ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 6, pp. 794–797. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © Z.B. Namsaraev, 2009, published in Mikrobiologiya, 2009, Vol. 78, No. 6, pp. 836–839.

## SHORT COMMUNICATIONS

# Application of Extinction Coefficients for Quantification of Chlorophylls and Bacteriochlorophylls

**Z. B. Namsaraev**[1]

*Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

Received December 04, 2008

**DOI:** 10.1134/S0026261709060174

Spectrophotometric analysis of the chlorophyll *a* and bacteriochlorophyll concentration in cell cultures and environmental samples is an efficient and relatively simple research method necessary for the physiological studies of phototrophic bacteria in cultures and for research in their environmental distribution.

Considerable amount of publications describes various techniques to extract pigments and to calculate the pigment concentration [1, 2]. In general, the procedure is as follows: (1) a sample of a known volume (in case of water or liquid culture samples) or area (bacterial mats and fouling) is collected; (2) the sample is concentrated on a filter, centrifuged, or homogenized; (3) a known volume of a solvent is introduced into the vessel containing the sample (the filter, precipitate, or homogenized biomass); (4) the sample is incubated in the dark in a fridge for complete extraction;( 5) cells are precipitated by centrifugation; (6) the supernatant is carefully transferred to a spectrophotometer cuvette; (7) $Q_y$ absorption peak of the extract is measured against the solvent; and (8) pigment concentration is calculated.

Experience shows that correct calculations of the concentration at the last step of the described procedure may be problematic. While for chlorophyll *a*, absorption coefficients and formula are well-known, problems arise when calculating bacteriochlorophyll (a pigment of anoxygenic phototrophic bacteria, APB) concentration. First, often publications reporting absorption coefficients of bacteriochlorophylls are not easily available to researchers. Secondly, errors may occur in the calculations. The aim of the present work is to help the investigators to solve these problems.

An important source of errors in the calculations of bacteriochlorophyll concentration is the use of the so-called "shortened" formula frequently used for chlorophyll *a*. For example, [Chl *a*] = 11.9 A, where A stands for absorption at 665 nm. It is rather tempting to replace a coefficient 11.9 by bacteriochlorophyll adsorption coefficient. However, it should be kept in mind that the coefficient 11.9 is not the adsorption coefficient of chlorophyll *a*. It is a value obtained by division of a unit by 84 and multiplying the result by 1000, where 84 is the true coefficient of absorption for chlorophyll *a* expressed in $l\ g^{-1}\ cm^{-1}$ (table). Therefore, the error may be significant.

Let's analyze the procedure of calculation. According to the Beer–Lambert–Bouguer law, absorbance (or extinction) A of a solution irradiated with monochromatic light is proportional to the concentration of an absorbing substance in the solution C and optical path length L:

$$A = a \cdot C \cdot L, \quad (1)$$

where a stands for the extinction (or absorptivity, or absorption) coefficient, a constant which characterizes a substance's absorbance at a given wavelength. It is expressed in $l\ g^{-1}\ cm^{-1}$ (specific absorption coefficient). If the concentration of the solution is expressed in $mol\ l^{-1}$, and the path length, in cm, then the coefficient a is designated as ε and called the molar absorption coefficient. It corresponds to a portion of light absorbed by 1 ml of the solution in a 1-ml thick cuvette and is expressed in $M^{-1}\ cm^{-1}$ or $l\ mol^{-1}\ cm^{-1}$. These two coefficients correlate according to the formula:

$$a\ (l\ g^{-1}\ cm^{-1}) \times M\ (g\ mol^{-1}) = \varepsilon\ (l\ mol^{-1}\ cm^{-1}), \quad (2)$$

where M is the molecular weight of a pigment. In practice, the extinction coefficient expressed in tens of thousands $l\ mol^{-1}\ cm^{-1}$ is divided by 1000; thus, the resulting value is expressed in $l\ mM^{-1}\ cm^{-1}$. As follows from equation (1),

$$C = A/(aL). \quad (3)$$

To calculate the concentration in the sample, C should be multiplied by the solvent volume v:

$$C(\text{in sample}) = (A\ v)/(a\ L). \quad (4)$$

Nonspecific adsorption at 750 or 850 nm ($A_{nonsp}$) is subtracted from the measured adsorption ($A_{meas}$). Thus, the final formula in μg per sample appears like

$$C(\mu g/\text{sample}) = ((A_{meas} - A_{nonsp})v\ (ml)1000/a(1\ g^{-1}\ cm^{-1})L(cm)). \quad (5)$$

