

Carotenoid-to-chlorophyll ratio as a proxy for assay of total fatty acids and arachidonic acid content in the green microalga *Parietochloris incisa*

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Abstract The relationships between pigment (carotenoid and chlorophyll) content with accumulation of total fatty acids (TFA) and arachidonic acid (AA) were studied in the green microalga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta) grown under different PFDs (35, 200, and 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and nitrogen availabilities. The growth of *P. incisa* under higher light and nitrogen deficiency was accompanied by accumulation of FA, an increase in carotenoid and a decline in chlorophyll content. It was found that the carotenoid-to-chlorophyll ratio (but not the individual pigment content) correlates closely with the volumetric content of both TFA and AA. Analysis of scattering-compensated absorption spectra of *P. incisa* suspensions revealed their tight relationship in the blue-green range of the spectrum with the carotenoid-to-chlorophyll ratio, TFA, and AA content. These findings allowed the development of algorithms for the non-destructive assay of TFA and AA in cell suspensions in the ranges of 0.09–3.04 and 0.04–1.7 $\mu\text{g mL}^{-1}$, with accuracy of 0.06 and 0.01 $\mu\text{g mL}^{-1}$, respectively, via analytically measured carotenoid-to-chlorophyll ratio and using the ratio of absorption coefficients at 510 and 678 nm, with accuracy of 0.07 and 0.02 $\mu\text{g mL}^{-1}$, respectively. The feasibility of obtaining essential spectral information con-

cerning the physiological condition of *P. incisa* using a standard spectrophotometer is also shown.

Keywords Absorption spectra · Non-destructive assay · Fatty acids · Photoadaptation · Scattering correction

Abbreviations

$\tilde{A}(\lambda)$	scattering-free absorption spectrum
AA	arachidonic acid
TAG	triacylglycerols
TFA	total fatty acids
PUFA	polyunsaturated fatty acids

Introduction

A regular sampling with subsequent ‘wet’ chemical analysis is employed traditionally for monitoring physiological condition and biochemical composition of algae in the course of their cultivation. These methods are precise but laborious and time-consuming, requiring complete extraction and chromatographic analysis of the compounds of interest. Therefore, the development of efficient and rapid techniques for monitoring physiological conditions of cultivated algae is of considerable importance in microalgal biotechnology (Vonshak 1985; Borowitzka and Borowitzka 1988; Gitelson et al. 2000; Borowitzka 2005). The changes in algal metabolism, both developmental and stress-induced, are often accompanied by dramatic and specific changes in pigment content and composition which subsequently affect optical properties of algal cells and cell suspensions. Therefore, non-destructive approaches based on application of optical spectroscopy often look promising for assessment of the algal culture condition (Gitelson et al. 1996, 2000; Rabbani et al. 1998;

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Merzlyak and Naqvi 2000; Merzlyak et al. 2008). These methods are suitable for on-line monitoring of culture growth, early detection of damage, and selection of optimal time for biomass harvesting.

The green oleaginous freshwater green microalga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta) is the richest plant source (Khozin-Goldberg et al. 2002) of the polyunsaturated ω -6 arachidonic acid (AA). Arachidonic acid is an essential, structural, and functional constituent of cell membranes, and is especially required for growth and function of the brain and vascular system of pre-term babies (Hansen et al. 1997; Crawford et al. 2003).

Previously, we found that long-term cultivation of the alga under nitrogen-deprivation and weak irradiation in batch culture induced both an accumulation of AA and remarkable changes in chlorophyll (Chl) and carotenoid (Car) content as well as in the optical spectra of cell suspensions. We found that parameters of Chl absorption, characteristic of so-called 'packing affect', strongly correlated with AA level in the cells during long-term growth under low light. Although a considerable decline in Chl was recorded on the background of Car retention, these processes showed only a weak correlation with TFA and/or AA accumulation (Merzlyak et al. 2007). Changes in the fatty acid and pigment composition and content of *P. incisa* were also studied as a function of irradiance and nitrogen availability under conditions providing for higher growth rates of the culture (Solovchenko et al. 2008a, b). The exposure of algal cells to high light and lack of nitrogen resulted in an increase in the relative Car content occurring in parallel with the accumulation of AA-rich triacylglycerols (TAG).

