

THE TWO-WAVELENGTH METHOD OF MICROSPECTROPHOTOMETRY

IV. A New Solution

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ABSTRACT

In connection with the potential development of automatic two-wavelength microspectrophotometry, a new version of the two-wavelength method has been formulated. Unlike its predecessors, the Ornstein and Patau versions, the new method varies the area of the photometric field seeking to maximize a relationship between distributional errors at the two wavelengths. Stating this distributional error relationship in conventional photometric terms, the conditions at the maximum are defined by taking the first derivative with respect to field size and setting it equal to zero. This operation supplies two equations; one relates the transmittances at the two wavelengths, and a second states the relative amount of chromophore in the field in terms of transmittance at one wavelength. With the first equation to drive a servomechanism which sets the appropriate field size, the desired answer can then be obtained directly and continuously from the second equation. The result is identical in theory with those of the earlier methods, but the technique is more suitable for electronic computing.

A decade ago the two-wavelength method was developed independently and in two different forms by Ornstein and Patau (4, 5). The method was clearly an improvement over one-wavelength techniques in that it reduced distributional error considerably and eliminated the need for micrometer measurements of the objects. Parallel refinements of scanning methods of photometry provided another solution for the distributional error problem. However, the two-wavelength method continues to hold a unique position since it alone is ideally suited for the measurement of intact nuclei and other irregularly shaped or heterogeneous objects which have thicknesses in excess of the depth of focus of an oil immersion objective (1, 3).

Perhaps the greatest deterrent to the utilization of the two-wavelength method has been the laboriousness of the procedure. The measurement

itself requires two transmittances, usually involving a pair of photometric readings at each wavelength and a resetting of the monochromator between. In suitable material and with great attention to detail, it is possible to measure 50 nuclei in one hour. Another hour is required to complete the calculations, although some time can be saved by the use of an appropriate set of tables (2).

An additional complication stems from the fact that an accidental mistake or a technical failure in any of the operations is difficult to recognize in the raw data. To guard against the inevitably bizarre final result which such errors produce, one can either make duplicate readings (doubling the time required) or identify the individual objects and have the option of later repeating the suspi-

cious entries. In any case, the labor involved encourages a strong reluctance to amass sizable samples or include adequate controls.

Clearly what is needed is a modification of the two-wavelength method which will reduce the operational steps and provide a definitive reading while the object is still in the field. As an approach to this end, the author, in collaboration with R. Q. Edwards and R. C. Bateson, explored the possibility of designing an analog computer for Patau's version of the two-wavelength method. Several solutions appeared to be feasible, but all were disturbingly awkward. Meanwhile, the

relationship between wavelengths, the two-wavelength method to be presented differs from its predecessors in that B is made a dynamic rather than a static parameter.

According to the Beer-Lambert Law, the relative amount of chromophore km_c within the area B_c can be stated in terms of transmittance T_c or absorbance A_c as

$$km_c = B_c A_c = -B_c \log T_c. \quad (1)$$

To find T , the transmittance through field B , one takes a weighted mean of the transmittances

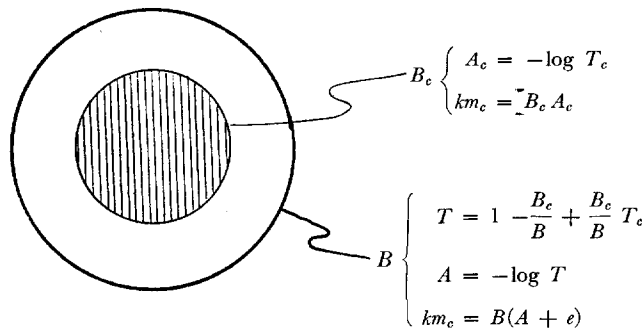


FIGURE 1

The model used for the two-wavelength methods. Some of the photometric relationships are summarized for the field containing only chromophore (B_c) and for the compound field of chromophore and background (B). See text for details.

author has examined the two-wavelength method itself and has uncovered a third solution. This new statement of the two-wavelength method is now presented as an alternative to the two methods already available and as a more convenient formulation for electronic computing.

As shown in Fig. 1, the model for the two-wavelength method is a photometric field B within which there is an area B_c of homogeneously distributed chromophore. In the Patau and Ornstein versions, the transmittance through B is determined for two wavelengths preselected to have absorptivity constants such that $k_1 = 2k_2$. By what is essentially a method for the solution of simultaneous equations coupled to the Beer-Lambert Law, the transmittances are used to define the relative amount of chromophore in B . Of course, a single transmittance reading through B would give an incorrect estimate because of distributional error.

