REVIEW ARTICLE

A CRITICAL ACCOUNT OF THE SPECTROPHOTOMETRIC ESTIMATION OF VITAMIN A

By H. H. Bagnall, B.Sc., F.R.I.C., and
F. G. Stock, B.Pharm., Ph.C., A.R.I.C.

City Analyst's Laboratory, Birmingham

It has been recognised for some time that, because of the presence of variable amounts of irrelevant absorption, the value obtained for the vitamin A content by merely multiplying the extinction coefficient at 328 mμ by a factor cannot be regarded, in the case of many oils, as a correct measure of their activity. A pure, reproducible primary standard, and some means of correcting the initial spectrophotometric measurements were required before the latter could be translated accurately into "units" of vitamin A.

These conditions were fulfilled when, in August, 1949, the Expert Committee on Biological Standardisation appointed by the World Health Organisation issued a report[1] of its Sub-Committee on Fat-Soluble Vitamins, which recommends the use of crystalline vitamin A acetate as the International Standard and outlines the requirements for a suitable physico-chemical method of assay. The new factor of 1900 specified for the conversion of $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328 mμ to international units is to be used only after a suitable correction for irrelevant absorption has been made.

The spectrophotometric methods outlined in the B.P. Addendum 1951[2] and in the U.S.P. XIV[3] comply with these requirements and recognise in suitable cases the use of a geometrical correction procedure as a means of estimating that portion of the gross absorption at 328 mμ attributable to vitamin A.

The geometrical correction procedure originates in the publications of Morton and Stubbs[4,5,6] being based upon the assumed linearity of the irrelevant absorption over a narrow range on either side of $\lambda_{max.}$ and more specifically on the linearity of three points termed the fixation points; the absorption in between these chosen points may vary irregularly. The absorption curve of pure vitamin A being known, determination of the actual absorption at the three fixation points is sufficient to enable that due to vitamin A to be calculated. By subtracting the "corrected" absorption from the gross the so called "irrelevant absorption" or the absorption due to substances other than vitamin A is obtained.

Before discussing the fundamental question of the absorption curve of pure vitamin A, the attention of the reader should be called to two publications, one by Gridgeman[7] which reviews the earlier literature on the estimation of vitamin A up to about 1944, and another by Morton[8] which brings us up to the time of the introduction of the new International Standard in 1949. The 1951 edition of "Methods of Vitamin Assay"[9]

includes an extensive bibliography and discusses the U.S.P. XIV method in detail. The present year has seen the publication of further important papers, which have introduced several complications.

Over the last few years the accepted standard curve has been that published by Morton and Stubbs.[4,6] The fixation points recommended for a *cyclo*hexane solution of vitamin A acetate were 313 mμ, 328 mμ, and 338·5 mμ and $E_{313\,m\mu} = E_{338\cdot5\,m\mu} = 6/7\,E_{328\,m\mu}$. The absorption curve of vitamin A alcohol differs slightly but significantly from that of the acetate and furthermore the fixation points vary with the solvent used. Chatain and Debodard[10] have suggested further modifications and their curves indicate that their vitamin A was purer than that of Morton and Stubbs.

Cama, Collins and Morton,[11] in what is probably one of the most important publications to date, derive new standard curves for the ester and the alcohol using both synthetic and natural vitmin A, and alter the positions of the fixation points. The spectroscopic properties of all-*trans* vitamin A alcohol and acetate are fully dealt with and the data given should become the accepted standard for these substances. The new fixation points for vitamin A acetate dissolved in *cyclo*hexane are 312·5 mμ, 327·5 mμ and 337·7 mμ, and $E_{312\cdot5\,m\mu} = E_{337\cdot7\,m\mu} = 6/7\,E_{327\cdot5\,m\mu}$. These are the points specified in the B.P., Addendum, 1951, and in addition the old fixation wavelengths, together with an entirely new set with equal wavelength intervals are given, with accompanying equations for correction of the gross E value, both based on the new absorption curve. Attention is drawn to the differences in magnitude of the extinction coefficients for pure vitamin A in different solvents, these being sufficiently great to necessitate the use of different conversion factors according to the solvent used. For vitamin A ester dissolved in *cyclo*hexane the factor becomes 1920.

At this stage it will be profitable to discuss the differences between the methods of the B.P., Addendum 1951, and the U.S.P. XIV, for it is noteworthy that while the U.S.P. method specifies the saponification of all preparations, so estimating vitamin A *via* the alcohol, the B.P. requires saponification only in the case of cod-liver oil, and the determination is made on the oil itself, i.e., via the ester, in the case of halibut liver oil and dilutions of vitamin concentrates. The unqualified inclusion of all vegetable oil dilutions of vitamin A concentrates under the same heading as halibut liver oil is undoubtedly a weakness of the B.P. method, for reasons which will appear shortly.