In this paper, we report the interrelationships between lipid accumulation, changes in pigment content, and suspension light absorption revealed using the quantitative data acquired previously for *P. incisa* grown under different PFDs (35, 200, and 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and nitrogen availability (Solovchenko et al. 2008a, b) and make an attempt to employ the observed changes for the development of techniques for non-destructive assessment of total fatty acids (TFA) and AA in *P. incisa*. In addition, we demonstrate the possibility of measuring scattering-corrected light absorption by the microalgal cell suspensions with a standard spectrophotometer.

Materials and methods

Parietochloris incisa cultivation conditions and patterns of the culture growth have been published previously (Solovchenko et al. 2008a, b). The total fatty acid (Solovchenko et al. 2008a) and pigment (Solovchenko et al. 2008b) contents were analyzed in the inoculum and following 3, 7, 10, and 14 days

of growth under three photon flux densities (PFDs) (35, 200, and 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) on complete (+N) or nitrogen-lacking (-N) BG-11 medium.

The absorbance spectra of *P. incisa* cell extracts and suspensions were recorded using a Cary 50 Bio spectrophotometer (Varian, USA). To eliminate influence of scattering, a technique similar to that suggested by Shibata (1973) for measuring turbid biological samples was used. For this purpose, two wet GF/F glass-wool filters (Schleicher & Schuell, Germany), as light diffusers, were mounted on the output windows of the cuvette compartment of the spectrophotometer. A 1-cm glass cuvette with cell suspension was placed as close as possible to the filter and measured against a cuvette with BG-11 medium. Since the measurements did not provide a complete collection of scattered light, the methodology developed by Merzlyak and Naqvi (2000) and Merzlyak et al. (2008) was adopted and the second spectrum of the suspension was taken with the cuvette placed at about 10 mm distance. The spectra were then used for calculation of the 'true' absorption spectra essentially free of scattering contribution according to Merzlyak and Naqvi (2000) and Merzlyak et al. (2008). The spectra obtained, $\tilde{A}(\lambda)$, were found to be compatible with the scattering compensated spectra presented for *P. incisa* (Merzlyak et al. 2007) and were used for further analysis.

Results

Relationships between the changes in lipid and pigment content

In all cultures of *P. incisa* the TFA content increased with time during 14 days of cultivation and with illumination intensity (Fig. 1), reaching 2.5 and 3.0 $\mu\text{g mL}^{-1}$ for +N and -N cultures, respectively. Generally, cultures grown on -N medium accumulated more FA than those grown on +N medium. In all cultures, there was an increase with time in the proportion of AA (from ca. 30 to 58% in -N) as well as in its volumetric content (from 0.02 to 0.80 and 1.62 $\mu\text{g mL}^{-1}$ for +N and -N cultures, respectively) (Fig. 1). The volumetric contents of AA and TFA exhibited a close linear relationship regardless of the illumination intensity and nitrogen availability. Since the AA proportion of TFA was always higher in the nitrogen-starved cultures, this relationship had a higher slope in the case of the -N cultures (Fig. 1).

According to our findings, a considerable increase in the Car/Chl ratio was recorded in response to high light and nitrogen starvation suggesting an increase in relative Car content (Fig. 2). Cultures grown on N-free medium had higher Car/Chl values in comparison to cultures

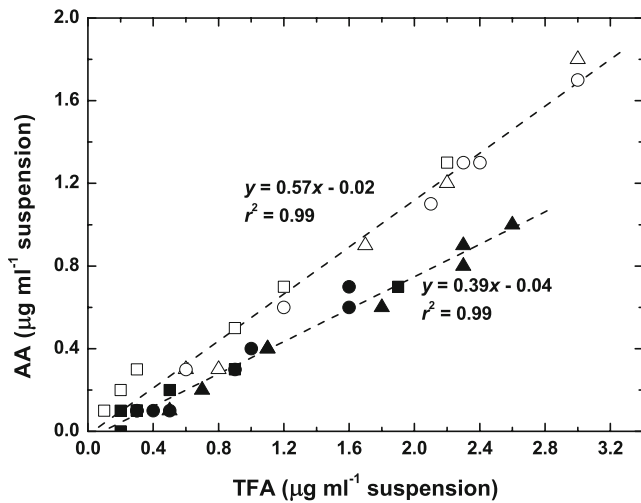


Fig. 1 Relationships between arachidonic acid and total fatty acid volumetric contents in *P. incisa* cells grown on complete (closed symbols) and nitrogen-free (open symbols) media under irradiance of 35 (■, □), 200 (○, ●), or 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (▲, Δ). Here and below, dashed lines represent the best-fit functions

grown on complete medium (0.30–1.20 vs 0.25–0.65). The Car/Chl values appeared to be normally distributed ($P=0.085$) in both +N and -N cells. The Car/Chl ratio exhibited a tight relationship with AA (Fig. 2) and TFA content in the suspension ($r^2=0.91$, $n=46$) regardless of the cultivation conditions. At the same time, a weaker ($r^2 < 0.4$) correlation was found between individual pigment (Car or Chl) content and the volumetric content of TFA or AA (data not shown).

