrate of same	—	—	—	—	—	0·68	0·62
,, (quick method)	—	—	—	—	—	0·68	0·66
Cod-liver oil 4	3·0	0·2	1·00	15·0	19·0	0·97	1·03
Cod-liver oil 5	2·0	0·0	0·80	3·0	18·0	0·94	0·92
Halibut-liver oil 1	1040	205	27·50	*ca.* 600	1600	47	49
Halibut-liver oil 2	35	5·6	6·00	*ca.* 100	1750	50	49
Herring oil concentrate	—	—	—	—	0·9	0·073	0·066
Ether extract of vitamin preparation (thick syrup; ether-soluble matter = 1·2 g./100 g., sp. gr. = 1·32)			Sample 3		3·3	0·124	0·117
			,, 24		15·0	0·38	0·39
			,, 73		10·5	0·25	0·23

The blue value, colour and Kreis test have all been determined in a standard Lovibond Tintometer. The last test has been carried out with 5 ml. cod-liver oil + 5 ml. concentrated hydrochloric acid + phloroglucinol and measured in a 2 cm. layer [Taffel and Revis, 1931; Notevarp and Pillgram-Larsen, 1932].

Practical applications of the vitameter.

$E_{328\,m\mu}$ of oils and their concentrates. The directions for the determination of vitamin A by means of the absorption at 328 $m\mu$ regard it as necessary to carry out the determination on the unsaponifiable fraction, except in the case of very potent preparations. It seems, though, as if the relation between the biological potency and spectrographic value is chiefly based on E of cod-liver oils themselves [Coward *et al.*, 1932; Chevallier and Chabre, 1933]. Separation of the unsaponifiable fraction is a considerable complication and greatly increases the demand for accuracy if the results are to be reliable. By the introduction of the photographically recording vitameter the measurement of absorption has become quite a simple operation with few sources of error, and if it were possible to avoid the preparation of the concentrate the determination of E would approach the determination of the blue value in simplicity. This latter is now in very extensive use for commercial and other mass analysis, but it is known to be a less good measure of the vitamin content than the absorption; it is messy and uses an unstable reagent, and it embraces a considerable number of sources of error. It would seem a great improvement if it were possible to replace it

by the vitameter. We have therefore carried out a number of determinations on oils and their concentrates to obtain an estimate of the intrinsic absorption of the oils. These values will be found in Tables III and IV. Table III gives the values for three oils determined both spectrographically and in the vitameter, the values in Table IV are from the vitameter alone.

All due care has been taken when preparing the concentrates, the values marked * refer to concentrates prepared by a rapid method we have developed, which will not be described in detail here. The principle is that only a small amount of oil is saponified and the soap solution extracted with a known volume of toluene. This is then washed, dried and used directly for the determination. All values have been corrected for the absorption of the solvent. The values for the concentrates are calculated so that they correspond with the amount of oil from which they have been prepared as the amount of concentrate is of no interest in this connection.

Table IV.

Sample	Quality	Colour 2 cm. layer Y.V.	Colour 2 cm. layer R.V.	Free fatty acid g./100 g.	Kreis test R.V.	B.V. calculated for 0·04 g. oil	$E^{10g./l.}_{328m\mu}$ Oil	$E^{10g./l.}_{328m\mu}$ Concentrate	$\frac{E \text{ conc.}}{E \text{ oil}}$
L. 14	Light medicinal	1·5	—	0·35	2·3	10·0	0·58	0·51*	0·88
L. 15	,,	1·5	—	0·45	2·0	12·0	0·65	0·62*	0·95
L. 28	,,	1·5	—	0·25	4·6	11·0	0·69	0·65*	0·94
567	,,	1·4	—	0·25	*ca.* 5·0	8·8	0·52	0·44	0·85
								0·47*	0·90
559	,,	1·7	—	0·35	5·0	8·0	0·50	0·44	0·88
A.J. 3	,,	1·5	0·0	0·95	3·0	7·7	0·46	0·39	0·85
								0·38*	0·83
P. 215 I	Dark medicinal	4·4	0·4	1·40	6·0	17·0	0·90	0·77	0·86
								0·77*	0·86
P. 132	Fresh	—	—	—	—	—	0·82	—	0·85
	Rancid	2·0	0·0	0·25	14·0	16·4	0·97	0·70*	0·72
272	Crude	7·0	1·2	3·20	9·6	15·0	0·75	0·66*	0·88
								0·62	0·83
P. 182	Poultry brown	30	3·0	0·50	31·5	8·5	0·73	0·42*	0·58
P. 323	Industrial	100	15	32·0	9·0	6·5	0·65	0·33*	0·51

It will be seen that the absorption values of concentrates of medicinal cod-liver oils of high quality are from 83 to 95 % of the corresponding absorption of the oil. Even crude oils with 3·2 g./100 g. free fatty acid have given absorptions in the concentrate of 83 and 88 % of that of the oil. To determine the $E_{328m\mu}$ of pure medicinal oils it would therefore appear unnecessary to separate the unsaponifiable fraction, and in the case of cruder oils it seems only necessary when a high degree of accuracy is needed. The intrinsic absorption of these oils must be small, especially as it cannot *a priori* be supposed that the concentrates can be prepared entirely without loss.