Although it is based on this model and the same

through B_c and the blank component of the field. Thus

$$T = 1 \left(\frac{B - B_c}{B} \right) + T_c \left(\frac{B_c}{B} \right) = 1 - \frac{B_c}{B} + \frac{B_c}{B} T_c \quad (2)$$

and

$$A = -\log T \quad (3)$$

where A is the absorbance for field B . Since the relative amount of chromophore in B_c and B is identical,

$$km_c = B_c A_c = B(A + e) \quad (4)$$

where e refers to distributional error due to the non-random distribution of chromophore in B . Rearranging equation (4),

$$e = \frac{B_c}{B} A_c - A. \quad (5) \quad \text{Combining equations (6) and (4), or equations (7) and (5),}$$

The conventional two wavelengths, designated by the subscripts 1 and 2, provide the following relations:

$$e_1 - 2e_2 = 2A_2 - A_1, \quad (9)$$

and combining equations (2), (3), and (9),

$$k_1 m_c = 2k_2 m_c \quad (6) \quad e_1 - 2e_2 = \log \left(1 - \frac{B_c}{B} + \frac{B_c}{B} T_{c_1} \right) \quad (10)$$

$$A_{c_1} = 2A_{c_2} \quad (7) \quad - 2 \log \left(1 - \frac{B_c}{B} + \frac{B_c}{B} T_{c_2} \right).$$

$$T_{c_1} = (T_{c_2})^2. \quad (8)$$

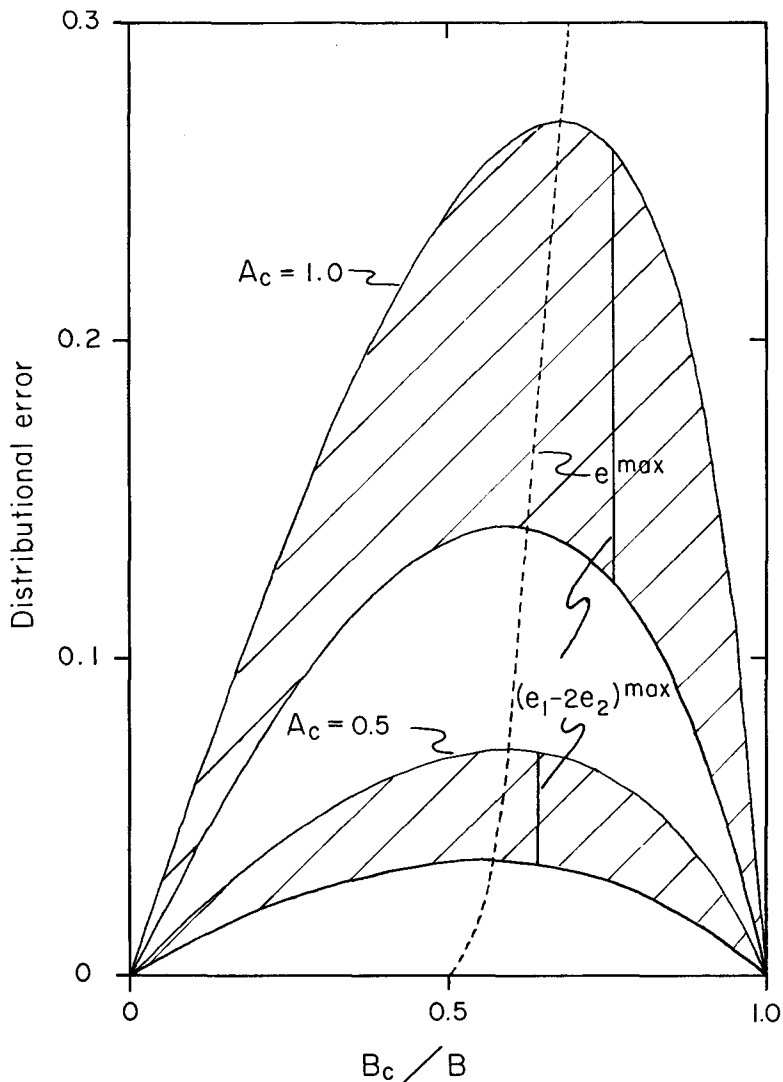


FIGURE 2

The relationships between distributational error, absorbance, and area of the photometric field. See text for details.

