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TECHNICAL COMMUNICATION

The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroides*

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Abstract

We have determined the molar extinction coefficient of bacteriochlorophyll (BChl) *e*, the main light-harvesting pigment from brown-coloured photosynthetic sulfur bacteria. The extinction coefficient was determined using pure [Pr,E] BChl e_F isolated by reversed-phase HPLC from crude pigment extracts of *Chlorobium* (*Chl.*) *phaeobacteroides* strain CL1401. The extinction coefficients at the Soret and Q_y bands were determined in four organic solvents. The extinction coefficient of BChl *e* differs from those of other related *Chlorobium* chlorophylls (BChl *c* and BChl *d*) but is similar to that of chlorophyll *b*. The determined extinction coefficient was used to calculate the stoichiometric BChl *e* to BChl *a* and BChl *e* to carotenoids ratios in whole cells and isolated chlorosomes from *Chl. phaeobacteroides* CL1401 using the spectrum-reconstruction method (SRCM) described by Naqvi et al. (1997) (Spectrochim Acta A Mol Biomol Spectrosc 53, 2229–2234). In isolated chlorosomes, BChl *a* content was ca. 1% of the total BChl content and the stoichiometric ratio of BChl *e* to carotenoids was 6. In whole cells, however, BChl *a* content was 3–4 %, owing to the presence of BChl *a*-containing elements, i.e. FMO protein and reaction centre. An average of 5 BChl *e* molecules per carotenoid was determined in whole cells.

Abbreviations: BChl – bacteriochlorophyll; [E,E]BChl e_F – 8-ethyl, 12-ethyl, BChl *e* esterified with farnesyl (F). Analogously: I – isobutyl; N – neopentyl; Pr – propyl (see Smith 1994); Car – carotenoids; Chl – chlorophyll; FMO – Fenna-Matthews-Olson BChl *a*-protein; Isr – Isorenie-

ratene; Rt – retention time; SRCM – spectrum-reconstruction method.

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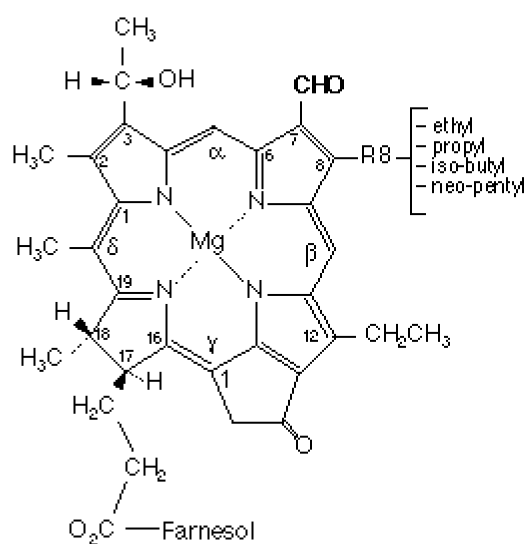
Introduction

Bacteriochlorophyll (BChl) *e* is the main light-harvesting pigment in brown species of green sulfur bacteria. It was first isolated and structurally characterised by Gloe and co-workers (1975). BChl *e*, like other related pigments found in photosynthetic green bacteria (BChl *c* and *d*), is a magnesium dihydroporphyrin with an isocyclic pentanone ring V and a propionic ester group at C17. However, BChl *e* differs from such pigments in a formyl group at C7 (Fig. 1), which relates BChl *e* to eukaryotic Chl *b*, as pointed out by several authors (Gloe et al. 1975, Otte et al. 1993; Richards 1994). Like other chlorosome chlorophylls, BChl *e* is not a single molecular form but a mixture of up to 15 homologs that mainly vary in the alkylation at positions C8 and C12, in the chirality of the $C3^1$-center, and in the nature of the esterified alcohol at C17 (Gloe et al. 1975; Brockmann 1976; Smith and Simpson 1986; Senge and Smith, 1995). These homologous forms are tightly packed inside the antenna complexes of green bacteria, referred to as chlorosomes (Blankenship et al. 1995 and references therein). Chlorosomes also contain variable amounts of carotenoids (Schmidt 1980) although no information is available on their precise location and function. In brown-coloured species of Chlorobiaceae isorenieratene and β-isorenieratene are the main carotenoids found (Overmann et al. 1992). Recent research suggests that carotenoids are not directly involved in pigment organisation in chlorosomes (Frese et al. 1997, Aschenbrücker et al. 1998). The hydrophobic nature of the chlorosome matrix permits coupling interactions between the chromophores, which form protein-free, rod-like BChl elements with a high level of organisation (Holzwarth and Shaffner 1994; Blankenship et al. 1995). As a result of the precise orientation and arrangement of these pigments, chlorosomes are one of the most efficient light harvesting and energy transduction structures found in photosynthetic organisms (Olson 1998).