Vitamin A concentrates may be prepared by a number of methods:— (i) saponification followed by molecular distillation to give the alcohol; (ii) treatment of this product with a fatty acid to give the ester; (iii) direct molecular distillation of the vitamin A esters; (iv) the Solexol process for the production of ester concentrates (see Stubbs,[12] Neale-May[13] and Hilditch[14]) and (v) synthetic preparations.

One of the largest manufacturing houses in this country prepares a concentrate by method (i), i.e., vitamin A alcohol, which is sold as such. Now if this concentrate were utilised to prepare "Concentrated Solution of Vitamin A, Solution of Vitamin A and D and products of similar

properties" and the correction equation specified under that heading used to estimate the vitamin A, erroneous figures would be obtained. Before attempting, therefore, to apply a geometrical correction procedure in the case of a diluted concentrate of unknown origin, it is important to know whether the vitamin is present in the form of alcohol or ester.

There are several ways of determining the relative proportions of alcohol and ester in a preparation and these are discussed by Kascher and Baxter,[15] who recommend the determination of the distribution between light petroleum and aqueous ethanol. The relative stability of vitamin preparations derived from ester and alcohol concentrates has incidentally been fully discussed by Lindholm and Terp,[16,17,18,19] and they show that the ester is without doubt far superior to the alcohol form in this respect. It is therefore rather surprising that the alcohol should still be commercially available for use in pharmaceutical preparations.

On account of this instability, vitamin A alcohol is seldom encountered in pharmaceutical preparations in the U.S.A., and the manufacturers offer a fair proportion of their concentrates in the form of the acetate. Synthetic vitamin A concentrates are also available in the same form. This complicates vitamin A analysis because vitamin A acetate has properties in between those of the alcohol and the esters of the higher fatty acids. Spectrophotometrically, however, the acetate behaves like the other esters.[20] It is on account of the possibility that vitamin A alcohol might be encountered in pharmaceutical products, some of which are of necessity saponified during the assay as part of the purification process, that the U.S.P. XIV has required saponification of all samples.

The U.S.P. XIV differs from the B.P. Addendum 1951 in using *iso*propanol as solvent, instead of *cyclo*hexane, and it also includes an identity colour test with antimony trichloride, the vitamin A being estimated from a standard curve prepared by the use of the saponified International Standard. The ratio between the values for vitamin A obtained from the colour test and the ultra-violet absorption test should be between 1·00 and 1·30. Most good quality fish oils and their concentrates give a ratio of about 1·15, the systematic discrepancy between the two methods apparently being due to the absence of the so-called neovitamin A from the standard. An allowance of $\pm 0{\cdot}15$ from the mean represents the variability inherent in the two methods.[21] Though less precise than ultra-violet absorption methods, blue colour assays are more specific and more sensitive, and for these reasons have wider applicability. The reproducibility of the method is from 3 to 10 per cent., depending upon the product tested.[9]

Some of the less satisfactory features of the U.S.P. XIV method are discussed by Cama *et al.*[11] For example, the conversion factor for vitamin A acetate in *cyclo*hexane is 1920 and for the alcohol in the same solvent 1910, but for the corresponding *iso*propanol solutions they are 1895 and 1820 respectively. The $E_{1\text{ cm.}}^{1\text{ per cent.}}$ $\lambda_{max.}$ value of the International Standard Preparation using *iso*propanol as solvent would be 5·28, and that of an equivalent solution of vitamin A alcohol 5·49. If this rise in the E value is ignored a solution actually containing 9620

I.U./g. would be accepted as containing 10,000 I.U./g. Cama *et al.*[11] further state that, although saponification in many cases removes most of the irrelevant absorption, with high potency material the process is much less effective, and stress that the more elaborate the chemical manipulation the greater will be the risk of loss. The correction equations can be applied much more successfully to untreated oils in *cyclo*hexane than to their unsaponifiable fractions. Moreover *cyclo*hexane is a more suitable solvent than *iso*propanol because the difference between the spectra of vitamin A and neovitamin A is least in the hydrocarbon solutions. Application of the correction procedure to the unsaponifiable fraction leads to low results, the irrelevant absorption is not strictly linear and there is reason to suspect that isomerisation takes place during saponification. Accordingly Cama *et al.*[11] recommend the use of unsaponifiable extracts for low potency oils of good quality which should then give a "normal" absorption curve, the E value of which needs to be corrected only for vitamin A_2. The "corrected" figure on the oil direct will agree closely if adjusted for the presence of neovitamin A. This implies a criticism of the application of correction procedures to cod-liver oil after saponification. For higher potency oils they advise that two or more of the correction formulae should be used. The results, when adjusted for the presence of neovitamin A, will be practically the same as those resulting from the most laborious elimination one by one of interfering substances.

The presence of neovitamin A, mentioned above, further complicates the problem. This isomer of vitamin A, discovered by Robeson and Baxter,[22] differs from vitamin A itself only in the spatial configuration about the double bond nearest the hydroxyl group. Its estimation is based on the more rapid formation of an adduct of maleic anhydride with all-*trans* vitamin A than with neovitamin A and the subsequent use of antimony trichloride; the all-*trans* isomer gives very little colour after this treatment.

