Corrections for Background in Spectrophotometry Using Difference-in-Absorbance Values. Application to Vitamin A

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▶A difference between two spectrophotometric absorbance values on a spectral curve designated as ΔA may be used to estimate the concentration of a compound, such as vitamin A, and to detect the presence of isomers and degradation products of vitamin A or other substances that absorb light at the test wavelengths. Furthermore, such an estimate of concentration may also be employed to expedite a subsequent more accurate spectrophotometric neutralization evaluation. This procedure is described, and results of its application to vitamin A-containing pharmaceuticals are given. The advantages of such a technique are not only that a molecular change may be detected, but more important, tedious and time-consuming purifications may not be required for adequate quantitative determination of a light-absorbing compound such as vitamin A.

ONE OF THE CHIEF PROBLEMS in analyzing complex systems by spectrophotometry is the interference of background absorption. A number of investigators have devised various techniques to eliminate or compensate for this interference.

In 1946, Morton and Stubbs, as well as others (*4*, *15–17*), reported comprehensive studies on correction factors as applied to assay of vitamin A. The Morton and Stubbs correction, when applied to certain sources of complex vitamin A, may lead to erratic results because such a correction depends upon the assumption of a linear background, which may not exist. This limitation is now generally recognized.

Another approach to the general problem of background absorption is the graphically or mathematically elaborated base line technique (*2*, *3*, *7*, *11*, *14*, *20*, *25*). Davidow and Woodard (*5*) and Rotondaro (*18*) have provided excellent practical examples of this kind of correction. Most workers who apply this technique in practice apparently position the baseline in a somewhat intuitive or empirical fashion. Although such results are on a tenuous basis, they appear to be valid enough for practical purposes.

In reality, certain baseline applications are essentially derived from values of ΔA, that is, the difference between any two absorbance values on the absorption curve of a compound. The baseline values can be considered as particular ΔA values extending from a more or less arbitrary baseline to another point at the peak of the curve. Thus the baseline technique can give valid results independently on a particular placement of the baseline itself.

Allen and Rieman (*1*) developed a more elaborate mathematical technique which combined baseline and multicomponent calculation and introduced original mathematical innovations. However, their complex procedure has a somewhat limited range of application.

In still another approach to the problem of background interference, Jones, Clark, and Harrow (*11*, *12*) developed a technique based on a variable reference or spectrophotometric neutralization in which the concentration of the reference (known) solution is varied during the determination until the absorption of the compound in the reference solution and that of the same compound in the unknown solution are equalized. The concentration of the compound of reference in the unknown solution is then identical with that of the compound in the reference (known) solution. This technique has been limited in its practical application because special equipment is required for the continual balancing of the samples being determined, which involves mechanically pumping and altering the strength of a solution of the pure compound through a comparison reference cell. In addition, the apparatus for use with ultraviolet spectrophotometry requires all-glass or quartz components.

After the development of a simple, practical spectrophotometric neutralization apparatus in these laboratories (*24*), we were able to use this technique in combination with horizontal corrections calculated from ΔA values for more accurate determinations. The scope and utility of the combined method have been demonstrated by analyses of vitamin A from a variety of sources. Throughout this paper, the work on vitamin A will serve as the chief example of the advantages and limitations of this technique.

ORIGINS AND RELATIONSHIP OF ΔA TO RELATIVE ABSORBANCE

Ordinary spectrophotometry for pure compounds is based upon the simplest application of Beer's law, namely, that the absorbance (usually at the peak) is proportional to the concentration. The present approach is based upon some simple, almost axiomatic derivations from Beer's law. Figure 1 shows the absorbance relationship of different concentrations of pure vitamin A. The lower concentrations represent, respectively, 10 and 50% of the highest concentration. Each of these curves has a peak as well as a substantially straight line section.

The use of the ΔA value as shown in Figure 1 provides an automatic horizontal background correction because the horizontal component of the background is equally incorporated into any absorbance value on the curve. Hence, the subtraction of any absorbance value from another eliminates such a horizontal component from the difference.