So far, BChl *e* was the only pigment from photosynthetic bacteria for which the molar extinction coefficient was unknown. However, this problem was traditionally solved by the application of extinction coefficients from other related pigments, either BChl *d* (Montesinos et al. 1983) or, more recently, Chl *b* (Otte et al. 1993). This solution has led to under- or

overestimation of the real concentrations of BChl *e*. In addition, the variability of the molar extinction coefficient values for the related (bacterio)chlorophylls found in the literature introduces more uncertainty in the determination of BChl *e* concentrations in photosynthetic preparations of brown-coloured species of green sulfur bacteria. Here, we provide a reliable molar extinction coefficient for BChl *e*, which can be useful in studies on eco-physiological, biochemical, and biophysical aspects of brown-coloured Chlorobiaceae. We also applied the molar coefficient of BChl *e* to calculate stoichiometric ratios between the different pigment pools in whole cells and isolated chlorosomes from *Chlorobium phaeobacteroides* strain



CL1401.

Figure 1. Chemical structure of bacteriochlorophyll *e*.

Materials and Methods

Organism and growth conditions

Chlorobium phaeobacteroides strain CL1401 isolated from Sisó Lake (Banyoles, Spain) was kindly provided by Dr. R. Guyoneaud. The culture was grown in standard Pfennig mineral medium (Trüper and Pfennig 1992) in a 5-litre glass bottle under continuous stirring. Illumination was continuously provided by two Philips SL25 fluorescent lamps giving an

averaged light intensity of 100 µmol m^{-2} s^{-1} at the surface of the culture bottle. The incident light was measured between 300 and 1100 nm using a LI-COR 1800-1W spectroradiometer with a remote cosine-corrected receptor (LI-COR 1800-11). Cells were harvested at the beginning of the stationary phase by centrifugation at 16,000 x *g* for 20 min at 4 °C in a Sorvall RC5B. Pellets were stored at –80 °C.

Isolation of chlorosomes

Chlorosomes were isolated as described by Gerola and Olson (1986), with some modifications. A 5 g aliquot of the original frozen cell pellet was thawed and resuspended in 50 mM Tris-HCl buffer, pH 8.0, and centrifuged at 23,500 x *g* for 15 min at 4 °C. The pellet was then resuspended in 10 mM potassium phosphate buffer, pH 7.4, with 10 mM sodium ascorbate. After homogenisation, cells were passed 3 times through an ice-cold French Pressure Cell operating at 20,000 psi. The broken cells were centrifuged at 39,000 x *g* for 15 min at 4 °C to remove intact cells and large pieces of debris. The supernatant was loaded onto a 20–50% (w/w) continuous sucrose gradient prepared in PA buffer and centrifuged overnight at 218,000 x *g* at 4 °C in a Sorvall O7D 75B ultracentrifuge. The fraction containing chlorosomes appeared as a dark brown band at about 25% sucrose. Membrane fragments banded at 40% sucrose as a reddish-brown band. Chlorosomes were further purified using sucrose flotation gradients according to Steensgaard et al. (1997). Gradients were centrifuged overnight as described above. Flotation gradients yielded chlorosome fractions at 20% sucrose, which were collected and stored at –80 °C.