Robeson and Baxter[22] report that neovitamin A occurs to the extent of 30 to 40 per cent. of the total vitamin A in a large number of oils examined by them. Meunier and Jouanneteau[23] give figures for the neovitamin A content of only 8 oils, which range from 18 to 65 per cent.; 5 of these vary only from 40 to 50 per cent. Figures of 55 per cent. and 65 per cent. were obtained on a tunny fish liver oil, the former on the oil direct and the latter on the unsaponifiable fraction of the same oil.

Cawley, Robeson, Weisler, Shantz, Embree and Baxter,[24] announcing the commercial synthesis of vitamin A, showed that the synthetic concentrates contain neovitamin A. Since there are a number of possible geometrical isomers of vitamin A it is of interest to note that only the naturally-occurring forms actually resulted. Assays of two synthetic concentrates by the maleic anhydride method indicated that the proportions of vitamin A and neovitamin A present were 1·5 : 1 and 2 : 1. These ratios closely approximate to those earlier reported for fish liver oils. The authors suggested that vitamin A either *in vivo* or *in vitro* is converted, in part, by catalytic agents into neovitamin A, and that therefore the occurrence of neovitamin A in fish liver oils is not necessarily indicative of any

peculiar requirements of the fish for this isomer. Instead, it appears that "vitamin A," physiologically speaking, must be considered as a mixture of the two geometrical isomers. Schwarzkopf, Cahnmann, Lewis, Swidinsky and Wüest[25] in their synthesis of vitamin A reported 36·9 to 40·9 per cent. of neovitamin in their products.

Dalvi and Morton[26] state that the absorption curves for neovitamin A esters closely resemble those of the all-*trans* isomer, except that on the long wave side of 310 mμ they are displaced by 2 to 3 mμ. They state that their experience shows that many natural products contain about 25 per cent. of the total vitamin A in the neo-form, and if this is confirmed it seems likely that for ester concentrates "over correction" need not reach 5 per cent. If for example, the fixation points appropriate to all-*trans* vitamin A acetate be used to "correct" the curve for 100 per cent. neovitamin A esters the result would be about 20 per cent. too low, using *iso*propanol as solvent, and 14 per cent. too low using *cyclo*hexane.

Until very recently it had been assumed that biologically neovitamin A and vitamin A were equivalent. Harris, Ames and Brinkman,[27] however, discussing the biopotency of neovitamin A in the rat, say that repeated bioassays of the two vitamin A isomers in pure form, both free and esterified, have indicated a significant difference in their biological potencies of the order of 20 to 28 per cent., the all-*trans* form having the higher activity. This is in line with recent researches on the carotenoids, the change from a *trans* to a *cis* configuration resulting in a decrease of physiological activity ranging from 25 to 75 per cent. The conversion factor, therefore, of a product containing 1 part of neovitamin A to 2 parts of all-*trans* vitamin A, would be about 5 per cent. to 8 per cent. lower than the conversion factor of a similar preparation of all-*trans* vitamin A. On this basis the correction procedure in the spectrophotometric estimation discounts neovitamin A to about the same extent as does the rat bioassay. More work needs to be done, involving large scale inter-laboratory assays using many more animals, before this statement can be universally accepted. It does, however, indicate that there is a distinct probability that neovitamin A is not so potent biologically as all-*trans* vitamin A.

The question of the so-called "irrelevant" absorption, i.e., that exhibited by other constituents of oils, apart from vitamin A, can now be considered. The principal contributions to this are made by vitamin A_2, kitol, anhydrovitamin(s) A and, most important from the point of view of the public analyst, oxidation products of the vitamin itself. The properties of the first three will be briefly considered.

Vitamin A_2 is present to a greater or lesser extent in most fish liver oils. Its presence gives rise to some distortion of the spectral absorption curves resulting in a "shoulder" effect on the long wave side of 328 mμ. Vitamin A_2 cannot be separated by chromatography but the absorption curve of the blue colour formed with antimony trichloride has a maximum at 693 mμ, which can be used for its estimation. The ultra-violet absorption at 328 mμ due to vitamin A_2 can then be calculated and allowed for. It is eliminated by the correction procedure as "irrelevant" absorption. Shantz[28] reported the isolation of pure vitamin A_2 and gave an account of

its spectroscopic characteristics. Recently Shantz and Brinkman[29] determined its biological activity and concluded that it has approximately 40 per cent. of the activity of crystalline vitamin A as determined by the U.S.P. rat growth procedure. It is stored in the liver less effectively than is vitamin A but does not appear to be converted to vitamin A *in vivo*. The B.P. Addendum 1951 states that the simple form of correction procedure may be affected by the presence of vitamin A_2 in unusual amounts. An indication of its presence is given by its maximum at 351 mμ in the subtraction curve; the amount is usually small and may be neglected, but oils containing abnormal amounts are occasionally met with and care should then be taken. The analyst may in this case be well advised to estimate it and allow for its biological activity, and Cama *et al.*[11] have suggested that the determination of vitamin A_2 may well have to be undertaken as a routine estimation.