The relationships ‘TFA] vs [Car]/[Chl]’ and ‘[AA] vs [Car]/[Chl]’ obeyed essentially the same linear law in cultures grown on either complete or nitrogen-free media (Fig. 2). Therefore, using the molar Car/Chl ratio as an indicator of FA accumulation, the algorithms for the assay of TFA and AA (see Table 1) were suggested in the following forms:

$$[\text{TFA}] = 4.39 \cdot [\text{Car}] \cdot [\text{Chl}]^{-1} - 1.10 \quad (1)$$

$$[\text{AA}] = 2.15 \cdot [\text{Car}] \cdot [\text{Chl}]^{-1} - 0.51 \quad (2)$$

The volumetric FA content values yielded by the algorithms (1) and (2) could be converted to percentages of cell dry weight which designate the accumulation of the lipids in the biomass:

$$\text{FA}_{\% \text{DW}} = [\text{FA}] \cdot \text{DW}^{-1} \quad (3)$$

where $\text{FA}_{\% \text{DW}}$ —FA percentage of biomass dry weight, [FA]—[TFA] or [AA] from equations (1) or (2), respectively, and DW—dry weight (mg mL^{-1}).

Relationships between lipid and pigment content and cell suspension spectral absorption

In the NIR region of the spectrum (710–800 nm) the scattering-free $\tilde{A}(\lambda)$ spectra of *P. incisa* cell suspensions were flat and did not show detectable absorption. To characterize the effect of Car on light absorption by *P. incisa*, the spectra of the cells grown on complete or nitrogen-free medium were divided into three approximately equal subsets ($n > 5$) with low, medium, and high Car/Chl ratios (see Fig. 2). The average spectra for each subset normalized to the red Chl absorption maximum in vivo (678 nm) are shown Fig. 3. The spectra of cell suspensions did not show a considerable change in the shape of the spectra in the red band of Chl absorption (Fig. 3) in contrast to growth in batch culture. The increase in Car/Chl was accompanied by a considerable increase in absorption in the blue region of the spectrum: the \tilde{A} in the blue region of the spectrum increased over that in the red, depending on the cultivation conditions. A weaker effect was found in the +N cultures (Fig. 3 A, curves 2–1 and 3–1) whereas in the -N cultures the increase in contribution of Car to the \tilde{A} in the blue region of the spectrum was higher (Fig. 3 B, curves 2–1 and 3–1). The difference, $\Delta\tilde{A}(\lambda)$, spectra obtained by subtraction of the average spectra for samples with different Car/Chl values (Fig. 3 A, curves 2–1 and 3–1) contained maxima near 480, 460, and 420 nm, characteristic of Car absorption in *P. incisa*. The amplitude of the $\Delta\tilde{A}(\lambda)$ spectra in the blue region was higher in the case of -N cultures possessing increased Car/Chl ratio that reflects a higher relative contribution of Car absorption in the blue region of the spectrum.

The relationships between $\tilde{A}(\lambda)$ and total Car or Chl content were weak (not shown); by contrast the correlation between Car/Chl ratio and $\Delta\tilde{A}(\lambda)$ normalized to the red Chl maximum,

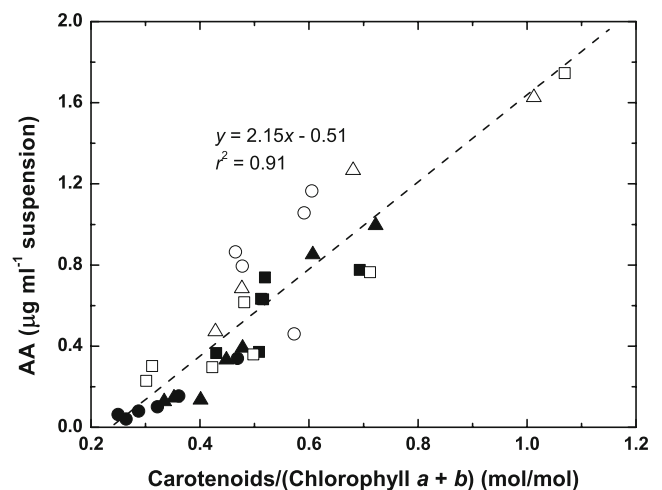


Fig. 2 Relationships between arachidonic acid volumetric content and carotenoid-to-chlorophyll ratio in *P. incisa* cells grown on complete (closed symbols) and nitrogen-free (open symbols) media under irradiance of 35 (■, □), 200 (○, ●), or 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (▲, Δ)