Extraction of pigments and HPLC analysis

For the determination of the extinction coefficient of BChl *e*, pigments were extracted from thawed cell pellets with 50 ml of acetone:methanol (7:2) (Scharlau, Multisolvent grade). The extract was stored at –30 °C for 24 h and then centrifuged at 16,000 x *g* for 15 min at 4 °C. The supernatant was dried under a stream of nitrogen and stored at –30 °C in the dark. Dry pigments were dissolved in 10 ml of methanol (Scharlau, HPLC grade) and passed through a 0.2

μm Dynagard syringe filter prior to reversed-phase HPLC analysis. Samples were analysed according to Borrego and Garcia-Gil (1994), with the following modifications: pigments were separated in a 10 x 250 mm, 10 μm particle size, Spherisorb ODS2 Semi Prep column at a flow rate of 2.0 ml min^{-1}. Prior to injection, 10% of ammonium acetate (1 M) was added to the sample as ion pairing agent to improve the resolution of the BChl homolog separation.

For stoichiometric measurements, pigments from whole cell pellets and isolated chlorosomes were extracted in ethanol (Scharlau, Multisolvent Grade) and centrifuged at 12,500 x *g* for 10 min at room temperature to pellet the unsolubilised proteins. Extracts were kept in the dark until the absorption spectra were recorded. BChl *e* and Isr were purified as described below. BChl *a* was extracted from the LH1 complex of *Rhodospirillum rubrum* S1 and then purified by thin layer chromatography. The molar extinction coefficient of BChl *a* was 62,000 l $mol^{-1}cm^{-1}$ in ethanol (Connolly et al. 1982). For Isr, a value of 109,800 l $mol^{-1}cm^{-1}$ in petroleum ether was used as extinction coefficient (Britton 1995). Stoichiometric ratios of BChl *e* to BChl *a* and BChl *e* to carotenoid(s) were determined using the spectrum-reconstruction method (SRCM) described by Naqvi et al. (1997). All spectra were recorded on a Milton Roy 3000 diode array spectrophotometer.

Results

Isolation of the [Pr,E]BChl e_F *homolog*

The HPLC analysis of the crude pigment extract from *Chlorobium phaeobacteroides* showed the typical elution pattern for BChl *e* homologs (Borrego and Garcia-Gil 1994) (Fig. 2A). BChl *e* eluted in two peak groups. The former consisted of a four-peak cluster (Rt from 24 to 27 min), assigned to the main farnesyl-esterified BChl *e* homologs, which eluted in increasing order of alkylation, i.e. [E,E], [Pr,E], [I,E], and [N,E]BChl e_F. The second group (Rt from 33 to 40 min) was composed of several peaks corresponding to minor BChl *e* homologs with esterifying alcohols other than farnesyl (Borrego and Garcia-Gil 1994; Francke and Amesz 1997). BChl *a* eluted at 39.5 min as a single peak traceable at 770 nm. Carotenoids eluted as two groups: the first, attributed to isorenieratene, eluting at 49.5 min, and the second, eluting at

53.5 min, corresponded to β-isorenieratene. Both carotenoids eluted as a cluster of two peaks, corresponding to the all *trans*- and *cis*- isomers, respectively.