In a family of curves, as shown in Figure 1, it is obvious that the curve segment between any two wavelengths increases as the peak height increases. It can also be demonstrated that the increment of absorption, $\Delta A^{\lambda 1}_{\lambda 2}$, proportionately increases with increase in peak height. This may be mathematically stated as $\Delta A^{\lambda 1}_{\lambda 2} \cdot K = A_{peak}$.

This is a general equation—i.e., it holds for any $\Delta A^{\lambda 1}_{\lambda 2} \cdot K$ (K, of course, will vary for each ΔA) and has many practical applications.

A further simple but important consequence of Beer's law and the characteristic properties of ΔA values is that the ratio of any two ΔA values on the same curve is independent of the concentration and is a constant for a solution of any pure compound that absorbs light.

Such a ratio will be referred to as a purity index, since variations from the constant indicate the extent of an im-

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Table I. Survey Series Showing Differences in Characteristics of Sample Type

Sample identification	Symbol identity	Relative A_{310}	Relative A_{325}	Relative $A_{332.5}$	Relative A_{334}	Relative A_{340}	Relative A_{355}	Index (#1) $\frac{\Delta A^{332.5}_{355}}{\Delta A^{325}_{332.5}}$	Index (#2) $\frac{\Delta A^{332.5}_{358}}{\Delta A^{332.5}_{340}}$	Index (#3) $\frac{\Delta A^{332.5}_{340}}{\Delta A^{325}_{332.5}}$	Fraction of spectrophotometric neutralization value (FSNC) From uncorrected value	From $\Delta A^{332.5}_{355}$ values	From $\Delta A^{332.5}_{340}$ value	From ΔA^{340}_{355} value	From Morton and Stubbs values
a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p
Pure vitamin A	A	0.848	1.000	0.896	0.866	0.733	0.347	5.28	5.37	1.56	1.0	1.0	1.00	1.00	0.962
Vitamin A spec. neut. std.	B	0.854	1.000	0.885	0.864	0.729	0.323	4.90	3.60	1.36	1.0	1.015	0.99	1.09	0.950
Acetate ester #1	C		1.000	0.890	0.860	0.724	0.332	5.14	3.39	1.50					
Acetate ester #2	D	08.52	1.000	0.883	0.853	0.721	0.330	5.00	2.33	1.50					
Deteriorated acetate ester #3	E	0.847	1.000	0.944	0.917	0.792	0.472	5.66	3.09	1.83	1.120	0.963	1.048	0.925	0.823
Neovit. A #1	F	0.809	1.000	0.971	0.948	0.543	0.410	18.80	3.03	5.20	1.15	1.14	1.06	1.17	0.816
Neovit. A #2	G	0.940	1.000	0.963	0.928	0.554	0.421	14.3	2.81	4.00	1.11	1.12	0.98	1.07	0.511
Kitol	H	1.90	1.000	0.764	0.631	0.401	0.145	2.62	3.5	1.54					No eval. minus
Kitol-like	I-15														No eval. minus
Low #1 index reg. run	J-41	1.66	1.000	0.840	0.807	0.659	0.295	3.40	3.01	1.13	1.07	0.934	1.19	1.01	0.816
High #1 index neolike reg. run		1.96	1.000	0.814	0.775	0.659	0.421	3.50	3.00	1.08	1.18	1.40	1.13	0.94	No eval. minus
Normal index	K-2	1.000	1.000	0.952	0.929	0.809	0.464	10.25	3.41	3.00	1.19	1.06	1.04	1.06	0.363
	L-45	0.54	1.000	0.897	0.860	0.748	0.351	5.30	3.65	1.45	1.03	1.02	0.94	1.05	0.613

purity or in some cases ranges of certain ΔA ratios can indicate alteration in a compound structure, for example, isomerization or deterioration.

A long recognized criterion of purity using absorption curve shapes is relative absorbance, defined as the ratio of absorbance at any wavelength ($A_{\lambda x}$) to the absorbance at the peak ($A_{\lambda peak}$). This ratio transposes all absorbances to values relative to a peak absorbance of unity, and, like the ΔA indices, reveals departures from the pure compound form but with the important difference that no correction for background is inherent. However, where Beer's law is valid, the value of the relative absorbance is independent of the concentration and is constant at any wavelength; therefore, it is useful in discriminating a departure from pure compounds—e.g., vitamin A. Such a ratio may even be used to calculate the percentage of a compound in a mixture under sufficiently controlled conditions (*3, 6, 9, 10*).

