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# A Discussion of Some Errors in Quantitative Spectrophotometry.*

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Recent advances in photoelectric spectrophotometry reported by Hogness, *et al.*,[1] and Smith[2] now permit precise measurements of absorption coefficients. The techniques described by these workers have made it possible for investigators in different laboratories to obtain concordant results. Kuhn,[3] Miller,[4, 5] and Van Der Hulst[6] have pointed out several analytical applications of spectrophotometry in biological chemistry. However, these analytical procedures are valid only when the investigator is cognizant of the limitations imposed by certain errors. This paper concerns anomalous transmission values (or *i. e.*, $I_o/I$)† that are caused by scattered radiation or absorption due to impurities.

Data presented in Graph 1 illustrate the discrepancies in carotenoid absorption curves of solutions containing 0.6% butter and solutions with equivalent carotenoid concentrations but no butter. In this experiment 20% diethyl ether and 80% ethanol (by volume) were employed as solvent. When the absorption curves were measured on aliquot portions of butter—carotenoid solutions at one hour intervals, the difference between the last curve, in the series, and the standard curve (no butter present) was always less than when similar comparisons were made with curves obtained on earlier runs. When the absorption curves were run on butter solutions that had stood for 15 to 24 hours, no deviation was observed from the standard curves.

In experiment 2, the transmittance was measured on butter-carotenoid solutions in spectral regions where the carotenoids do not absorb. The $I_o$ deflections were adjusted to 100.0 ± 0.5 cm. Table I summarizes the I deflections for butter-carotenoid solutions at

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1 Hogness, T. R., Zscheile, F. P., Jr., and Sidwell, A. E., Jr., *J. Phys. Chem.*, 1937, **41**, 379.

2 Smith, J. H. C., *J. Am. Chem. Soc.*, 1936, **58**, 247.

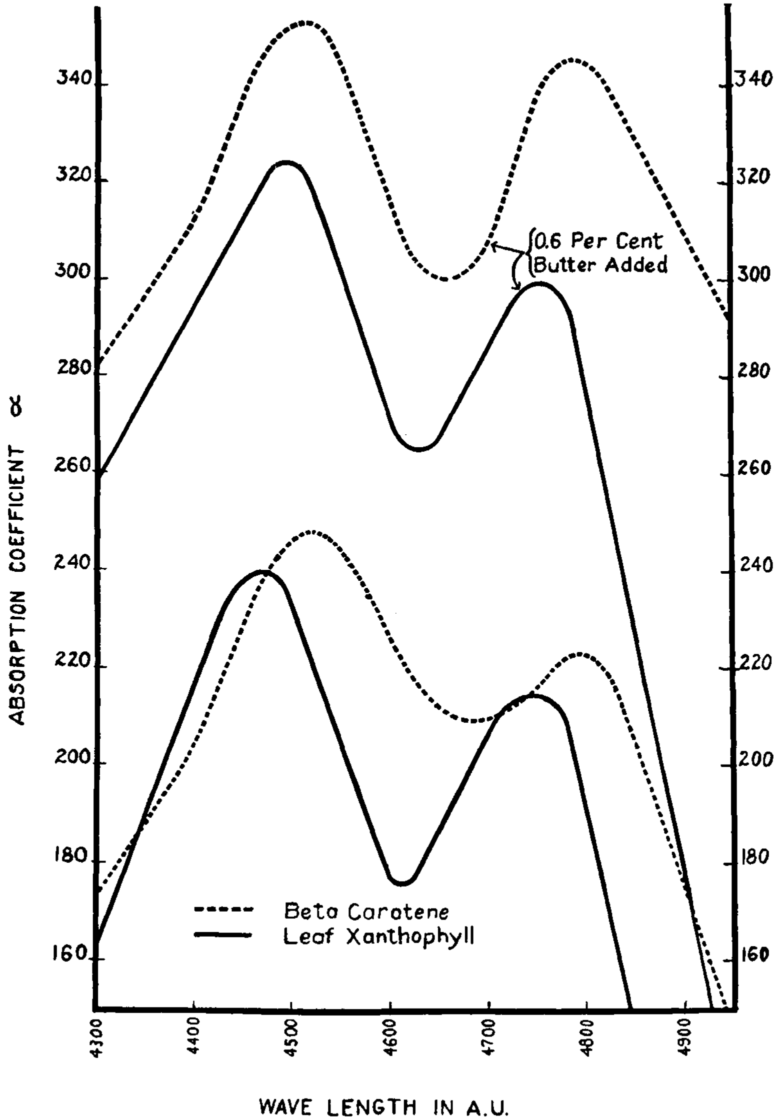
3 Kuhn, Richard, and Smakula, Alexander, *Z. Physiol. Chem.*, 1931, **197**, 161.