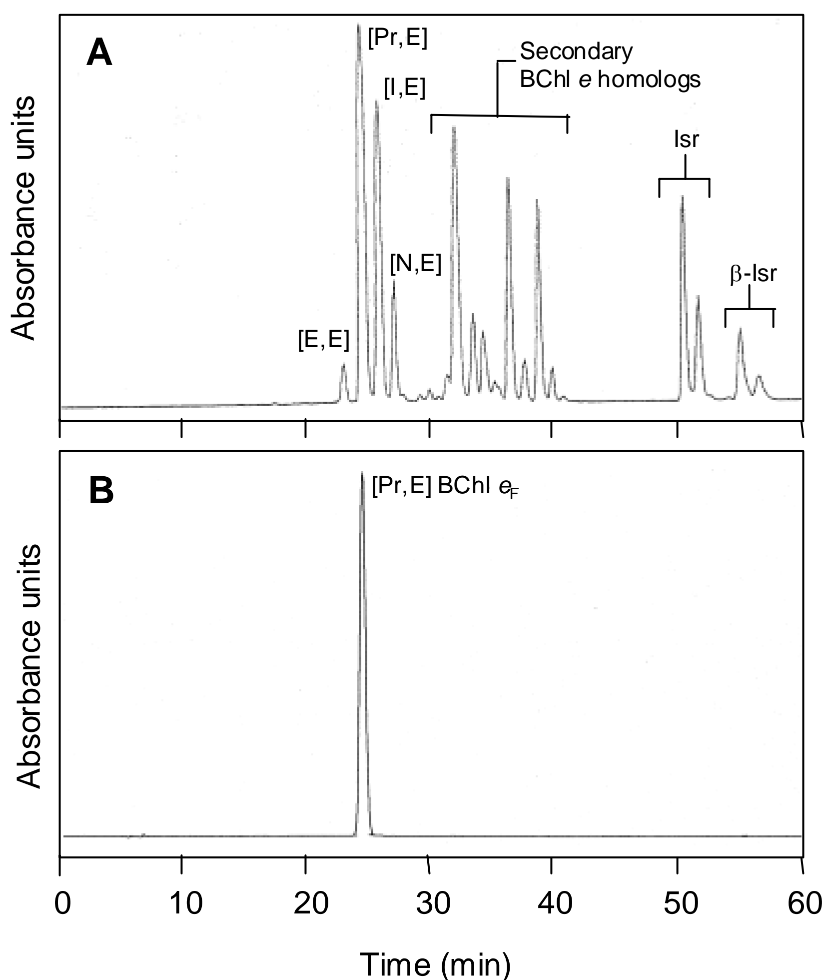


Figure 2. (A) HPLC trace at 473 nm of the crude extract of *Chlorobium phaeobacteroides* pigments where the different BChl *e* homologs are shown. Carotenoids (Isr and β-Isr) eluted at the end of the run. (B) HPLC trace at 473 nm of a pure sample of the isolated [Pr,E] BChl e_F homolog.

The crude pigment extract was repeatedly injected in the chromatographic system in 1 ml aliquots. Fractions corresponding to the second BChl *e* homolog —identified as [Pr,E]BChl e_F— were collected in a glass vial wrapped in aluminium foil. The pigment fraction was immediately dried and stored under a stream of nitrogen. Several HPLC runs were carried out until the crude extract ran out. After the first series of HPLC runs the [Pr,E]BChl e_F pools were redissolved in methanol and re-injected into the chromatographic system in the same identical reversed-phase conditions to further purify BChl *e*. This second analysis confirmed that the manipulation of the sample had been satisfactory since neither detectable traces of the other

BChl *e* homologs nor degradation products, e.g. bacteriopheophytins, were detected (Fig. 2B). The retention time and the absorption spectrum of [Pr,E]BChl e_F did not change during manipulation. The molar extinction coefficient of BChl *e* was determined as soon as sufficient material from the HPLC purification had been collected.

Calculation of the molar extinction coefficient

Pure [Pr, E]BChl e_F was thoroughly dried under a stream of nitrogen and weighed in a HR60 analytical scales (A&D Instruments). Then, 2 mg of [Pr,E]BChl e_F was poured into a volumetric flask and dissolved in acetone. Several dilutions were prepared from the sample solution and the absorption spectrum of each one was recorded in a 1-cm path-length, quartz cuvette. The molar extinction coefficients of [Pr,E]BChl e_F were then determined at the maximum absorbance peaks of the Soret and Q_y bands using a molecular weight of 835.1. Several organic solvents such as acetone, methanol, ethanol, and the mixture acetone:methanol (7:2) were used too. The values of the molar extinction coefficients at the Soret and Q_y bands of [Pr,E]BChl e_F in all the solvents studied are given in Table 1.

Table 1: Molar extinction coefficients for BChl *e* at the Soret and Q_y bands in the different solvents used.