[1] Corresponding author; e-mail: zorigto@gmail.com

Extinction coefficients of chlorophylls and bacteriochlorophylls

| Pigment | Solvent | Wavelength, nm | a, l $g^{-1}$ $cm^{-1}$ | ε, l $mM^{-1}$ $cm^{-1}$ | Reference |
|---|---|---|---|---|---|
| Chl *a* (893.5*) | Ethanol | 665 | 83.4 | | [8] |
| | Methanol | 665 | 79.95 | 71.43 | [3] |
| | Acetone (80%) | 663 | 85.95 | 76.79 | [3] |
| Chl *b* (907.5) | Methanol | 652 | 42.48 | 38.55 | [3] |
| | Acetone (80%) | 646 | 51.84 | 47.04 | [3] |
| Bchl *a* (911.5) | Methanol | 771 | 60 | 54.8 | [9] |
| | Acetone (80%) | 770 | | 69.3 | [9] |
| | Ethanol | 774 | | 59.6 | [9] |
| | Acetone–methanol (7 : 2) | 771 | | 65.3 | [10] |
| | Diethyl ether | 773 | 105 | | [11] |
| Bchl *b* | Diethyl ether | 791 | | 106 | [10] |
| Bchl *c* (859.5) | Acetone–methanol (7 : 2) | 666 | 86 | 74 | [12] |
| | Methanol | 667 | 86.0 | 69.7 | [13] |
| | Acetone (80%) | 662 | 92.6 | | [14] |
| Bchl *d* (801.9**) | Methanol | 657 | 82.3 | 66.0 | [13] |
| | Acetone (80%) | 654 | 98.0 | | [14] |
| Bchl *e* (834.5***) | Acetone (80%) | 649 | 58.6 | 48.9 | [15] |
| | Acetone–methanol (7 : 2) | 651 | | 41.4 | [15] |
| | Methanol | 660 | | 35.5 | [15] |
| | Ethanol | 654 | | 41 | [15] |
| Bchl *g* (819.5) | Methanol | 762 | 92.7 | 76 | [16] |

Notes: * Molecular mass of the pigment is given in parentheses.
** Recalculated according to [13].
*** Recalculated according to [15].

Note that the factor of 1000 is used to recalculate from parts of milligrams to tens of micrograms. Absorbance A is a nondimensional value; however, it is often estimated in optical density units (ODU) or absorbance units (AU). The formula may be used in the absence of considerable amounts of degradation products and a single pigment present in a sample.

Bacteriochlorophylls *a*, *b*, and *g* in solvents possess characteristic *Qy* peaks at 770, 791, and 762 nm respectively, and thus may be easily determined on a spectrum. However, determination of chlorophylls *a* and *b* (peaks at 663 and 646 nm) and bacteriochlorophylls *c*, *d*, and *e* (667, 657, and 660 nm) present simultaneously in a sample requires additional analysis.

