

Technical communication

A routine method to determine the chlorophyll *a*, pheophytin *a* and β -carotene contents of isolated Photosystem II reaction center complexes

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Abstract

The most simple way in which the stoichiometry of chlorophyll *a*, pheophytin *a* and β -carotene in isolated Photosystem II reaction center complexes can be determined is by analysis of the spectrum of the extracted pigments in 80% acetone. We present two different calculation methods using the extinction coefficients of the purified pigments in 80% acetone at different wavelengths. One of these methods also accounts for the possible presence of chlorophyll *b*. The results are compared with results obtained with HPLC pigment analysis, and indicate that these methods are suitable for routine determination of the pigment stoichiometry of isolated Photosystem II reaction center complexes.

Abbreviations: Car – carotene; Chl – chlorophyll; HPLC – high-performance liquid chromatography; Pheo – pheophytin; PS – Photosystem; RC – reaction center

Introduction

The smallest unit of Photosystem II (PS II) capable of primary charge separation is the D1–D2-cytochrome *b*-559 reaction center complex (Nanba and Satoh 1987). This complex is structurally related to the well-characterized RC complex of photosynthetic purple bacteria and binds Chl *a*, Pheo *a* and β -Car molecules as co-factors involved in the primary energy and electron transfer reactions. Despite intensive research, the exact number of bound pigment molecules per complex has for long been a matter of debate. Kobayashi et al. (1990) and Gounaris et al. (1990) suggested that the PS II RC complex binds 6 Chl *a*, 2 Pheo *a* and 2 β -Car molecules. Other groups, however, suggested that the PS II RC complex in its most pure form binds 4 Chl *a*, 2 Pheo *a* and 1 β -Car molecules (Montoya et al. 1991) and that higher contents of Chl *a* and β -Car arise from contamination with the core antenna protein CP47 (Chang et al. 1994; Pueyo et al. 1995). It is important to solve the question of the pigment

content of the PS II RC complex, since the number and energies of bound chlorophylls seriously affect the interpretations of functional studies of the PS II reaction center, such as those on the primary energy and electron transfer reactions (van Gorkom 1995).

In a previous report we analyzed several HPLC and spectroscopic methods for the determination of the pigment stoichiometry of the PS II RC (Eijkelhoff and Dekker 1995) and concluded that in some reports the Chl to Pheo ratio has been underestimated to very significant extents. In particular some of the applied spectroscopic methods were suggested to yield rather unreliable results. This is unfortunate, because generally spectroscopic methods are well-suited for routine analysis of PS II RC preparations (they are easy to perform and do not require special equipment). An additional advantage of spectroscopic methods is the relative ease by which PS II RC complexes prepared in different laboratories can be compared.

In this report, we describe a very simple spectroscopic method well-suited for routine analysis to deter-

mine the Chl *a*, Pheo *a* and β -Car contents of PS II RC preparations from the room temperature absorption spectra of their extracts in 80% acetone. The results are in line with the conclusion of a number of recent detailed studies (Eijkelhoff et al. 1996; Zheleva et al. 1996; Kurreck et al. 1997) that the isolated PS II RC complex in its most stable and pure form binds 6 Chl *a*, 2 Pheo *a* and 2 β -Car molecules.

Experimental

The aim of this work is to determine the concentrations of Chl *a*, Pheo *a* and β -Car of PS II RC preparations from their extracts in 80% acetone. For each wavelength λ the absorption A_λ of the extract is given by:

$$A_\lambda = (c_{\text{chl}} \times \epsilon_{\text{chl},\lambda}) + (c_{\text{pheo}} \times \epsilon_{\text{pheo},\lambda}) + (c_{\text{car}} \times \epsilon_{\text{car},\lambda}) \quad (1)$$

So, if the molar extinction coefficients ϵ of the three pigments are known at three different wavelengths, the concentrations c of the three pigments in the extract can be determined. Because for some preparations contamination of Chl *b* can not always be totally excluded, we present an additional method including this pigment.