As is developed in this paper, the automatic correction inherent in the ΔA approach generally provides greater accuracy for this type of calculation.

ANGULAR CORRECTION: ORIGIN AND PROPERTIES

The angular correction (assuming background linearity) may be derived by first equating an index (ratio of two ΔA values) to its constant value as established from the curve of the pure compound. Thus from Table I, Index #3:

$$\frac{\text{Pure } \Delta A^{332.5}_{340}}{\Delta A^{325}_{332.5}} = 1.56 \qquad (1)$$

$$\frac{\Delta A^{332.5}_{340} - Y}{\Delta A^{325}_{332.5} - KY} = 1.56 \qquad (2)$$

where Y and KY are the angular correction values in the respective spectral regions in which the ΔA's are measured.

Because we are dealing theoretically with the linear background absorbancy, Y and KY are proportional to each other in the same way that the corresponding $\Delta\lambda$'s are proportional, since they are corresponding sides of similar triangles. Therefore they can be used as a correction in Equation 2. In this particular equation, $K = 1$ and therefore $Y = KY$, since $(340 - 332.5) = (332.5 - 325)$.

This is the theoretical angular correction. After substitution of actual values a final usable formula is derived:

$$\text{Pure } A_{325} = K\,[\Delta A^{332.5}_{340} - (2.777 \times \Delta A^{325}_{332.5} - 1.772\ \Delta A^{332.5}_{340})] \qquad (3)$$

where K is the horizontal Beer's law proportionality constant; $\Delta A^{332.5}_{340}$ is the impure horizontal correction value;

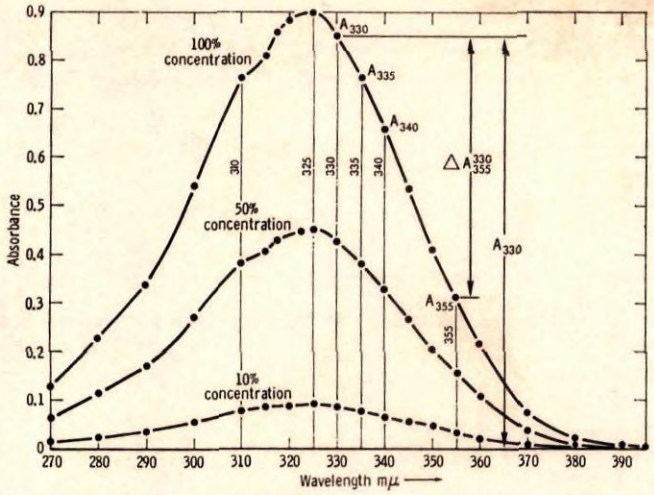

Figure 1. Vitamin A absorbance curves showing dilution effects

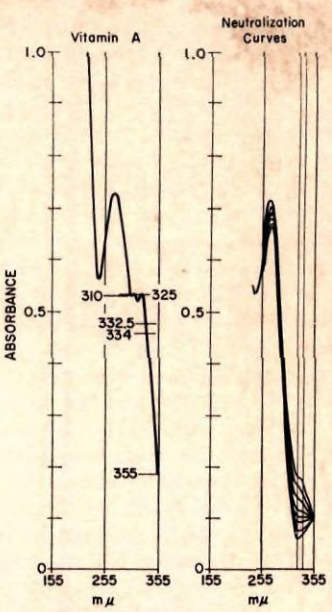

Figure 2. A comparison of a typical impure vitamin A curve from a sample with the corresponding set of neutralization curves

and (2.777 $\Delta A^{325}_{332.5}$ − 1.772 $\Delta A^{332.5}_{340}$) is the angular correction value. While there is a theoretical validity for the use of this type of angular correction, the lack of linearity in practice and also the mathematical and graphical properties resulting in increased error indicate that it is useless for practical application to vitamin A samples. This indication is confirmed by the tabulated results to be considered later.

However, a purity index by itself has other uses beside providing an angularly corrected value, because one or a combination of such indices may aid in delineating the nature of the background. This is demonstrated for vitamin A samples.