4 Miller, Elmer S., *J. Am. Chem. Soc.*, 1935, **57**, 347.

5 Miller, Elmer S., *J. Cereal Chem.*, in press Jan. issue, 1938.

6 Van Der Hulst, L. J. N., *Rec. Trav. Chim.*, 1935, **54**, 639.

† Same nomenclature as in reference 1.




GRAPH 1.
Absorption spectra of beta carotene and "leaf xanthophyll" in solutions containing 0.6% butter. The lower two curves are included for comparison purposes.

wave lengths 5600 and 6000Å. The deflections (I) for the control cell are recorded in the last column.

Data presented in Table I show that turbidity caused sufficient scattering of light to account for the anomalous absorption coefficients presented in Graph 1.

TABLE I.
Scattered Radiation Due to Turbidity.

| Readings made at following intervals Hours | Scattered butter at 5600 Å cm. | Radiation by wavelengths 6000 Å cm. | Scattered radiation in control at λ {5600, 6000} Å cm. |
|---|---|---|---|
| Initial | 33.9 | 31.0 | —0.4 |
| 0.05 | 33.0 | 29.0 | +0.2 |
| 0.2 | 31.6 | 27.4 | —0.1 |
| 1.0 | 26.4 | 24.8 | +0.2 |
| 3.0 | 25.8 | 22.0 | 0.0 |
| 16.0 | 0.8 | 1.0 | +0.5 |
| Solution shaken at end of exp. | 35.0 | 30.6 | + .4 |

The following data concerns the variations in error in absorption coefficient measurements introduced by impurities in the sample. Table II summarizes the errors in absorption coefficients for beta carotene in the visible and ultraviolet region.[7]

TABLE II.
Errors in Absorption Coefficients for Beta Carotene.

| Maxima at wavelengths | Specific $a$ | Maximum deviation ± 3 runs | Error % |
|---|---|---|---|
| 4800 | 222 | 1.0 | 0.9 |
| 4525 | 249 | 1.4 | 1.1 |
| 2790 | 43 | 0.5 | 2.6 |
| Minima at wavelengths | | | |
| 4700 | 210 | 1.0 | 2.0 |
| 3200 | 10 | 0.5 | 10.0 |
| 2200 | 17 | 1.0 | 12.0 |

Hogness, *et al.*,[8] have published the molecular absorption coefficients for ferriheme cyanide (Table III).

Although the data in Tables II and III are for different kinds of substances, the variations of the percent error are in good agreement. From the view point of practical spectrophotometry, it is important to recognize that a 1% impurity on weight basis may introduce a 10% error in absorption coefficients, especially if the coefficient is very small.

Data presented in Graph 1 and Table I show that solutions should be optically clear before accurate transmission measurements are made. If the foreign substance cannot be removed, the amount of

[7] Miller, Elmer S., *Plant Physiology*, 1937, **12**, 667.

[8] Hogness, T. R., Zscheile, F. P., Jr., and Sidwell, A. E., Jr., *J. Biol. Chem.*, 1937, **118**, 1.

TABLE III.
Absorption Data for Ferriheme Cyanide in a Solution Containing 0.093 M. NaOH and 0.5 M. KCN per liter.

| Maxima at wavelengths | Molecular $a \times 10^{-3}$ | Maximum deviation ± (2 runs) | Error % |
|---|---|---|---|
| 5450 | 11.0 | 0.1 | 1.8 |
| 4225 | 85.5 | 0.8 | 1.9 |
| 3650 | 37.5 | 0.5 | 2.7 |
| 2300 | 32.1 | 0.8 | 5.0 |
| Minima at wavelengths | | | |
| 5000 | 7.0 | 0.7 | 20.0 |
| 3825 | 33.6 | 0.3 | 1.8 |
| 3000 | 12.7 | 0.2 | 3.1 |

scattered radiation due to turbidity can be determined by measuring I (the transmission) in adjacent spectral regions where absorption by the substance investigated is zero. Data in Table I indicate that the error in $a$ after such a correction is made, can be reduced to 2-5%. Under proper conditions, transmission measurements of certain turbid solutions are reproducible, *i. e.,* if readings are made at corresponding time intervals after shaking the solution. When scattered radiation is taken into account, Beer's law is obeyed by carotenoid solutions containing 0.6% butter.

Miller[9] in preliminary attempts to measure the absorption curve for Spirogyra between wavelengths 5000 and 7000Å, found that 2-3% variations insignificant because so much of the absorption was due to scattered radiation.

In spectrophotometry, errors introduced by impurities may be the limiting factor. A simple calculation is presented as an illustration. Many lipids have a specific coefficient (grams per liter) less than 0.5. If the impurity has a specific coefficient of 250, only a 0.02% contamination of the sample will introduce a 10% error in the lipid coefficient.

[9] Miller, Elmer S., unpublished data.