For chlorophylls *a* and *b* (cyanobacteria, higher plants, and green algae are present), a correction is recommended according to the equations proposed by Porra [3] (in μg/ml methanol):

$$[\text{Chl } a] = 16.29\ A_{665} - 8.54\ A_{652},$$
$$[\text{Chl } b] = 30.66\ A_{652} - 13.58\ A_{665}, \quad (6)$$
$$[\text{Chl } a + b] = 22.12\ A_{652} + 2.71\ A_{665}.$$

In μg/ml 80% ethanol, pH 7.8:

$$[\text{Chl } a] = 12.25\ A_{663} - 2.55\ A_{646},$$

$$[\text{Chl } b] = 20.31\ A_{646} - 4.91\ A_{663},$$

$$[\text{Chl } a + b] = 17.76\ A_{646} + 7.34\ A_{663}.$$

In case of simultaneous presence of chlorophylls *a*, *b*, and *c1* + *c2* (diatomic algae and dinoflagellates are also present), correction is required according to the equations of Jeffrey and Humphrey [4] in μg/ml 90% acetone:

$$[\text{Chl } a] = 11.85\ A_{664} - 1.54\ A_{647} - 0.08A_{630},$$

$$[\text{Chl } b] = 21.03\ A_{647} - 5.43\ A_{664} - 2.66\ A_{630}, \quad (7)$$

$$[\text{Chl } c1 + c2] = 24.52A_{630} - 7.60A_{647} - 1.67A_{664}.$$

In equations (6) and (7), readings at 750 nm should be initially subtracted from the optical density values. Then, the values obtained in equations (6) and (7) may be introduced into the final formula where v is the solvent volume in ml and L is thickness of a cuvette in cm:

$$C(\mu g/\text{sample}) = [\text{Chl}]\ v(\text{ml})/L(\text{cm}). \quad (8)$$

In case of simultaneous presence of chlorophylls *a* and *b*, and bacteriochlorophyll *c* in the sample (for example, hydrothermal cyanobacteria mats containing an APB *Chloroflexus aurantiacus*), Madigan and Brock suggest that the following procedure should be followed: (1) measure the peak absorption at 665 nm in a solvent; (2) isolate the pigments by chromatography; (3) measure the absorption of isolated pigments at 665 nm; (4) and divide the common peak height in proportion to the concentrations of purified pigments [5]. Castenholz proposes an even simpler calculation method: (1) measure the absorbance of the common peak at 665 nm in a solvent; (2) using the in vivo spectrum of cellular membranes in 50% glycerol, determine the peak height for chlorophyll *a* at 680 nm and that for bacteriochlorophyll *c* at 740 nm; and (3) divide the height of the common peak in proportion to the in vivo peak height [6]. To quantify bacteriochlorophyll *d* (of green APB of the genus *Chlorobium*) in a mixture with chlorophylls *a, b*, and *c*, Gorbunov and Umanskaya proposed the following formula (absorbance at 850 nm should be initially subtracted) [7]:

$$\text{Bchl } d = -12.88A_{664} + 23.45A_{654} - 9.54A_{647} + 0.86A_{633}. \quad (9)$$

At the final step of calculation the obtained values are recalculated with respect to the initial (before concentrating) sample volume or area.

## ACKNOWLEDGMENTS

The work was supported by grants of the Russian Foundation for Basic Research (project no. 07-04-00651a) and the Program of the Presidium of the Russian Academy of Sciences “Biosphere Origin and Evolution”.