Table 1 Algorithms for the assay of volumetric TFA and AA contents ($\mu\text{g mL}^{-1}$) in *P. incisa* cell suspensions via analytically determined molar Car/Chl ratio

	Range ($n=46$)	Estimation algorithm	RMSE	r^2
Total fatty acids	0.09–3.04	$[\text{TFA}] = 4.39 \cdot [\text{Car}] [\text{Chl}]^{-1} - 1.10$	0.06	0.89
Arachidonic acid (see Fig. 2)	0.04–1.7	$[\text{AA}] = 2.15 \cdot [\text{Car}] [\text{Chl}]^{-1} - 0.51$	0.02	0.91

$\tilde{A}(\lambda) [\tilde{A}(678)]^{-1}$, was high. As a result of a tight interrelationship between Car/Chl, TFA, and AA (Figs. 1 and 2), a strong correlation was found between the volumetric content of AA and $\tilde{A}(\lambda) [\tilde{A}(678)]^{-1}$. The correlation spectra for the ' $\tilde{A}(\lambda)$ vs [AA]' relationship, $r\{\tilde{A}(\lambda) \text{ vs } [\text{AA}]\}$, computed for the spectra of all samples studied regardless of cultivation conditions are presented in Fig. 4. For both +N and -N cultures, the highest correlation ($r > 0.93$) was found in the blue-green range of the spectrum (Fig. 4). Negative peaks were observed in the red region of the spectrum governed by Chl absorption. In the NIR, the region where pigments exhibit low absorption, a weak correlation ($r < 0.2$) was recorded. The $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{Car}]/[\text{Chl}]\}$ and $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{TFA}]\}$ correlations spectra were essentially the same as $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{AA}]\}$ (not shown).

Algorithms for non-destructive assay of fatty acid content

Taking into account the findings above and the maximum near 510 nm in the correlation spectrum (Fig. 4), the algorithm for estimation of the volumetric content of AA in *P. incisa* cell suspensions was suggested in the form:

$$[\text{AA}] = 1.94 \cdot \tilde{A}(510) \cdot [\tilde{A}(678)]^{-1} - 0.95 \quad (4)$$

where [AA] is the volumetric content of AA in $\mu\text{g/ml}$ suspension; $\tilde{A}(510)$ and $\tilde{A}(678)$ are the \tilde{A} values in the

region of maximum and minimum of $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{AA}]\}$ spectrum, respectively. This algorithm allowed the assay of AA in the range of 0.04–1.70 $\mu\text{g mL}^{-1}$ with an accuracy of 0.02 $\mu\text{g mL}^{-1}$ (Fig. 5, Table 2). A similar model was proposed for TFA determination:

$$[\text{TFA}] = 3.74 \cdot \tilde{A}(510) \cdot [\tilde{A}(678)]^{-1} - 1.96 \quad (5)$$

This model enabled the assessment of TFA in the range of 0.09–3.04 $\mu\text{g mL}^{-1}$ with an error of 0.07 $\mu\text{g mL}^{-1}$ (Table 2). The FA volumetric content values yielded by algorithms (4) and (5) could be converted to percentages according to Eqn. (3).

Discussion

In our spectral measurements, the influence of scattering has been eliminated after application of a recently developed scattering-correction methodology (Merzlyak and Naqvi 2000; Merzlyak et al. 2008), which yielded an $\tilde{A}(\lambda)$ spectra bearing almost no features of scattering (Fig. 3). These spectra were essentially flat and close to the baseline in the NIR region where pigment absorption is nearly absent, the peaks attributable to Car and Chl were fairly resolved (Fig. 1, curves 1–3), and the ratio of the maxima in the red and blue regions of the spectrum was close to that of