EXPERIMENTAL

Apparatus. The basic apparatus used in the experimental work was a Beckman DK1 spectrophotometer equipped with specially developed modifications for rapid and convenient spectrophotometric neutralization (*24*).

Reference Material. Pure crystalline vitamin A and unsaponifiable material from U.S.P. Standard vitamin A acetate in oil and National Bureau of Standards potassium chromate were used.

Procedure. The experimental part of this study is based upon conventional spectrophotometric curves as well as upon the use of pure vitamin A solutions and vitamin A extracts from pharmaceutical samples, using the accepted U.S.P. saponification and extraction prior to the spectrophotometric steps. The spectrophotometry consisted of a conventional absorption curve followed by a set of neutralization curves as shown in Figure 2. The curve on the left is the common absorption curve, while the set of curves on the right is composed of the neutralization curves. The vertical pair of lines centrally located and crossing the neutralization curves are index lines machine placed ±3 mμ on each side of the 325 mμ vitamin A peak. The concentration of the standard vitamin A solution where the peak levels out between the vertical index lines is the neutralization point and establishes the spectral A_{325} of the standard and consequently the concentration of the vitamin A in the sample. In executing an optical neutralization, the standard in the reference cell may be either concentrated or diluted to attain the neutralization point. In the work here reported, the initial reference solution was purposely made more concentrated than the vitamin A in the sample, and the reference was then diluted progressively to match the sample. This expedites the manipulation, since the device for introducing solution needs no cleaning or substitution between successive sample solutions. It has always been possible, by using the ΔA values from the absorption curve, to calculate adequately the necessary excess of vitamin A in the sample solution to prepare it for neutralization without resorting to any trial dilutions. The curves given in Figure 2 are typical. No real uncertainty was ever noted in ascertaining the neutralization point, even though the appearance of curves varied greatly among solutions from different samples. Further insight into the performance of such curves may be obtained by referring to previously mentioned papers (*8*, *11*, *12*, *19*, *20*, *23*) as well as to the papers of Tardif (*21*) and Tunnicliff (*22*).

SURVEY SERIES OF SAMPLES

Table I serves to furnish a perspective for the experimental part of this study, in that it presents the actual experimental values. Other tabulated results of this work have been subjected to statistical treatment and show relationships. The only statistical term of reference in this paper is relative standard deviation (rel. std. dev.). Columns i, j, and k in Table I include several of the purity indices previously discussed. When the values from unmodified vitamin A forms, neovitamin A and Kitol in column i are compared, a correlation can be noted between the index and the type of vitamin A product. Indices in column k show some of the distinguishing differences but are not as pronounced as the indices in column i. All indices in column j show insignificant differences. The indices in column i are apparently unique in that they reveal isomerization or other molecular alterations. That these changes are revealed only in the region of the peak is indicated by the indices in column j, where the index is composed only of increments in the straight line segments of the curve.

Columns l, m, n, o, and p illustrate all results in relation to the neutralization value, which is assumed to be the most accurate value and is used as the reference. [(FSNC) is the chosen symbol for this fractional neutralization comparison relating any ΔA peak evaluation (A_{325} for vitamin A) to the ΔA peak evaluation derived from the neutralization technique.] Columns n and o, derived from $\Delta A^{332.5}_{340}$ and ΔA^{340}_{355}, respectively, provide the greatest consistency with the reference optical neutralization value, while the Morton and Stubbs values generally are more erratic and are always on the low side. In the chosen series of ΔA values, values embodying the peak value A_{325} were avoided because of the selective flattening noted in this region and evidenced in column i by the index values. Table II deals exclusively with relative absorbance (for 51 samples), which has been defined and discussed previously. This table is presented in this form mainly to indicate reliability in making measurements on the slope of the curve. The last line, rel. std. dev., indicates those wavelengths

which are most precise and therefore useful for the ΔA measurements of this study. The importance of a particular ΔA value relative to a single A value for the vitamin A determination will be considered in more detail later. Since relative absorbance has been so frequently used in the past to indicate impurity in a compound, it is enlightening to compare these values with the ratio of two ΔA values featured in this study. Tables II and III illustrate these results. Table II shows that relative absorbance possesses a dispersion of 3.4–18% *vs.* 18–23% for the lower two ratio ΔA values in Table III. These two ranges cannot be regarded as significantly different. However, the rel. std. dev. of the index No. 1 in Table III is 39.5% and significantly larger than for either index No. 1 (18%) or for index No. 2 (23%). This value, 39.5%, is also significantly larger than that resulting from any of the relative absorbance values given. Hence, this index No. 1 provides an important and revealing tool for demonstrating variability found in the vitamin A occurring in current samples. Further study should provide further insight into the matter. The practical utility associated with index No. 1 in the routine analysis of vitamin A samples is already apparent.

