Quantitative spectrophotometry using integrating cavities

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Abstract

Absorption spectrophotometry, a standard tool for quantitative analysis, suffers from two major drawbacks: lack of sensitivity and vulnerability to scattering. It has been pointed out earlier that the solution to these problems lies in using a reflecting cavity as a sample holder. Due to multiple reflections at the cavity wall, the effective pathlength becomes considerably larger than the diameter of the cavity, and scattering losses are eliminated because scattered light is prevented from escaping the detector. Though much effort has been spent in analysing and improving the performance of such a device, often called an integrating cavity absorption meter (ICAM), a simple strategy for deducing the absorbance of the sample is still lacking. It is shown here that the absorbance \( A_0 \) measured by using an ICAM exhibits a sublinear increase with the solute concentration \( C \). The physical reason for this departure from linearity is explained, and a straightforward procedure for converting \( A_0 \) to the true absorbance \( A \) (proportional to \( C \)) is established. The reliability of the procedure is demonstrated by comparing the ICAM absorption spectrum of dilute dye solutions with the spectra of more concentrated solutions recorded in a conventional spectrophotometer. The ability of the device to cope with scattering was tested by filling the ICAM with a suspension of chloroplasts, and the spectrum was found, as expected, to be free from scattering artefacts.

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1. Introduction

Absorption spectroscopy is a convenient and reliable technique for measuring the concentration of optically clear samples. One of its chief drawbacks is that the limit of detectability is set by the lowest absorbance that can be reliably measured (typically 0.005 for a modern commercial instrument). Since the absorbance \( A \) of a single-component sample equals the product \( \varepsilon CL \) (where \( \varepsilon \) is the molar absorption coefficient, \( C \) is the molar concentration and \( L \) is the pathlength), and \( \varepsilon \) seldom exceeds \( 10^5 \text{ M}^{-1}\text{cm}^{-1} \) [1], while \( L \) is usually 1 cm, it is obvious that submicromolar concentrations present a challenge to the technique. If the analyte concentration cannot be increased, the only recourse is to increase the pathlength directly or indirectly by using multiple traversal of the monitoring light through the absorbing specimen [2,3]. A second disadvantage of conventional absorption spectroscopy is its inability to deal with turbid samples, for the measured attenuation reflects losses due to absorption as well as scattering \[4–6\]. Though scattering losses can be removed by placing the cuvette inside an integrating cavity, the shape of the measured absorption spectrum no longer coincides with that recorded by using a conventional (single pass or multipass) arrangement [7]. The main reason for the disagreement lies in the fact that the pathlength distribution depends on whether or not the photons pass through an absorbing media. To overcome this problem, an alternative method was suggested, which entails positioning the cuvette outside the sphere. Although

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some of the scattered light escapes the detector, the losses can be compensated as described in [6].

An article by Fry et al. [8] has generated considerable interest in the use of the so-called integrating cavity absorption meter (ICAM), where a reflecting cavity is completely filled with the absorbing solution, and acts thereby as a multi-pass cuvette. They traced the idea of using a cavity to Elterman [9], who “emphasized that, if a sample is in an isotropic homogeneous field, then the absorbed radiant power is independent of scattering effects.” It seems fair to point out that the use of an integrating cavity as the cuvette, as well as the calibration procedure devised by Fry et al., should be credited to Ketskeméty and co-workers [10,11], whose main interest was in recording the absorption spectra of weakly absorbing substances. Though much effort has been spent on deducing, from intensity measurements, the absorption coefficient of the sample within the cavity, the agreement between the calculated value and that measured directly by using a conventional spectrophotometer is far from satisfactory. We have developed a convenient and reliable calibration procedure which provides distortion-free spectra in a broad concentration range of dilute solutions and also for scattering samples.