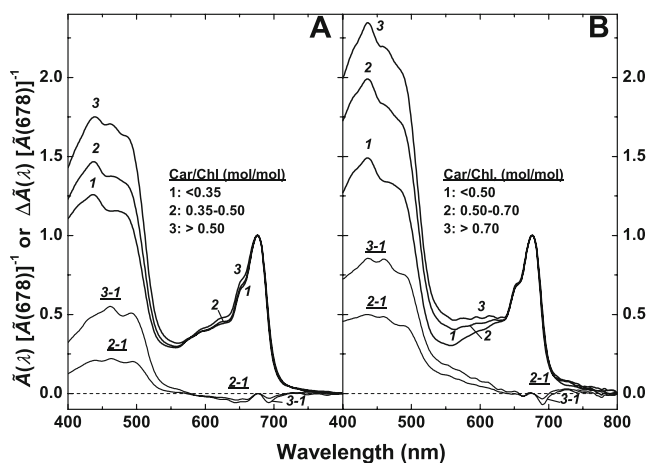


Fig. 3 Average scattering-corrected absorption spectra (1–3) of *P. incisa* cell suspensions differing in carotenoid-to-chlorophyll ratio grown on complete (A) and nitrogen-free (B) media and their differential spectra (2–1, 3–1)

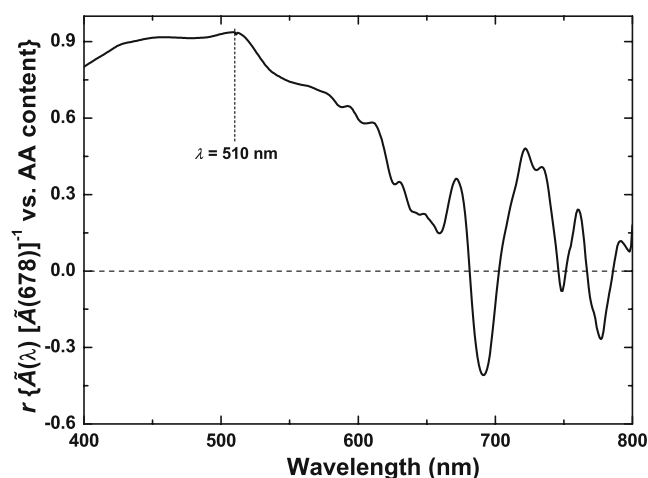


Fig. 4 Spectrum of coefficient of correlation between arachidonic acid content and spectral absorption of cell suspensions of *P. incisa* normalized to 678 nm, calculated for all samples

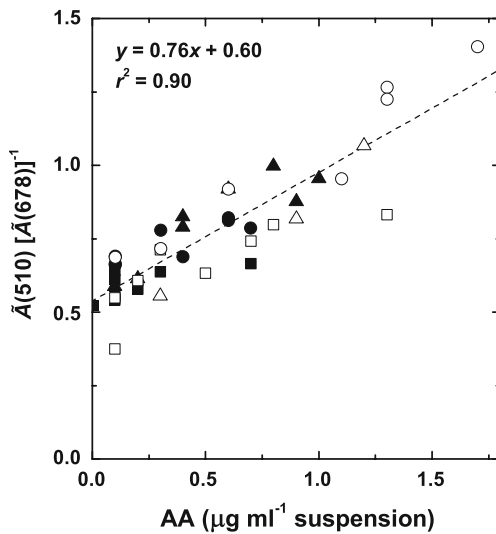


Fig. 5 Relationship between arachidonic acid volumetric content and the index $\tilde{A}(510) [\tilde{A}(678)]^{-1}$ in *P. incisa* cells grown on complete (closed symbols) and nitrogen-free (open symbols) media under irradiance of 35 (■, □), 200 (○, ●), or 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (▲, △), $n=46$

P. incisa spectra measured with the use of an integrating sphere (Merzlyak et al. 2007). These findings establish the feasibility of using simple spectrophotometers without complex and expensive accessories for obtaining routine spectral information, at least for *P. incisa* cell suspensions under laboratory conditions.

It has previously been found that stresses induced by high light (Cheng-Wu et al. 2002; Solovchenko et al. 2008a, b) and lack of nitrogen (Khozin-Goldberg et al. 2002; Solovchenko et al. 2008b) trigger distinct responses in *P. incisa*, including dramatic changes in pigment composition. The common feature of the stress-induced pigment changes was a conspicuous increase in the Car/Chl ratio (Fig. 2) due to accumulation of high amounts of Car, during cultivation on complete medium, or retention of Car on a background of a decline in Chl on nitrogen-free medium (Merzlyak et al. 2007; Solovchenko et al. 2008b). It turned out that the increase in the Car/Chl ratio in *P. incisa* is closely related with biosynthesis and accumulation of AA, which occurs under high light and/or nitrogen-lack conditions. The coordinated synthesis of lipids and Car under stress conditions is known to take place in some other microalgal species such as *Dunaliella salina* (Ben-Amotz