Kitol is a major constituent of whale oil and its separation by chromatographic methods is necessary before the vitamin A content of an oil containing it can be estimated. The B.P. monograph on halibut liver oil specifies that the absorption at 300 mμ must not be greater than 75 per cent. of that at 328 mμ (absence of whale-liver oil). It should be noted that in a badly oxidised oil the absorption at 300 mμ may well exceed 75 per cent. of that at 328 mμ even in the absence of whale-liver oil. Gridgeman, Gibson and Savage[30] described a method for the estimation of vitamin A in whale-liver oil, involving a chromatographic separation after saponification, while Barua and Morton[31] preferred to work on the original oil. Kitol is not normally encountered in halibut-liver oil and the two references mentioned are possible methods to follow if a chromatographic method is indicated for reasons other than the presence of kitol.

Anhydrovitamin A_1 is characterised by its three maxima at 351, 371 and 392 mμ. The subsidiary band at 392 mμ is always well defined, but the maximum at 351 mμ is in some samples a pronounced peak and in others only a sharp inflexion. If present to any great extent it causes a "shoulder" effect on the long wave side of 328 mμ but is readily identified by its 392 mμ maximum. It is not removed by saponification and is eliminated by the correction procedure. It is biologically practically inactive.[32]

The vitamin A content of an oil decreases on storage, and this fact is reflected in a corresponding decrease in the gross $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 328 mμ and an increase in absorption on the short wave side of $\lambda_{max.}$ It is believed that vitamin A epoxide is formed, having an absorption maximum in the 270 to 280 mμ region. An interesting feature of the oxidative process, which may more clearly be demonstrated by its artificial acceleration, is the appearance of a so called isosbestic point, or point of constant absorption, at about 290 mμ. Bolomey[33] has already called attention to this phenomenon, which in our opinion might conceivably be used to give an approximate idea of the original vitamin A in an oxidised oil. In some cases in our experience, it would have been extremely interesting to be able to say whether or not the amount of vitamin declared had ever been

present in an oil before oxidation. As oxidation proceeds, vitamin A epoxide in its turn decomposes and at the same time the isosbestic point becomes indefinite.

A rough indication of the quality of an oil is given by the "persistence," defined as the difference between $\lambda_{max.}$ and. $\lambda_{min.}$ on the short wave side. As the irrelevant absorption due to oxidation increases, the persistence decreases, and since most of the oils found by a public analyst to be deficient in vitamin A also show evidence of oxidation, it follows that the possibility of accurate analysis is least when it is most important that it should be greatest. This fact must be borne in mind when the respective merits of the two alternative methods of calculation of vitamin A in high potency oils ($E^{1\%}_{1\,cm.}$ 328 mμ greater than 4·0), viz.: gross $E^{1\%}_{1\,cm.}$ 328 mμ × 1600 and corrected $E^{1\%}_{1\,cm.}$ 328 mμ × 1900, are considered.

It has been shown empirically that, within the limits of error of the animal experiments, $E^{1\%}_{1\,cm.}$ 328 mμ × 1600 gives a figure for potency which agrees with the biological value, and Morton[8] states that subsequent work has shown that no better factor for fish-liver oils in general could even to-day be chosen for converting gross $E^{1\%}_{1\,cm.}$ values to international units, although oils showing little irrelevant absorption would be somewhat undervalued and oils exhibiting more irrelevant absorption would be a little over-valued.

The points of view of the manufacturers and the public analyst on this question of potency are essentially different. The former deal with great numbers of oils which are normally fresh and unoxidised, and the gross $E^{1\%}_{1\,cm.}$ 328 mμ × 1600 will *on average* give the same results as the corrected $E^{1\%}_{1\,cm.}$ 328 mμ × 1900. They will therefore not need to introduce the question of correction of the gross $E^{1\%}_{1\,cm.}$ 328 mμ at all. On the other hand, a large number of the samples submitted to a public analyst under the Food and Drugs Act will have deteriorated owing to oxidation of the vitamin and gross $E^{1\%}_{1\,cm.}$ 328 mμ = 1600 will therefore give a distorted picture, and cannot be recommended for checking label claims, or for testing the compliance of a sample with B.P. requirements. Furthermore, a public analyst has to give a reasonably accurate opinion on a single sample and this would be difficult, not to say impossible, without the use of some sort of correction procedure or separation.

We have discussed this question with a number of manufacturers and find that they prefer to use the gross $E^{1\%}_{1\,cm.}$ 328 mμ × 1600, partly because of its simplicity and partly on account of the controversy which has arisen over geometrical correction procedures in connection with the basic assumption of the effective linearity of the "irrelevant" absorption curve over the critical range, not to mention the further complications which have been discussed above. In any case, a manufacturer dealing with fresh oils would, taking into account the large quantity passing through his hands, arrive at approximately the same figure for the total vitamin content expressed in international units in the whole bulk whichever method of calculation he used. The consumer, however, with whose interests the public analyst is vitally concerned, purchases a small quantity

of oil, the potency of which calculated from gross $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 328 mμ × 1600 might be either greater or less than the actual value.