The relationship in equation (5) between e , A_c , and B_c/B is depicted in Fig. 2. The curves labeled $A_c = 1.0$ and $A_c = 0.5$ demonstrate the striking increase in distributional error as absorbance increases. In following any one curve, and beginning at the right hand extreme ($B = B_c$; $e = 0$), it is evident that as B increases the error increases. Eventually distributional error goes through a maximum, and then falls back to zero when B is infinite. The dotted line, e^{\max} , describes the position of the maximum as a function of A_c .

The shaded parts of the graph represent $e_1 - 2e_2$. In each case the lower limit of the shaded area is defined by the curve for twice the distributional error at the second wavelength. The vertical lines are the maxima for $e_1 - 2e_2$. The graphs indicate that the position of the maximum is a function of A_c and that there is only one maximum for each pair of curves.

The conditions at the maximum for $e_1 - 2e_2$ can be obtained from equation (10) by taking the partial derivative of $e_1 - 2e_2$ with respect to B . Thus,

$$\frac{\partial(e_1 - 2e_2)}{\partial B} = \frac{\left(\frac{B_c}{B^2} - \frac{B_c}{B^2} T_{c_1}\right) \log e}{1 - \frac{B_c}{B} + \frac{B_c}{B} T_{c_1}} - \frac{\left(\frac{B_c}{B^2} - \frac{B_c}{B^2} T_{c_2}\right) 2 \log e}{1 - \frac{B_c}{B} + \frac{B_c}{B} T_{c_2}} \quad (11)$$

Setting the derivative equal to zero, and discarding the solution when B is infinite,

$$0 = 2T_{c_2} - T_{c_1} - 1 + \frac{B_c}{B} (1 - T_{c_2} + T_{c_1} T_{c_2} - T_{c_1}) \quad (12)$$

Appropriate solutions for equation (12) are obtained by substitution. For example, using equation (8) to eliminate the T_{c_1} terms, the expression simplifies to

$$\frac{B_c}{B} = \frac{1}{1 + T_{c_2}} \quad (13)$$

Starting again with equation (12) and using equation (2) to substitute for T_{c_1} and T_{c_2} , one obtains

$$T_2 = \frac{2T_1}{1 + T_1} \quad (14)$$

Using equations (13) and (14), the substitution for T_2 and T_{c_2} in equation (2) yields

$$\frac{B_c}{B} = \frac{1}{1 + T_1} \quad (15)$$

From equations (13) and (15),

$$T_1 = T_{c_2} \quad (16)$$

and hence

$$A_1 = A_{c_2} \quad (17)$$

It follows from equations (1), (15), and (17) that when $e_1 - 2e_2$ is maximal,

$$k_2 m_c = A_{c_2} B_c = A_1 B_c = \frac{A_1 B}{1 + T_1} \quad (18)$$

The particular advantage of these equations for automatic computing lies in the relationship between equations (14) and (18). In equation (14) an equality is stated between the two measured transmittances. Since this relationship exists only at the maximum for $e_1 - 2e_2$, it can be used to drive a servomechanism which varies B until the measured transmittances comply with the formula. The photometric field is then uniquely set for the maximum for $e_1 - 2e_2$. Under these conditions, equation (18) supplies the relative amount of chromophore in terms of the transmittance at one wavelength. The entire operation could be performed by the computer, thus giving a direct read-out of the desired answer.

An additional point of some practical significance is that the effect of varying B can be achieved electronically and need not involve a mechanical operation on the photometer itself. One can readily conceive of distributions of chromophore which would not allow a circular (or other regular-shaped) B sufficient latitude to find the maximum without encroaching on B_c . Since the variable component in B is blank field, the appropriate changes in B can be represented by adding or subtracting equal increments to the two measured components of transmittance, I and I_0 . As indicated by equation (15), the desired B has readily defined limits, ranging from B_c to $2B_c$; these are well suited for a simple addition

operation prior to the computation of transmittance.

This solution for the two-wavelength relationship is mathematically equivalent to the two earlier solutions. Like its predecessors, it is an exact solution for the model shown in Fig. 1 and only an approximate solution for the situation in which the chromophore in B_c is not uniformly distributed. However, both Patau and Ornstein have clearly shown that the two-wavelength methods greatly reduce distributional error for a wide variety of conceivable distributions within

B_c . The residual error depends largely on the type of distribution present in B_c and the absorptivity. By choosing chromophores or wavelengths or material such that the peak absorbances are kept below 1.0, the residual distributional error is then small when compared with the other errors of cytophotometric technique.

This work was supported by grant C-3896 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

Received for publication, July 12, 1961.

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