In this connection it is interesting to note that Morgan *et al.* [1934] state that the biological activity of concentrates is 85 to 95 % of the corresponding value of the oils and that Evers and Smith [1934] find values of E for concentrates of fresh medicinal cod-liver oils which are 83–94 % of the values for the oils themselves.

E *determined in the vitameter in relation to the blue value.*

We have obtained hundreds of parallel determinations of E and the blue value, as these are carried out simultaneously as a rule, and have found a satisfactory proportionality between the two. We are aware that this is not usually found for the oils by other authors [Coward *et al.*, 1932, *etc.*], whereas it is found

for the concentrates [*e.g.* McWalter, 1934]. Our evaluation of the blue value differs from the usual, however, so it is necessary to explain this.

It is a well-known fact that the number of Lovibond glasses necessary for matching the antimony trichloride blue value is not proportional to the concentration of chromogen. If it is not possible to obtain a direct match for 0·04 g., owing to the depth of the colour, I have found that the true blue value, *i.e.* the value that would have been found if the matching of 0·04 g. in a 1 cm. cell had been possible, can be calculated by a formula of the following type:

$$B = b.d^x,$$

where b is the blue value actually measured, and d the dilution factor. x was originally determined to be 0·7 from a very great number of blue values carried out at different concentrations [Notevarp and Hjorth-Hansen, 1932]. Later on it was possible to check the formula over a much greater range (B.V. = 4 to B.V. = 150) in relation to spectrographic measurements, and it was found that $x = 0{\cdot}8$ gave the best agreement [Notevarp and Weedon, 1933]. Detailed publications dealing with the subject are in preparation.

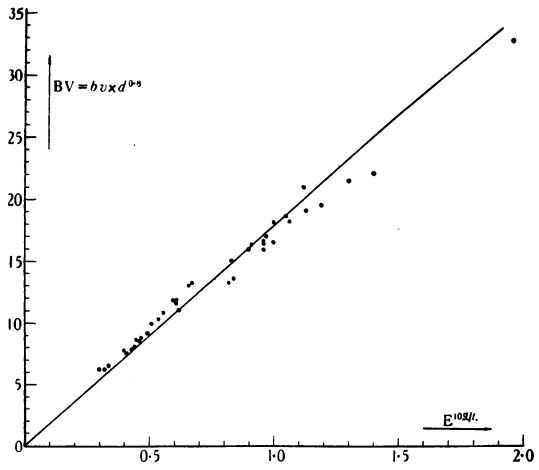

Fig. 3.

Fig. 3 shows how blue values calculated in this way give proportionality with E determined in the vitameter.

Destruction of the vitamin. Differential determinations.

The intrinsic absorption of absorbing oils may be eliminated by separating the unsaponifiable fraction, but this is of course not possible when the absorbing substances are of the same chemical nature as the vitamin. This will often be the case with oils and fats of a low vitamin potency, such as herring oils, muscle oils of other fat fishes, vitaminised margarine, butter *etc.*

To estimate the absorption due to vitamin A it will then be necessary to remove either the foreign absorbents or the vitamin. The removal of pigments by means of adsorbent carbon is mentioned by Gillam *et al.* [1933].

It has been attempted to remove the vitamin in various ways. McWalter [1934] describes the effect of heat and aëration; irradiation has also been mentioned. We have tried these various methods of destruction, with the result that

oxidation and heat have been rejected as uncertain. Oxidation will often give rise to substances that absorb light at 328 $m\mu$, especially when there are minute quantities of fat present. Irradiation seems to give much better results; even when an oil is dissolved direct in alcohol, it appears to be possible to destroy vitamin A by light without the formation of appreciable amounts of absorbing substances. The difference agrees with what would be expected from the absorption of the concentrate or the blue value.

This subject will also be treated more in detail in a later publication, as there are still points that need closer investigation. Thus we have observed a hitherto inexplicable rise in the absorption of concentrates after destruction in a few cases. I will therefore only mention a few differential determinations here. The destruction has been effected through irradiation with a 500 watt Osram Vitalux lamp, the solutions being placed about 4 cm. from the surface of the lamp, usually for about 6 hours. The samples were cooled by a strong current of air, yet the temperature of the solution rose to about 40°.