In addition to the work with vitamin A, the ΔA ratio or index has other uses. This has been shown in previous applications in this laboratory (*13*, *14*). In one of these instances (*13*) the correlation between such an index and the quantities of vitamin D_2 and D_3 in a mixture obtained by chromatography was established by using ΔA values in the infrared.

Also, such an index is potentially useful for determining the thoroughness of a chromatographic separation. For example, it would have a timely use in estimating the purity of an absorbing compound separated as a spot in thin layer chromatography. Such a spot would need only to be eluted and transferred in a proper solvent to a spectrophotometer for obtaining the absorbance values required for the index.

Table II. Statistical Evaluation of Relative Vitamin A Absorbances at Various Wavelengths as Derived from 51 Pharmaceutical Samples

	A_{310}	$A_{332.5}$	A_{334}	A_{340}	A_{355}
Pure vitamin A	0.848	0.896	0.866	0.733	0.347
Mean of 51	1.06	0.89	0.853	0.725	0.359
Average fraction of pure vitamin A	1.25	0.996	0.986	0.988	1.03
Rel. std. dev.	18	3.4	3.8	7.05	11.0

Table III. Range of Purity Indices Derived from 51 Pharmaceuticals

Sample	No. 1 $\frac{\Delta A_{355}^{332.5}}{\Delta A_{332.5}^{325}}$	No. 2 $\frac{\Delta A_{355}^{332.5}}{\Delta A_{340}^{332.5}}$	No. 3 $\frac{\Delta A_{340.}^{332.5}}{\Delta A_{332.5}^{325}}$
Pure vitamin A	5.28	3.37	1.57
Mean of 51	5.34	3.22	1.66
Fraction of pure	1.01	0.96	1.06
Rel. std. dev.	39.5	18.01	23.0

Table IV. A_{325} Values from Optimum ΔA Method Compared with Uncorrected and Morton and Stubbs Method Using a Series of Ten Pharmaceutical Vitamin A Samples

Identity of sample	A_{325} calcd. from A_{325} uncorr. 1	A_{325} calcd. from $\Delta A_{355}^{332.5}$ 2	A_{325} calcd. from Morton and Stubbs (correction) 3	A_{325} calcd. from spectral neut. data 4	Fraction of spectral neutralization value (FSNC) A_{325} from uncorr. A_{325} 5	A_{325} from $\Delta A_{355}^{332.5}$ 6	A_{325} from Morton and Stubbs (correction) 7
Pure vitamin A	1.000	1.000	0.962	1.000	1.00	1.00	0.962
1	0.670	0.638	0.221	0.622	1.08	1.03	0.35
2	0.750	0.756	0.298	0.730	1.03	1.04	0.40
3	0.970	0.893	0.531	0.850	1.14	1.05	0.62
4	0.500	0.428	0.337	0.422	1.18	1.01	0.80
5	0.940	0.960	0.664	0.910	1.03	1.05	0.72
6	0.382	0.331	−0.012	0.314	1.22	1.05	...
7	0.492	0.437	0.392	0.420	1.14	1.02	0.92
8	0.660	0.656	0.873	0.632	1.04	1.04	1.38
9	0.390	0.423	−0.989	0.410	0.95	1.03	...
10	0.790	0.793	0.502	0.780	1.01	1.02	0.603
Mean					1.07	1.03	0.57
Rel. std. dev.					8.32	1.26	62.30

PERFORMANCE OF TECHNIQUE BASED UPON OPTIMUM CHOICE OF ΔA

Table IV refers to a consecutive set of routine vitamin A samples used in this study. It is shown in greater detail than in the previous tables and with emphasis on the optimum choice of $\Delta A_{355}^{332.5}$ value. The results from the Morton and Stubbs procedure are ncluded for comparison only. The mean Morton and Stubbs values of 57% of the spectrophotometric value, together with its 62.3% rel. std. dev., indicate its unsuitability for dealing with the type of samples represented. The extreme variability of absorbance values in the 310 mμ region, as previously observed, undoubtedly has much to do with the discrepancies noted.