Solvent	Soret band (nm)	$_{Soret}$ (mM^{-1} cm^{-1})	Q_y band (nm)	$_{Qy}$ * (mM^{-1} cm^{-1})
Acetone	462	185.0 ± 4.0	649	48.9 ± 2.3
Acetone:Methanol (7:2)	466	155.6 ± 0.1	651	41.4 ± 0.7
Methanol	476	130.7 ± 2.6	660	35.5 ± 0.8
Ethanol	469	142.3 ± 4.3	654	41.0 ± 1.2

*: Molar extinction coefficients ($_{mM}$) at the Q_y band in 100% acetone for other related (bacterio)chlorophylls:
Chl *b* = 47 at 645 nm (MacKinney 1940); 49.3 at 647 nm (Vernon, 1960); 49.3 at 644 nm (Ziegler and Egle 1965); 46.9 at 644.8 nm (Lichtenthaler 1987).
BChl *a* = 69 at 770 nm (Sauer et al., 1966); 71.5 at 770 nm (Connolly et al. 1982); 84 at 768 nm (Korthals and Steenbergen 1985)
BChl *c* = 74 at 662.5 (Stanier and Smith 1960); 75.4 at 662.5 nm (Oelze 1985).
BChl *d* = 78 at 654 (Stanier and Smith 1960); 78.9 at 654 nm (Oelze 1985).

Pigment stoichiometry in cells and chlorosomes from Chlorobium phaeobacteroides

Having determined the molar extinction coefficient of [Pr,E]BChl e_F we performed the pigment analysis of the whole cells and chlorosomes isolated from *Chl. phaeobacteroides* using

the SRCM. Chlorosomes have three groups of pigments: (i) The BChl *e* group, which includes the main four homologs, i.e. [E,E], [Pr,E], [I,E], and [N,E]BChl e_F, plus several non-farnesyl esterified BChl *e* forms. Since the absorption properties of a chromophore are mainly derived from the conjugated π system rather than the macrocycle substituents, all the BChl *e* homologs were assumed to have the same molar extinction coefficient. This assumption was supported by the observation that the different BChl *e* homologs have identical absorption spectra in organic solvents (Otte et al. 1993; Borrego and Garcia-Gil 1994; Blankenship et al. 1995). (ii) The carotenoids, composed of Isr and β-Isr, which had been assumed to have the same extinction coefficient of 107,000 l $mol^{-1}cm^{-1}$ in ethanol. This value was determined from a solution of Isr in petroleum ether, whose absorbance was previously known. The petroleum ether was then dried under a stream of nitrogen and the Isr was dissolved in ethanol. Changes in absorption peaks keeping the same Isr concentration were attributed to different molar extinction coefficients of Isr in these two solvents. (iii) The baseplate BChl *a*, which acts as an intermediate in the energy transfer from antenna pigments to FMO proteins in the membrane (Gerola and Olson 1986).

Chlorosome and whole cell pigments were extracted in ethanol as indicated in the Materials and Methods section. The absorption spectrum of the sample was recorded between 320 and 880 nm and denoted as $A_p(\lambda)$ according to the notation given by Naqvi and co-workers (1997). The solutions of pure BChl *e,* BChl *a*, and Isr were prepared in ethanol to give an absorbance between 0.5 and 1.0 at the corresponding peak maximum. The absorption spectra of these solutions, denoted by $F_{BChle}(\lambda)$, $F_{Car}(\lambda)$ and $F_{BChla}(\lambda)$, were recorded. The function:

$$A_m(\lambda) = \alpha F_{BChle}(\lambda) + \beta F_{Car}(\lambda) + \gamma F_{BChla}(\lambda) \quad (1)$$

was then fitted by using a linear unweighted least-squares routine to $A_p(\lambda)$ in the 320–880 nm region. The peak absorbance values of the spectra $\alpha F_{BChle}(\lambda) \equiv A_{Bchle}(\lambda)$, $\beta F_{Car}(\lambda) \equiv A_{Car}(\lambda)$, and $\gamma F_{BChla}(\lambda) \equiv A_{BChla}(\lambda)$ were determined from the best fit obtained; a prime will henceforth be used to designate the peak values of the absorbances and the corresponding extinction