The problem is to ascertain whether the correction procedure introduces a larger error or greater uncertainty than is implicit in the multiplication of gross $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 328 mμ by an arbitrary factor, and on balance the present position seems to be that if the new official B.P. Addendum 1951 process using a geometrical correction procedure can be shown to give the same result, within reasonable limits, when carried out on the same oil in different laboratories, then it will become acceptable. If, however, an operator is thinking in terms of a possible variation of ±15 per cent.[34] he will probably conclude that the method is not worth the trouble. On the other hand the public analyst has need of a method which, in the case of a partially oxidised sample, would not necessitate multiplying absorption due to oxidised vitamin by 1600 and returning the quotient as units of vitamin A, but rather would enable him to arrive at a reasonably accurate figure for the vitamin content, which can be compared with the claim made on the label, and with the requirements of the B.P.

As has already been mentioned, the validity of geometrical correction procedures depends on the assumption that the irrelevant absorption at the fixation points is linear, and the controversy over this question arises on account of the fact that this assumption cannot be tested experimentally. Gridgeman,[35] in a very recent publication, discusses the theory of the method, and deals fully with the effect on the reliability of the method of departures from the basic assumptions. It is unfortunate in some respects that the new knowledge made available by Cama *et al.*[11] in their recent publication, and now used as the basis of the method given in the B.P. Addendum 1951 was not accessible when this paper was published. Gridgeman,[35] however, deals with the method as outlined by Morton and Stubbs in 1946-8 and although Cama *et al.*[11] put forward a number of alternative equations and the spectroscopic properties of all-*trans* vitamin A acetate and alcohol given in their paper became standard data, the basic theory of the method remains unaltered. Gridgeman[35] discusses possible errors under the headings of "observational" and "assumptive." Dealing with observational errors, he states that a result which is a function of three *E* values will clearly have wider limits of error than a result depending on one, proceeds to a statistical analysis of the problem, and concludes that for every 1 per cent. of observational error in a gross reading, an error of the order of 8 to 10 per cent. is to be expected in a corrected reading. Assumptive errors are defined as those introduced by departures from the assumed shapes and positions of the two absorption curves, those of vitamin A and the irrelevant material. The departures from the assumed characteristics of the vitamin A curve are discussed under "displacement" and "distortion." The displacement may be artificial due to maladjustment of the wavelength scale or, on the other hand, the natural ester may be slightly different chemically or stereoisometrically, and therefore spectrophotometrically, from pure all-*trans* vitamin A acetate. In our own experience, the wavelength scale can easily get out of adjustment and it is important frequently to check the position of the 4861 Å hydrogen line.

Gridgeman[35] finds that displacement of 1 mμ to the longer wavelengths results in a corrected *E* value 4 per cent. above the true value, while displacement to the shorter wavelengths is not so serious, as 1 mμ implies a corrected *E* value just over 1 per cent. less than the truth. The question of stereoisometric differences and the natural occurrence of neovitamin A have now been dealt with by Dalvi and Morton[26] and can be allowed for. Distortion effects would be produced by errors in engraving on the wavelength scale and we are of course entirely in the makers' hands in this respect, although some check can be made by the use of the mercury lines. The bulk of the evidence on this point, however, indicates it to be very unlikely that the wavelength at any particular point on the scale of a modern photoelectric instrument over the range 310 to 340 mμ will be more than ±0·1 mμ in error. Gibson and Balcom[36] of the U.S.A. National Bureau of Standards publish data on the Beckman instrument and state that the errors are so small that they may probably be neglected in most of the work for which the instrument is used, and can moreover be determined only by the most careful work and after installing an auxiliary indicator line over the wavelength scale to eliminate parallax.

The question as to whether the irrelevant absorption over the critical range is effectively linear can still not be decided, but the effect of possible departures from linearity may be minimised by the use of a number of correction equations instead of only one. Gridgeman[35] discusses this possibility and Cama *et al.*,[11] as mentioned above, deduce a number of equations.

In a consideration of the mechanism of the correction, Gridgeman[35] compares the irrelevant absorption curves obtained from a Solexol concentrate and a halibut-liver oil, and notes the similarity in their general shape. He says that this seems surprising since one is a natural oil and the other a processed concentrate and goes on to discuss the possibility of some degree of error in the assumption made in the correction method, that the absorption curves of the vitamin A fatty acid esters in these oils are identical with the published vitamin A acetate curve. The consensus of opinion seems to be that variations in the fatty acid portion of the molecule will not materially alter the absorption curve. Saponification would avoid any possibility of error in this respect but would introduce disadvantages which have already been discussed. Stereoisomerism causes bigger differences, and the effect of neovitamin A and the method of making allowance for it were described by Dalvi and Morton[26] as mentioned before. Although vitamin A has 4 possible geometric isomers only 2 have been reported as occurring naturally, and it seems highly significant that it is these 2 only that are present in the synthetic vitamin, and furthermore in approximately the same proportions as in the naturally occurring vitamin. Gridgeman[35] concludes that the reliability of the method is *sub judice* and its use requires caution. We should not like to comment on this statement beyond expressing our opinion that the reliability is indeed closely linked with the caution mentioned in the latter half of the statement. Cama *et al.*[11] mention that the simple correction procedure of Morton and Stubbs (1946-1948) has been very widely used,

sometimes with less caution than is required by the plainly stated assumptions on which it rests.