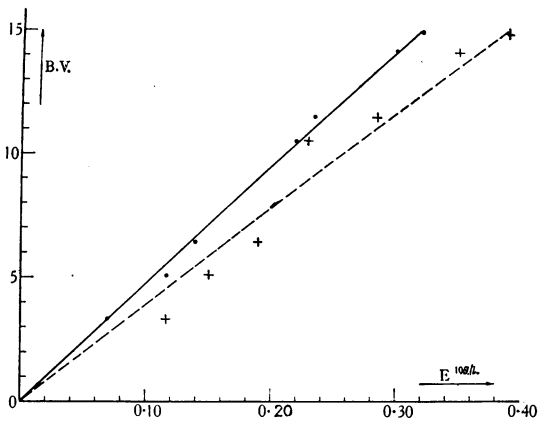

Fig. 4. + Gross values. • Net values (differential).

Fig. 4 shows the results for the ether extracts of samples of a vitamin preparation intended as a substitute for cod-liver oil. The gross absorption values do not agree very well with the blue values, while the differential determinations show as good agreement as may be expected from these methods. The blue values and E have been determined absolutely independently.

I would further mention that concentrates of a pure light cod-liver oil, or a pure halibut-liver oil, give values after irradiation from 5 % of the original value down to 0. This would appear to indicate that no substances that absorb light at 328 $m\mu$ are produced during the irradiation.

Destruction of the vitamin in the undiluted oil.

A sample of cod-liver oil was left on the roof for about 6 months (April 4th to October 10th, 1934) in a full, stoppered bottle of white glass. The following results were found:

	Colour 2 cm. Y.V.	Free fatty acid g./100 g.	Kreis value R.V.	Peroxide value	B.V. 0·04 g.	$E^{10\,g./l.}_{328\,m\mu}$
Oil before irradiation	2·6	0·85	10·5	5·5	9·0	0·52
Oil after irradiation	0·3	1·00	1·4	6·3	0·5	0·027

The irradiated oil has only about 5 % of the original E value, which agrees with the blue value. This may indicate that the light has destroyed the vitamin without forming new absorbing substances, but the possibility exists that the irradiation has destroyed other absorbents.

E *for the unsaponifiable fraction of herring oil, mackerel oil and margarines.*

As a last instance of determinations of E by means of the vitameter I will mention some values for concentrates of herring and mackerel oils and margarines, in Table V. The values are calculated so as to correspond with the amount of original substance.

Table V. *Concentrates of different oils and margarines.*

Sample		$E_{328\,m\mu}$ calc. for 10g. oil/l. Gross value (initial)	After destruction	Net value difference	Blue value calc. for 0·04 g. oil ($B=b.d^{0\cdot8}$)
Herring oils:					
From fresh herrings	I	0·081	0·014	0·067	2·4
	II	0·119	0·059	0·060	2·4
Salted	Males	0·072	0·031	0·041	1·6
	Females	0·057	0·018	0·039	1·8
Herring meal		0·066	0·029	0·035	0·9
Mackerel oils:					
From frozen mackerel	I	0·173	0·072	0·101	4·0
	II	0·157	0·030	0·127	5·0
Margarines:					
Vitaminised	I	0·019	0·012	0·007	0·3
	II	0·013	0·009	0·004	0·15

The unsaponifiable fractions have been prepared in the usual way or by the rapid method mentioned earlier. E and the blue value have been determined in the concentrate, and then the solution has been irradiated until the E value was constant.

Summary.

The Hilger vitameter A has been equipped with a simple device for photographic recording of the readings, which makes it possible to determine $E_{328\,m\mu}$ of oils, concentrates and the like with considerable accuracy.

The vitameter values give good agreement with spectrographic values, both for oils and their concentrates. Determinations have been carried out on pure and crude cod-liver oils, halibut-liver oils, concentrates of herring oil and extracts of commercial vitamin A preparations.

The vitameter values agree with the blue values found. The absorption of the unsaponifiable matter of pure cod-liver oils approaches that of the oil itself; normally 85–90 % is found. The reduction in absorption may be chiefly due to unavoidable losses during the preparation of the concentrate.

Vitamin A may be destroyed by irradiation, and it is thus possible to determine the vitamin by the difference in absorption before and after exposure to strong light. This eliminates any absorption due to substances other than vitamin A.

The Hilger vitameter A equipped with a device for photographic recording has proved to be a very useful instrument for the rapid determination of $E_{328\,m\mu}$ as a measure of vitamin A, and may be expected in many cases to replace the tintometer for rapid assay of the vitamin.

The author wishes to express his sincerest thanks to H. W. Weedon for much valuable help, especially in carrying out the spectrographic determinations and translating the manuscript. Further to L. Aure, who has carried out the majority of the vitameter determinations and a considerable part of the analytical work. Several other members of the staff have also contributed interested and helpful work.

Our thanks are due to Prof. B. Helland-Hansen, of the Geophysical Institute, Bergen, for placing their spectrograph at our disposal.

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