coefficients. The BChl *e*:BChl *a* and BChl *e*:Car ratios can then be calculated, by using these peak absorption values and the molar extinction coefficients (ε) for the respective pigments in ethanol, by means of the following relations:

$$\frac{C_{BChle}}{C_{Car}} = \frac{A'_{BChle}\varepsilon'_{Car}}{A'_{Car}\varepsilon'_{BChle}} \tag{2}$$

$$\frac{C_{BChle}}{C_{BChla}} = \frac{A'_{BChle}\varepsilon'_{BChla}}{A'_{BChla}\varepsilon'_{BChle}} \tag{3}$$

The total absorption spectrum $A_p(\lambda)$ of the isolated chlorosomes and whole cells from *Chlorobium phaeobacteroides* in ethanol and the fitted function $A_m(\lambda)$ match all along the 320–880 nm region of the absorption spectrum, proving the reliability of the fit (Fig. 3). The average BChl *a* content was 3–4 % and 0.9 % of the total BChl content in whole cells and isolated chlorosomes, respectively. The BChl *e* to carotenoid ratios was 5:1 in whole cells and 6:1 in chlorosomes.

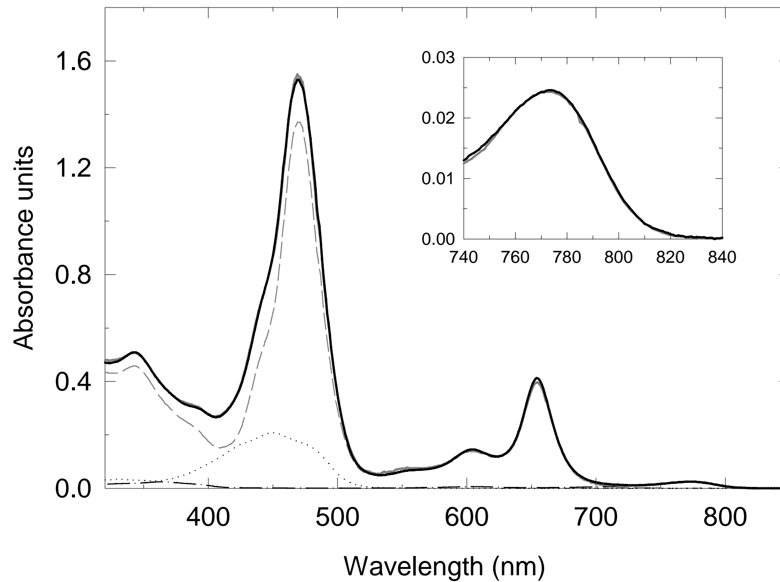


Figure 3. Absorption spectrum of the pigment extract of *Chlorobium phaeobacteroides* CL1401 cells in ethanol, $A_p(\lambda)$ (thick-black), given along with the reconstruction spectrum $A_m(\lambda)$ (thick-grey). The $F_{BChle}(\lambda)$ (grey-dashed), $F_{Car}(\lambda)$ (black-dotted), and $F_{BChla}(\lambda)$ (black dash-dotted) are the absorption spectra of the individual pigments in ethanol. The insert shows the fit of BChl *a* in the 740–840. See text for more details.