Adamson, Elvidge, Gridgeman, Hopkins, Stuckey and Taylor[34] discuss the precision of the three point correction method. 7 laboratories assayed each of 5 vitamin A oils, readings being made in duplicate on photoelectric instruments. The gross $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 328 mμ values were geometrically corrected for irrelevant absorption, and the conclusion arrived at from a statistical analysis of the results was that the limits of error of a determination of vitamin A content in duplicate by any one of the 7 laboratories were about ± 15 per cent. for $P = 0{\cdot}05$, the corresponding figures for gross E values being ± 2 per cent. As the authors state that the paper is concerned with the reproducibility of the method, the apparent lack of precision implied by this conclusion is, from the public analyst's point of view, somewhat disconcerting, since any statement of vitamin A content which he gives on his official certificate is open to challenge by the defendant's analyst.

Some doubt, however, may be felt about the value of this inter-laboratory test from the point of view of assessing the *possible* precision of the method, if we consider some of the material that was put into the statistical machine in relation to the meaning or value to be attached to what emerges from it. As a practical example of material of very doubtful value, may be mentioned a sample of halibut-liver oil which was submitted to each of the 7 laboratories, the average percentage of irrelevant absorption of which was found to be 10·7 per cent. The figure submitted by one of the laboratories, however, was *minus* 4 per cent., and we cannot help feeling that any results obtained by a laboratory which returns such a figure for an oil which may, on the available evidence, be assumed to show about 10 per cent. irrelevant absorption, would be somewhat unreliable. In any case the inclusion of a nonsense result is unjustified since in practice no conclusions would be drawn from such a result. Possibly insufficient attention was paid to the variability of the results from the laboratory furnishing the nonsense result and from one other. While a precise statement cannot be made in the absence of the original data, it would seem that an analysis based on the more consistent results of the 5 other laboratories might yield a precision estimate significantly lower than that given. As an experiment carried out by a number of laboratories in order to discover *what would happen* if particular oils were analysed by the use of the Morton and Stubbs (1946-48) correction procedure for their vitamin A content, the test is fair enough, but the ± 15 per cent. limits of error in no way indicate the ultimate precision attainable by the use of geometrical correction procedures, since at this stage laboratories differ widely in care, expertise and experience. What in fact the paper of Adamson *et al.* does strongly suggest is the need for certain laboratories to re-examine their methods, with particular attention given to rigid standardisation of technique. In this connection it may be mentioned that the use of three different correction equations as suggested by Cama *et al.*[11] and an increase in the number of replicates would materially add to the accuracy of the estimation and would in fact of itself reduce the limits of error.

The calibration of the spectrophotometer is a necessary basis for all reliable work, and its importance cannot be over emphasised; greater attention paid to this side of the picture will result eventually in errors being reduced to a minimum and the attainment of far better agreement between laboratories. The discussion of results obtained using geometrical correction procedures should have particular reference to the precautions which a laboratory has taken to ensure accuracy.

Gibson and Balcom[36] give an excellent account of the Beckman instrument and the precautions to be taken to attain results of high reliability. Rawlins and Wait[37] also discuss factors affecting its reliability and precision and conclude that by careful observance of all the variations the determination of the *E* value may be made with considerable precision, in their case, for example, with a standard deviation of 0·22 per cent. They suggest the use of control charts for laboratories doing large numbers of vitamin A determinations, which not only serve as a check on the technique of the operator but are invaluable in detecting incipient trouble in the equipment.

The best known and most popular solutes in use for calibration purposes are potassium chromate and dichromate. The United State Department of Commerce National Bureau of Standards, however, discusses methods of checking the calibration of spectrophotometers in Letter Circular L.C.929[38] and the reader is alse referred to Mellon's[39] very useful book which contains much valuable information.

The reproducibility in any one laboratory can be made to approach the $\pm$0·4 per cent. mentioned in the B.P. Addendum 1951 by giving careful attention to all the relevant factors. The trouble begins with inter-laboratory assays, and the results obtained by the Photoelectric Spectrometry Group,[40] using 28 Beckman instruments on two solutions of potassium nitrate, make rather startling reading, but it does seem that with improved techniques the variation can be reduced. Preliminary reports[41] of the second P.S.G. collaboration test, using potassium dichromate, reveals slightly lower errors than in the first test, but the position is still far from satisfactory. For vitamin A work it is advisable that the instrument be checked against the International Standard Preparation of vitamin A acetate.

