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 microspectro-
photometry, 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 photo-
metric 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 equa-
tion 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 refine-
ments of scanning methods of photometry pro-
vided 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 heteroge-
neous 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 la-
boriousness of the procedure. The measurement
itself requires two transmittances, usually involv-
ing a pair of photometric readings at each wave-
length 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 $k_{mc}$ within the area $B_c$ can be stated in terms of transmittance $T$, or absorbance $A$, as

$$k_{mc} = B_c A_c = -B_c \log T_c.$$  

(1)

To find $T$, the transmittance through field $B$, one takes a weighted mean of the transmittances through $B_c$ and the blank component of the field. Thus

$$T = 1 - \frac{B_c}{B} + \frac{B_c}{B} T_c,$$  

(2)

and

$$A = -\log T$$  

(3)

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

$$k_{mc} = B_c A_c = B(A + \epsilon)$$  

(4)

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

![Figure 1](attachment://image.png)

**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.
\[ \epsilon = \frac{B_2}{B} A_2 - A. \]  

(5) Combining equations (6) and (4), or equations (7) and (5),

\[ \epsilon_1 - 2\epsilon_2 = 2A_2 - A_1, \]  

(9)

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

\[ k_{\text{rel}} = 2k_{\text{rel}}, \]  

(6)

\[ \epsilon_1 - 2\epsilon_2 = \log \left( 1 - \frac{B_2}{B} + \frac{B_2}{B} T_{c1} \right) \]  

(7)

\[ T_{c1} = (T_\alpha)^2. \]  

(8)

\[ -2 \log \left( 1 - \frac{B_2}{B} + \frac{B_2}{B} T_\alpha \right). \]  

(10)

FIGURE 2

The relationships between distributional error, absorbance, and area of the photometric field. See text for details.
The relationship in equation (5) between $e$, $A_e$, and $B_e/B$ is depicted in Fig. 2. The curves labeled $A_e = 1.0$ and $A_e = 0.5$ demonstrate the striking increase in distributional error as absorbance increases. In following any one curve, and beginning at the right hand extreme ($B = B_e$, $e = 0$), 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, $e_{	ext{max}}$, describes the position of the maximum as a function of $A_e$.

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 $A_e$ 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,

$$\frac{\partial(e_1 - 2e_2)}{\partial B} = \left(\frac{B_e - B_t}{B^2 T_2} + B_e T_1\right) \log e \left[1 - B_e + \frac{B_e}{B} T_2\right]$$

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

$$0 = 2T_2 - T_1 - 1$$

Starting again with equation (12) and using equation (2) to substitute for $T_2$ and $T_1$, one obtains

$$T_2 = \frac{2T_1}{1 + T_1}$$

Using equations (13) and (14), the substitution for $T_2$ and $T_1$ in equation (2) yields

$$B_e = \frac{1}{1 + T_1}$$

From equations (13) and (15),

$$T_1 = T_2$$

and hence

$$A_1 = A_2.$$
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 $B_e$ 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 $B_e$. The residual error depends largely on the type of distribution present in $B_e$ 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.

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REFERENCES