2. Derivation of basic relations

In what follows, we will set \( \kappa = \varepsilon C \) and \( \alpha = \ln 10 \times \kappa \), and refer to \( \alpha \) as the absorption coefficient. Assume that a cavity is prepared by coating the external surface of a closed hollow bulb (of arbitrary shape) with a diffusely reflecting material with reflectivity \( \rho \) close to unity. Let \( S \) be the area of the internal surface and let \( V \) be the volume bounded by this surface, respectively; let \( s \) be the total surface area of the openings used for allowing the passage of light and the specimen, thereby converting the above cavity into an ICAM. We will follow Ketskeméty and Kozma [10] and take advantage of the fact that one uses an ICAM only when one is dealing with a weakly absorbing sample; we will therefore assume that the condition \( x r \ll 1 \), is satisfied, where \( r \) denotes a typical length specifying the dimensions of the cavity; if the cavity happens to be spherical, we will identify \( r \) with its radius. This assumption justifies the neglect of absorption before a photon suffers its first reflection at the surface of the cavity, and constitutes a great simplification in the theoretical analysis which now follows. Ketskeméty and Kozma [10] argued, that since reflection at the cavity is supposed to be diffuse, the radiation field within the cavity can be considered, to an excellent approximation, as a homogenous and isotropic gas of number density \( \rho \), which satisfies the following equation:

\[
V \frac{d\rho}{dr} = N_i - N_a - N_r - N_s = 0,
\]

where \( N_i, N_a, N_r, N_s \) denote the rates (in photons per unit time) of entry into the cavity, absorption by the material within (with a refractive index \( n \)), losses due to imperfect reflection at the surface, and escape through the orifices, respectively. According to elementary kinetic theory, the rate at which photons will strike a unit area of the cavity equals \( \rho \bar{c} / 4 \), where \( \bar{c} = c/n \), and \( c \) denotes the speed of light in vacuum; this implies that the output of a detector placed so as to receive the photons leaving the observation window will be proportional to \( \rho \). Since a photon travels a distance \( \bar{c}dr \) during a time \( dr \), the probability that it will be absorbed during this interval is simply, \( \alpha \bar{c}dr \), which means that \( N_a = \rho V \bar{c} \). We are interested in the expression for \( \rho \), which comes out to be

\[
\rho = \frac{N_i}{\varepsilon V^2} \left( \frac{1}{\alpha + \frac{S(1 - \rho)}{4V} + \frac{sp}{4V}} \right).
\]

We will add the suffix zero to denote the quantities pertaining to a reference measurement (made by filling the cavity with only the solvent). Let \( I_0 \) (proportional to \( p_0 \)) and \( I \) (proportional to \( p \)) denote the detector signals obtained when the cavity is filled with the solvent and the test sample, respectively, and define the transmittance of the sample as

\[
T = \frac{I}{I_0}.
\]

The absorption coefficient of the added solute can be expressed as

\[
\alpha = K \left( \frac{1}{T} - 1 \right) + 2, \quad K = K_1 + K_2, \quad K_1 = \frac{S(1 - \rho)}{4V}, \quad K_2 = \frac{sp}{4V}.
\]

If we now consider a spherical cavity and set \( S = 4\pi r^2 \) and \( V = 4\pi r^3 / 3 \), we should recover the results found by Ketskeméty and Kozma [10] and by Kirk [12], but this is not the case. Ketskeméty and Kozma made a small error that resulted in setting \( \rho = 1 \) into their expression for \( K_2 \), whereas Kirk argued that since \( S \gg s \), one may set \( K_2 = 0 \). It is worth noting that the neglect of \( K_2 \) will be valid only if the inequality \( S/s \gg \rho / (1 - \rho) \), is satisfied. In a practical instrument \( \rho \geq 0.9 \), which means that \( \rho / (1 - \rho) \gg 9 \), and the signal-to-noise ratio might become too low to permit making \( S/s \gg 10^3 \). One approach to the determination of the constants \( K_1 \) and \( K_2 \) is illustrated by the work Lerebourg and co-workers [13], who determined \( \rho \) and inserted it into the pertinent expressions; the calculated values of \( \alpha \) are not sufficiently close to the real values. Ketskeméty and Kozma [10], who set \( x_0 = 0 \), took a more pragmatic approach and calibrated their instrument by using a standard (dye with known absorption coefficient) and determining \( K \) experimentally by using the following relation:

\[
x_s = K \left( \frac{I_0}{I} - 1 \right).
\]

Once the value of \( K \) became available, they could find the absorption coefficients of other samples. We will show that this method is also unreliable.
3. Experimental