The results obtained by us on our Unicam instrument are given in Table I.

These results were obtained without previous knowledge of the figures of Cama *et al.*,[11] which were not available at the time. The International Standard Preparation, using the diluent oil as compensator and *cyclo*hexane as solvent would be expected to have an $E^{1\,\text{per cent.}}_{1\,\text{cm.}}$ 328 mμ value of 5·21 ($\times$ 1920 = 10,000 I.U./g.). Three determinations were made, using separate weighings from different capsules and, rather than assume that each capsule contained exactly 0·250 g. of material, we weighed a known amount from each capsule and compensated with the same concentration of diluent oil. The results were 5·104, 5·099 and 5·112, having a mean of 5·11 which agrees closely with the value of 5·09 on a weighed amount, obtained by Cama *et al.*[11] The International Standard Preparation

TABLE I

	Vitamin A acetate in *cyclo*hexane (Cama, Collins and Morton, 1951)	International Standard Preparation Compensator: cotton seed oil Solvent: *cyclo*hexane	
λ mμ	$\frac{E\lambda}{E\lambda_{max.}}$	$\frac{E\lambda}{E\lambda_{max.}}$	
295	0·448	0·447	
300	0·555	0·555	
305	0·670	0·667	
310	0·806	0·806	
311	0·830	0·829	
312	0·846	0·846	
312·5		0·857	
313	0·867	0·867	
315	0·894	0·890	
317·5		0·916	
320	0·935	0·937	
322·5		0·965	
325	0·985	0·988	
326	0·993	0·995	
327	1·000	0·998	
328	1·000	1·000	
330	0·989	0·991	
335	0·915	0·914	
338	0·853	0·857	
338·5		0·843	
340	0·811	0·814	
345	0·695	0·700	
350	0·556	0·562	

according to these figures is apparently 2 per cent. deficient in activity. There is certainly something abnormal about it, because continuation of the absorption curve over the short wave side of 300 mμ shows a very marked departure from the Standard vitamin A acetate curve. In these circumstances the International Standard cannot be considered as an absolute reference standard for spectroscopic purposes and it would not be feasible to consider the use of a factor to raise the apparent 98 per cent. to 100 per cent. Nevertheless the Standard over the range 300 to 340 mμ is very useful as a means of checking the ratio $E\lambda/E_{max.}$ at a particular wavelength and the values obtained agree very closely with the figures of Cama *et al.*[11] for pure all-*trans* vitamin A acetate.

In our view the spectroscopic procedure given in the B.P. Addendum 1951 is not to be regarded as an absolute standard method, but rather as indicating the broad principles which must be followed. One thing is certain—the analyst must allow for irrelevant absorption before using the 1900 factor; whether he does this by geometrical correction or by a separation method is left to his own particular fancy. In point of fact the onus is now upon the analyst to ascertain the true vitamin A content by using the method appropriate to the sample in hand, for each one may exhibit its own peculiar problems and must be treated on its own merits.

We believe the method has actually been indirectly official for some time. The B.P. 1948 stated that "an expression of the content of vitamin A . . . in Units per g. is obtained by multiplying the *ultra-violet absorption* $E_{1\,cm}^{1\ per\ cent.}$ by the factor declared by the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations as the factor to be used for this purpose." Since the World Health Organisation adopted the 1900 factor in August, 1949, it indirectly became official in the B.P. from that date, conditions under which the

factor is applicable and an indication of how, in principle, irrelevant absorption may be allowed for, including the use of geometrical correction procedures, being embodied in the report of the W.H.O.

For the estimation of vitamin A in halibut-liver oil and vegetable oil dilutions of concentrates we have followed the B.P. Addendum 1951 method with the following modifications, viz., all 3 correction equations recommended by Cama *et al.*[11] were used with each of 3 separate weights of oil dissolved in *cyclo*hexane so obtaining 9 "corrected" values for $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 327·5 mμ. The use of more than one set of fixation points goes some way to meet Gridgeman's criticism that the irrelevant absorption may not be strictly linear, for departures from linearity will be reflected in an increase in the fiducial limits of the mean, and so the effect will be minimised to some extent. The P = 0·05 fiducial limits of the mean of the 9 values obtained for $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 327·5 mμ were then calculated.

We found the reproducibility of a gross *E* value on our Unicam instrument to be about $\pm$0·5 per cent. and the P = 0·05 fiducial limits of the mean of 9 corrected *E* values in the region of $\pm$1 to 2 per cent.

The correction procedure eliminates the effects of irrelevant absorption and the resulting corrected *E* value only needs to be adjusted for neovitamin A by the use of a correction factor of 1·04. It is not possible to be dogmatic about this factor, but it has a reasonably factual basis. If, of course, it is subsequently shown, as seems possible, that neovitamin A is less potent biologically than vitamin A, the correction would no longer be necessary. Since we used *cyclo*hexane as solvent the conversion factor of 1920 was employed and our figures are therefore about 5 per cent. higher than those obtained by the strict use of the B.P. Addendum 1951 method, but we feel that we should in fairness to manufacturers give them every possible advantage. On the other hand, they will probably find it advisable to use the B.P. Addendum 1951 procedure, secure in the knowledge that they have a possible additional overage in reserve.