Discussion

BChl *e* was first characterised by Gloe and co-workers in the mid-seventies (Gloe et al. 1975). However, the molar extinction coefficient of this pigment has not been determined. This has hindered calculation of its concentration in brown-coloured photosynthetic sulfur bacterial samples. To overcome this drawback, the molar extinction coefficients of other related pigments (BChl *d* and Chl *b*) have been used instead (Montesinos et al. 1983; Guerrero et al. 1985; Otte et al. 1993). This solution led to a critical underestimation of up to 40% of the BChl *e* concentration when the molar extinction coefficient of BChl *d* at the Q_y band was used. This error arises from the great spectral differences between BChl *d* and BChl *e*. A more precise approach was later proposed by Otte et al. (1993), who suggested the use of the extinction coefficient of eukaryotic Chl *b* because of the spectral similarities between this pigment and BChl *e*. Since then, the Chl *b* extinction coefficient has been used in most of the studies dealing with brown-coloured green photosynthetic bacteria (Borrego and Garcia-Gil 1995, Borrego et al. 1997; Francke and Amesz 1997). However, some structural differences between Chl *b* and BChl *e* can still be distinguished, for example, the presence of a vinyl group in ring I of Chl *b*, which is absent in BChl *e*. The contribution of this vinyl group to the π-conjugation system of Chl *b* could be responsible for differences in the extinction coefficient of the two pigments. In addition, although the substituents at positions C8 and C12 do not influence the absorption spectra, the methyl group at C20 of the BChl *e* might introduce steric hindrance and distort the planarity of the porphyrin ring. This steric hindrance, rather than the contribution of the methyl group itself to the overall π-conjugation system, might be responsible for the differences in the spectral properties of BChl *e* and Chl *b*, in the same way as BChl *c* differs spectrally from BChl *d*.

Our results are consistent with the previous proposal by Otte and co-workers concerning the similarity of the extinction coefficient of BChl *e* to that of Chl *b*, although it differs greatly from those of other *Chlorobium* chlorophylls (Table 1). However, the application of the molar extinction coefficient of Chl *b* still led to errors in the determination of BChl *e* concentration.

An overestimation of up to 4% and an underestimation of up to 10% were obtained using the Q_y extinction coefficient values of Chl *b* reported in Table 1. We are confident about the further use of the new coefficient for future studies on biochemical, biophysical, and ecological aspects of brown Chlorobiaceae.

Once determined, the BChl *e* extinction coefficient was used to calculate pigment ratios in whole cells and chlorosomes from *Chl. phaeobacteroides* strain CL1401. Stoichiometric pigment ratios were calculated using the SRCM, which has been applied to biological preparations of higher plants to analyse the stoichiometric ratio of Chl *a*/Chl *b* in the light harvesting complex II (LHC II) of pea leaves (*Pisum sativum*) and to determine the total pigment content in oat leaves (*Avena sativa*) (Naqvi et al. 1997). Recently, the application of this method in preparations of purple bacteria provided information about the number of carotenoids per α, β-monomer in the LH1 and LH2 light harvesting complexes of purple bacteria (Arellano et al. 1998). SRCM is, therefore, suitable for preparations of green bacteria of known pigment composition. The BChl *a* content determined in the chlorosomes was 0.9 % of the total BChl content. This is consistent with values reported elsewhere for baseplate-BChl *a* (Gerola and Olson 1986, Olson 1998). The higher percentage of BChl *a* found in whole cells (3–4 %) is attributed to the presence of FMO proteins and reaction centres in the membrane. The mean stoichiometric ratio of BChl *e* to carotenoids was 5:1 and 6:1 in whole cells and isolated chlorosomes, respectively. The bulk of carotenoids (~ 80 %) is then present in chlorosomes. The remaining 20 % may correspond to carotenoids in the reaction centre, newly synthesised carotenoids which have not yet been incorporated into chlorosomes, or oleosome-like structures similar to those found in *Chloroflexus aurantiacus* (Beyer et al. 1983).

The pigment ratios reported here for *Chl. phaeobacteroides* CL1401 were dependent on light conditions and growth phase at which the bacterial cultures were harvested (data not shown). Since the cell pigment composition in green photosynthetic bacteria is greatly dependent on the bacterial species and growth conditions (Oelze and Golecki 1995 and references therein), some variations in pigment ratios should be expected when cells grow in

other conditions. Future work may provide more information about the content of different pigments and their variability range in green photosynthetic bacteria.

In conclusion, we have determined the extinction coefficient of BChl *e* in a few organic solvents for the first time. These experimental extinction coefficients will be a very valuable resort for all those research works related to this pigment of brown-coloured photosynthetic sulfur bacteria.

Acknowledgements

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