Sample 6, column 5 of Table IV, shows the uncorrected A_{325} value to be 22% larger than the corresponding neutralization value while the A_{325} value derived from the $\Delta A_{355}^{332.5}$ for the same sample, column 6, is only 5% larger than the corresponding neutralization value. Other comparative individual values in this table can be examined with consistent differences of the same order. The mean values reflect the same differences to a lesser degree (7% for the uncorrected values *vs.* 3% for the $\Delta A_{355}^{332.5}$ derived values).

The rel. std. dev. of the FSNC's from the $\Delta A_{355}^{332.5}$ values also reflect the thoroughness of elimination of error by the procedure (8.3% from the uncorrected values *vs.* 1.26% from the $\Delta A_{355}^{332.5}$ values). Thus the superiority of the A_{325} value from the $\Delta A_{355}^{332.5}$ is statistically demonstrated.

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LITERATURE CITED

(1) Allen, E., Rieman, W., III, ANAL. CHEM. **25**, 1325 (1953).
(2) Banes, F. W., Eby, L. T., IND. ENG. CHEM., ANAL. ED. **18**, 535 (1946).
(3) Beroza, M., ANAL. CHEM. **22**, 1237 (1950).
(4) Cama, H. R., Collins, F. D., Morton, R. A., *Biochem. J.* **50**, 48 (1951).
(5) Davidow, B., Woodard, G., *J. Assoc. Offic. Agr. Chemists* **32**, 25 (1949).
(6) Gibson, M. R., Schwarting, T. A., *J. Am. Pharm. Assoc., Sci. Ed.* **37**, 206 (1948).
(7) Heigel, J. J., Bell, M. F., White, S. W., ANAL. CHEM. **19**, 293 (1947).

(8) Hiskey, C. F., *Ibid.*, **33,** 927 (1961).
(9) Hoskins, A. L., Sherman, A. I., Allen, W. M., *J. Biol. Chem.* **182,** 429 (1950).
(10) Hotchkiss, R. D., *Ibid.*, **175,** 315 (1958).
(11) Jones, J. H., Clark, G. R., Harrow, L. S., *J. Assoc. Offic. Agr. Chemists* **34,** 135 (1951).
(12) *Ibid.*, p. 149.
(13) Morris, W. W., Haenni, E. O., *Ibid.*, **45,** 92 (1962)
(14) Morris, W. W., Wilkie, J. B., Jones, S. W., Friedman, Leo, ANAL. CHEM. **34,** 381 (1962).
(15) Morton, R. A., Stubbs, A. L., *Analyst* **71,** 348 (1946).
(16) Morton, R. A., Stubbs, A. L., *Biochem. J.* **41,** 525 (1947).
(17) Morton, R. A., Stubbs, A. L., *Ibid.*, **42,** 195 (1948).
(18) Rotondaro, F. A., *J. Assoc. Offic. Agr. Chemists* **40,** 824 (1957).
(19) Schiaffino, S. S., Ph.D. thesis, Georgetown Univ., Washington, D. C., 1956.
(20) Schiaffino, S. S., Loy, H. W., Kline, O. L., Harrow, L. S., *J. Assoc. Offic. Agr. Chemists* **59,** 180 (1956).
(21) Tardif, Real, *J. Pharm. Sci.* **50,** 693 (1961).
(22) Tunnicliff, D. D., Rasmussen, R. S., Morse, M. L., ANAL. CHEM. **21,** 895 (1949).
(23) Wilkie, J. B., *J. Assoc. Offic. Agr. Chemists* **32,** 455 (1949).
(24) Wilkie, J. B., *Ibid.*, **46,** 920 (1963).
(25) Wright, Norman, IND. ENG. CHEM., ANAL. ED. **13,** 1 (1941).

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