It should be noted as a matter of strict accuracy, that vitamin A_2 has some biological activity, but that its effect on the *E* value is eliminated by the use of the method described. If therefore it be present in abnormal amounts it should be estimated and its equivalent in terms of vitamin A determined and included in the final figure.

Finally we should like to express our thanks to Professor R. A. Morton, F.R.S., and his colleagues at Liverpool University, for their kindness in allowing us access to the recent paper by H. R. Cama, F. D. Collins and R. A. Morton on all-*trans* vitamin A and another by P. D. Dalvi and R. A. Morton on neovitamin A, in both cases prior to publication.

References

1. *World Hlth. Org. techn. Rep. Ser.*, 1950, 3.
2. *Addendum* 1951 *to the British Pharmacopœia*, 1948, *Appendix XVA*, pp. 92–93.
3. *United States Pharmacopœia XIV*, Vitamin A Assay—Spectrophotometric Method, p. 784.
4. Morton and Stubbs, *Analyst*, 1946, **71**, 348.
5. Morton and Stubbs, *Biochem. J.*, 1947, **41**, 525.
6. Morton and Stubbs, *ibid.*, 1948, **42**, 195.
7. Gridgeman, *The Estimation of Vitamin A*, Lever Bros., and Unilever Ltd., 1945.

8. Morton, *J. Pharm. Pharmacol.*, 1950, **2**, 129.
9. The Association of Vitamin Chemists, Inc., *Methods of Vitamin Assay*, Interscience Publishers, Inc., New York, 1951.
10. Chatain and Debodard, *C.R. Acad. Sci., Paris*, 1950, **231**, 1102.
11. Cama, Collins and Morton, *Biochem. J.*, 1951, **50**, 48.
12. Stubbs, *The Propane Segregation of Glyceride Oils, South African Industrial Chemist*, January, 1951.
13. Neale-May, *Production and Processing of Marine Drying Oils, ibid.*, August, 1950.
14. Hilditch, *Industrial Chemistry of Fats and Waxes*, Ballière Tindall and Cox, London, 1949.
15. Kascher and Baxter, *Ind. Engng. Chem., Anal. Ed.*, 1945, **17**, 499.
16. Terp, *Arch. Pharm. Chem.*, 1948, **55**, 513.
17. Nilou, Lindholm and Terp., *ibid.*, 1950, **57**, 332.
18. Lindholm and Terp, *ibid.*, 1950, **57**, 480.
19. Lindholm and Terp, *ibid.*, 1950, **57**, 661.
20. Lehman, *private communication.*
21. "*U.S.P. XIV and the Vitamin A Business*," *Distillation Products Industries N.Y.*, December, 1950.
22. Robeson and Baxter, *J. Amer. chem. Soc.*, 1947, **69**, 136.
23. Meunier and Jouanneteau, *Bull. Soc. chim. biol. Paris*, 1948, **30**, 260.
24. Cawley, Robeson, Weisler, Shantz, Embree and Baxter, *Science*, 1948, **107**, 346.
25. Schwarzkopf, Cahnmann, Lewis, Swidinsky and Wüest, *Helv. chim. Acta.*, 1949, **32**, 443.
26. Dalvi and Morton, *Biochem. J.*, 1951, **50**, 43.
27. Harris, Ames and Brinkman, *J. Amer. chem. Soc.*, 1951, **73**, 1252.
28. Shantz, *Science*, 1948, **108**, 417.
29. Shantz and Brinkman, *J. biol. Chem.*, 1950, **183**, 467.
30. Gridgeman, Savage and Gibson, *Analyst*, 1948, **73**, 662.
31. Barua and Morton, *Biochem. J.*, 1949, **45**, 308.
32. Shantz, Cawley and Embree, *J. Amer. chem. Soc.*, 1943, **65**, 901.
33. Bolomey, *J. biol. Chem.*, 1947, **169**, 331.
34. Adamson, Elvidge, Gridgeman, Hopkins, Stuckey and Taylor, *Analyst*, 1951, **76**, 445.
35. Gridgeman, *ibid.*, 1951, **76**, 449.
36. Gibson and Balcom, *J. opt. Soc. Amer.*, 1947, **37**, 593.
37. Rawlings and Wait, *Oil and Soap*, 1946, **23**, 83.
38. *U.S. Dept of Commerce, National Bureau of Standards, Letter Circular*, LC929, November 26, 1948.
39. Mellon, "*Analytical Absorption Spectroscopy*," John Wiley & Sons, Inc., New York, 1950.
40. *Photoelectric Spectrometry Group Bulletin*, No. 1, April, 1949.
41. *Ibid.*, No. 3, October, 1950.