Most of the results reported here were carried out with a spherical ICAM made of glass of ~0.5 mm thickness and a diameter of 76 mm; it has a 15 mm long neck (internal diameter 5 mm), pointing upwards, which serves a dual purpose: an exit port for the emerging light, and a passageway for the sample (Fig. 1). The outer surface of the ICAM was coated with a silver layer, which provides almost uniform reflectivity over the whole visible range. A window (8 mm in diameter) on the great circle of the sphere was left uncovered for light input, while the light output was picked up via a light-guide inserted into the neck. In order to protect the silver coating the sphere was covered with a white vitrified paint and then mounted into a box which held it fixed and also provided access to the optical window. Experiments were also performed by using an ICAM whose outer surface was coated with a diffusely reflecting white paint.

A 24 V, 150 W tungsten filament lamp served as a light source which was focussed onto the input window of the sphere. The light emerging via the neck was collected by a light-guide inserted into the neck. In order to protect the silver coating the sphere was covered with a white vitrified paint and then mounted into a box which held it fixed and also provided access to the optical window. Experiments were also performed by using an ICAM whose outer surface was coated with a diffusely reflecting white paint.

The dyes rose bengal and malachite green (pure A.R.) were purchased from EGA-Chemie (Germany) and Koch-Light Laboratories Ltd. (England), respectively. Chloroplasts were isolated from spinach leaves as described in [14].

4. A new treatment

The object of the calibration procedure is to find a relation between the measured and the true absorption values, which holds at every wavelength. In order to obtain the true spectrum one has to apply a correction method. It seems obvious, that after the correction: (a) the shape of the spectra should be the same within experimental error and (b) the ratio of the spectra should be equal to the ratio of the concentrations of the given solutions.

The calibration was carried out as follows. The ICAM was filled with distilled water and \( I_d(\lambda) \), the spectral intensity of the light reaching the detector, was recorded; a series of spectra, \( I(\lambda; C) \), were then recorded by changing \( C \), the concentration of the absorber, through stepwise addition of small quantities of a stock solution of the dye rose bengal. The quantity \( A'(\lambda; C) = \log[I_d(\lambda)/I(\lambda; C)] \), hereafter called apparent absorbance, was plotted against wavelength, as in Fig. 2. The inset, where the peak apparent absorbance is plotted against \( C \), shows deviations from Beer’s law. Our main task is to explain this behaviour and to propose a correction procedure, whereby \( A' \) can be converted to a true absorbance \( A \) that is proportional to \( C \) (for an arbitrary value of \( I \)).

For practical purposes, a simple relation between \( A \) and \( A' \) is highly desirable. It turns out that the data plotted in Fig. 3 can be fitted to the expression

\[
A' = a_0 \ln(1 + a_1 A),
\]

which can be inverted to obtain

\[
A = \frac{1}{a_1} \left[ \left( \frac{I_0}{I} \right)^{\gamma} - 1 \right], \quad \gamma = \frac{\log e}{a_0}.
\]

Given \( A' \) and the fitting parameters \( a_0 \) and \( a_1 \), one can determine the true absorbance \( A \). It should be noted that the value of \( a_1 \) depends on the choice of \( I \), but the product \( a_1 A \) does not; we have set \( I = 1 \) cm. The calculated true absorbances should all be superposable, when multiplied by a normalising factor which accounts for the concentration...
dependence. That this is indeed the case can be seen by examining Fig. 4. The normalisation factors \((N)\) are found to be equal (within 5\% error) to the concentration ratios and the effective pathlength comes out to be 166 cm.

One can see, after comparing Eqs. (1) and (2) that the calibration method presented in this paper gives a different function from that found by Ketskeméty and Kozma [10]. Our derivation of Eq. (2) relies on fitting the data points in a wider concentration range than that used earlier in [10]. However, even in the case of very dilute solutions, using Eq. (1) can result in a relatively large error, if \(\gamma\) differs significantly from unity.