## REFERENCES

1. Fedorov, V.D., *O metodakh izucheniya fitoplanktona i ego aktivnosti.* (Techniques for Investigation of Phytoplankton and Its Activity), Moscow: Mosk. Gos. Univ., 1979.
2. Khromov, V.M. and Semin, V.A., *Metody opredeleniya pervichnoi produktsii v vodoemakh.* (Techniques for Determination of Primary Production in Water Bodies), Moscow: Mosk. Gos. Univ., 1975.
3. Porra, R.J., Thompson, W.A., and Kriedemann, P.E., Determination of Accurate Extinction Coefficients and Simultaneous Equations for Assaying Chlorophylls *a* and *b* Extracted with Four Different Solvents: Verification of the Concentration of Chlorophyll Standards by Atomic Absorption Spectrometry, *Biochim. Biophys. Acta*, 1989, vol. 975, pp. 384–394.
4. Jeffrey, S.W. and Humphrey, G.F., New Spectrophotometric Equations for Determining Chlorophylls *a*, *b, c1* + *c2* in Higher Plants, Algae and Natural Phytoplankton, *Biochem. Physiol. Pflanzen.*, 1975, vol. 167, pp. 191–194.
5. Madigan, M.T. and Brock, T.D., Quantitative Estimation of Bacteriochlorophyll *c* in the Presence of Chlorophyll *a* in Aquatic Environments, *Limnol. Oceanogr.*, 1976, vol. 21, pp. 462–467.
6. Castenholz, R.W., Composition of Hot Spring Microbial Mats: A Summary, in *Microbial Mats: Stromatolites*, Cohen, Y., Castenholz, H.O., and Halvorson, R.W., Eds., 1984, New York: Alan R. Liss, pp. 101—119.
7. Gorbunov, M.Yu. and Umanskaya, M.V., Anoxygenic Phototrophic Bacteria in the Water Reservoirs of Protected Territories in the Samara Oblast, in *Ekologicheskie problemy zapovednykh territorii Rossii* (Ecological Problems of Protected Territories in Russia), Saksonov, S.V., Ed., Tolyatti: IEVB RAN, 2003, pp. 136–144.
8. Wintermans, J.F.G.M. and DeMots, A., Spectrophotometric Characteristics of Chlorophylls *a* and *b* and Their Pheophytins in Ethanol, *Biochim. Biophys. Acta*, 1965, vol. 109, pp. 448–453.
9. Permentier, H.P., Schmidt, K.A., Kobayashi, M., Akiyama, M., Hager-Braun, C., Neerken, S., Miller, M., and Amesz, J., Composition and Optical Properties of Reaction Centre Core Complexes from the Green Sulfur Bacteria *Prosthecochloris aestuarii* and *Chlorobium tepidum, Photosynth. Res.*, 2000, vol. 64, pp. 27–39.
10. Oelze, J., Analysis of Bacteriochlorophylls, *Methods in Microbiology*, 1985, vol. 18, pp. 257–284.
11. Clayton, R.K., Absorption Spectra of Photosynthetic Bacteria and Their Chlorophylls, in *Bacterial Photosynthesis. Symp. on Bacterial Photosynthesis, Yellow Springs, Ohio, March 18–20, 1963*, Gest, H., San Pietro, A., and Vernon, L.P., Eds., Ann Arbor: Edwards Brothers, pp. 495–500.
12. Feick, R.G., Fitzpatrick, M., and Fuller, R.C., Isolation and Characterization of Cytoplasmic Membranes and Chlorosomes from the Green Bacterium *Chloroflexus aurantiacus, J. Bacteriol.*, 1982, vol. 150, pp. 905–915.
13. Maresca, J.A., Chew, A.G.M., Ponsatí, M.R., Frigaard, N.-U., Ormerod, J.G., and Bryant, D.A., The *bchU* Gene of *Chlorobium tepidum* Encodes the C-20 Methyltrans-

ferase in Bacteriochlorophyll *c* Biosynthesis, *J. Bacteriol.*, 2004, vol. 186, pp. 2558–2566.

14. Stanier, R.Y. and Smith, J.H.C., The Chlorophylls of Green Bacteria, *Biochim. Biophys. Acta*, 1960, vol. 41, pp. 478–484.
15. Borrego, C.M., Arellano, J.B., Abella, C.A., Gillbro, T., and Garcia-Gil, L.J., The Molar Extinction Coefficient of Bacteriochlorophyll *e* and the Pigment Stoichiometry in *Chlorobium phaeobacteroides, Photosynth. Res.*, 1999, vol. 60, pp. 257–264.
16. Van de Meent, E., Kobayashi, M., Erkelens, C., van Veelen, P.A., Amesz, J., and Watanabe, T., Identification of 81-Hydroxychlorophyll *a* as a Functional Reaction Center Pigment in *Heliobacteria, Biochim. Biophys. Acta,* 1991, vol. 1058, pp. 356–362.