et al. 1982; Pick 1998; Mendoza et al. 1999; Borowitzka and Siva 2007) and *Haematococcus pluvialis* (Boussiba 2000; Zhekisheva et al. 2002; Wang et al. 2003). The enhanced synthesis of lipids could facilitate the adaptation to excessive PFDs providing the sink for excessive photosynthates (Rabbani et al. 1998; Solovchenko et al. 2008a). In *P. incisa*, most of the lipids synthesized in response to the stresses are deposited in cytoplasmic oil bodies (Khozin-Goldberg et al. 2002; Merzlyak et al. 2007), which also serve as a depot for accumulation of β -carotene (Solovchenko et al. 2008b). Within oil bodies, Car could have an antioxidative role (Edge et al. 1997) protecting unsaturated lipids from peroxidation, and could participate in the screening and trapping of excessive light otherwise absorbed by the chloroplast. These facts could probably explain the strong inter-correlation between pigment composition and lipid volumetric content (Fig. 2) and hence between lipids and spectral properties of *P. incisa* cells (Fig. 4), which has been exploited here for the development of an algorithm for non-destructive assay of TFA and AA contents.

Analysis of the correlations $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{AA}]\}$ and $r\{\tilde{A}(\lambda) [\tilde{A}(678)]^{-1} \text{ vs } [\text{TFA}]\}$ showed that the spectral regions that are most sensitive to variation in the Car/Chl ratio and therefore to the FA volumetric content (regardless of illumination intensity and nitrogen availability) are situated in the region of 500–520 nm (Fig. 4). The strength of the correlation with \tilde{A} values was insufficient ($r_{\text{max}}^2 = 0.79$ and 0.56 for +N and -N cultures, respectively) for a reliable assay of PUFA. The presence of the negative peaks in the red region of the correlation coefficient spectra (Fig. 4) suggested that the correlation of \tilde{A} with PUFA content could be impaired by interference from Chl, presumably Chl *b*. According to the methodology previously developed for the analysis of Car, the $\tilde{A}(678)$ band from the region governed by Chl was used to compensate for the interference of these pigments. As a result, the correlation has improved considerably ($r^2=0.90$; see Fig. 5).

The correlation between extensive accumulation of Car and the changes in spectral absorption in the blue region of the spectrum was observed only in *P. incisa* cells grown under high irradiance (Fig. 3). Growth under lower light conditions in the absence of nitrogen had also induced an increase in relative contribution of Car into *P. incisa* absorption in the blue due to a remarkable decline in Chl content (see Merzlyak et al. 2007). In this case, only a weak

Table 2 Algorithms for non-destructive assay of volumetric TFA and AA contents ($\mu\text{g mL}^{-1}$) in *P. incisa* using absorption measurements of cell suspensions

	Range ($n=46$)	Estimation algorithm	RMSE	r^2
Total fatty acids	0.09–3.04	$[\text{TFA}] = 3.74 \tilde{A}(510) \cdot [\tilde{A}(678)]^{-1} - 1.96$	0.07	0.84
Arachidonic acid	0.04–1.7	$[\text{AA}] = 1.94 \tilde{A}(510) \cdot [\tilde{A}(678)]^{-1} - 0.95$	0.02	0.90

correlation was found between TFA or AA content and the absorption in the blue-green region; the fatty acid content was much more closely related with the spectral features in the red which were ascribed to the change in the so called 'packaging' effect. Similar changes were found in the 650–750 region of suspension absorption spectra (Fig. 3, curves 2–1 and 3–1), but their magnitude was 2–3 times lower than that observed in the case of long-term nitrogen starvation under low light (Merzlyak et al. 2007). This could, at least in part, explain the existence of the correlation peak near 725 nm (Fig. 4).

In conclusion, the results of this work support the possibility of employing spectral data on light absorption by cell suspensions recorded on a standard spectrophotometer, after compensation for scattering, for obtaining essential information concerning the physiological condition of *P. incisa* cells. The use of this approach and the cross-correlation between changes in key pigment and PUFA content allowed us to develop simple algorithms for the non-destructive assay of TFA and AA volumetric and biomass content via Car/Chl ratio with a reasonable precision. We suggest that such algorithms are of potential use in laboratory routine and for the development of efficient non-destructive techniques for on-line monitoring of algae grown in photobioreactors.

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