Although the calibration was performed by using the absorption values of rose bengal at a certain wavelength, its outcome is a general relation that is applicable to other solutes. In order to substantiate this remark, we show (in Fig. 4) the corrected spectra of malachite green at different concentrations (all normalised to the same peak value). One can see that, even when the dye concentration is as low as 1 \(\mu\)g/l \((1.1 \times 10^{-5} \text{ M})\), it can be detected without any special treatment of the sample, unlike the situation in [15], where the authors applied magnetic solid phase extraction in order to preconcentrate the solution. Malachite green is widely used in the fish farming industries as a fungicide, although it is cytotoxic to mammalian cells [16]. Our method can be a cheap and painless way to control and determine water quality and reduce health risks associated with the use of this and similar dyes.

The very construction of the ICAM confers an important advantage, namely the possibility to record an absorption spectrum that is essentially devoid of scattering. The size of isolated chloroplasts is in the range of 10 \(\mu\)m in diameter, therefore scattering (and also sieving) can distort the measured absorption spectrum. Even if one is using a (conventional) integrating sphere, these distortions cannot be completely eliminated [7]; however, a method has been suggested to compensate for losses due to scattering [6,17].

In Fig. 5, we present the absorption spectrum of isolated chloroplasts, recorded by using a commercial instrument (Hitachi U-3010) equipped with an integrating sphere. The spectrum was corrected for scattering losses as described in [6,17]; the correction eliminated the long tail outside the absorption band and reduced the distortions due to scattering. In the same figure, we have also displayed the absorption spectrum, recorded with the aid of the ICAM, of chloroplasts after a dilution of 1:750; in this range of absorption the correction for the difference in the pathlength has very little effect. One can see that the two spectra, after adjusting their height to the same absorbance at 677 nm, are superposable in the range above 550 nm, proving that the spectrum so obtained is indeed scattering free.

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Fig. 3. Peak apparent absorbance values from Fig. 2 are plotted against the corresponding true values (for a 1 cm pathlength). The symbols show the average values of five independent experiments; standard deviations from the mean values are represented by the error bars. The function \(f(A,\lambda) = a_0 \ln(1 + a_1 A(\lambda))\) was fitted to the data points (solid line) with the parameters \(a_0 = 0.99\) and \(a_1 = 166.3\).

Fig. 4. Corrected and normalised absorption spectra of malachite green (dissolved in distilled water), recorded using the ICAM, compared with the absorption spectrum of the concentrated solution \((10 \text{ mg/l, } 1.1 \times 10^{-5} \text{ M})\) as obtained using a \(1 \times 1 \text{ cm}^2\) cell in a standard spectrometer (Shimadzu UV-1601 PC). The concentration of the dye was 1, 5 and 10 \(\mu\)g/l \((1.1 \times 10^{-9}, 5.5 \times 10^{-9} \text{ and } 1.1 \times 10^{-8} \text{ M})\), respectively. The curves were normalised to the peak absorbance, with \(N\) as indicated.

Fig. 5. Absorption spectra of isolated chloroplasts. Thick grey curve, recorded in a Hitachi U-3010 spectrophotometer equipped with a (conventional) integrating sphere, with further corrections to reduce scattering effects [6,17]. Black curve, recorded using an ICAM, after a dilution of the suspension by a factor of 750. The grey curve was normalised to match the peak absorbance at 677 nm.
The calibration procedure described above was found to be applicable also to an ICAM whose outer surface was covered by a diffuse reflector. This insensitivity of steady-state spectral measurements to the nature of coating is mainly due to the fact that the monitoring beam is not collimated. A detailed comparison, showing that time-resolved measurements are sensitive to the ICAM coating, will be presented elsewhere.

5. Conclusion

The feasibility of using the ICAM for quantitative absorption spectroscopy of non-scattering solutions has been analysed, and a correction procedure for obtaining the true absorption spectra has been developed. It was shown that the correction function depends on the absorbance of the solution, instead of the concentration of the sample alone. The corrected, true spectra agree, within 5%, over a wide range of the concentration. The calibration and correction method presented in this paper is general, but every ICAM–detector system pair needs, it must be concluded, its own calibration procedure. We have also demonstrated that, in the case of scattering samples, the use of the ICAM not only enhances the sensitivity but also reduces scattering-related spectral distortions. As the sample concentration in the sphere increases, the intensity of the monitoring light is attenuated both directly (absorption) and indirectly (effective pathlength), therefore this method is best suited for examining weakly absorbing samples.

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