The extraction procedure of PS II RC preparations from spinach in 80% acetone was described in detail before (Eijkelhoff and Dekker 1995). Chl *a*, Chl *b*, Pheo *a* and β -Car were obtained from such extracts with > 99.9% purity by reversed phase HPLC with 100% methanol as mobile phase (Eijkelhoff and Dekker 1995). The peak fractions were collected, dried by flushing with nitrogen gas and re-solubilized in 80% acetone, after which their absorption spectra were recorded on a Cary 219 spectrophotometer. The spectra of Chl *a*, Chl *b* and β -Car were normalized to published extinction coefficients in 80% acetone ($86.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 663 nm for Chl *a*, $134.14 \text{ mM}^{-1} \text{ cm}^{-1}$ at 460 nm for Chl *b* and $140 \text{ mM}^{-1} \text{ cm}^{-1}$ at 454 nm for β -Car – data from Lichtenthaler 1987), based on which we obtained extinction coefficients of these pigments in 80% acetone at all recorded wavelengths (see Eijkelhoff and Dekker 1995, for the spectra of Chl *a* and β -Car in 80% acetone after normalization according to these extinction coefficients).

In our previous report we also presented the spectrum of Pheo *a* in 80% acetone after normalization according to the extinction coefficients as determined by Lichtenthaler (1987), but for the work described in this report we decided to redetermine these coefficients. The reason for this is that small systematic

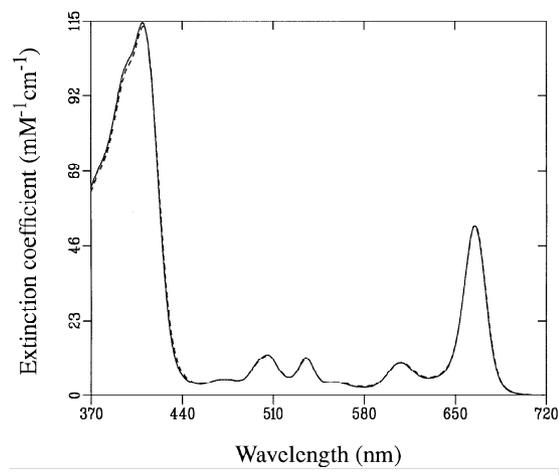


Figure 1. Room temperature absorption spectra of HPLC-purified Pheo *a* (full line) and Pheo *a* obtained by adding 0.75 mM HCl to purified Chl *a* (dashed line) in 80% acetone. The spectra were normalized as described in the text.

errors in the estimation of the Pheo *a* extinction coefficients will have relatively large effects on the calculated Chl/Pheo ratio of the extract. We prepared Pheo *a* by adding 0.75 mM HCl to purified Chl *a* in 80% acetone and recorded the absorption spectrum. Figure 1 (dashed line) shows the spectrum of the obtained fraction, which in view of the spectral shape almost exclusively consists of Pheo *a*. The spectrum of HPLC-purified Pheo *a* in 80% acetone is shown as well (Figure 1, full line). Both spectra are very similar. Nevertheless, in the spectrum of the acidified fraction a very small amount (<1%) of Chl *a* may be present, because the absorption near 430–435 nm is slightly stronger in case of the acidified fraction, whereas the absorption near 410–415 nm is slightly reduced. The spectrum of HPLC-purified Pheo *a* in Figure 1 (full line) was normalized to the integrated intensity of the acidified spectrum, which is based on the assumption that the total absorption between 370 and 720 nm of the <1% contamination of Chl *a* will result in a very similar total absorption if this fraction is converted into Pheo *a*. The spectrum of Pheo *a* in Figure 1 (full line) can therefore be regarded as the spectrum of highly purified Pheo *a* in 80% acetone, carefully normalized to the spectrum of Chl *a* in 80% acetone. We note that the extinction coefficient at the peak of the Q_y band of Pheo *a* (665 nm) calculated by this method is $51.95 \text{ mM}^{-1} \text{ cm}^{-1}$, which is identical to the value given by Lichtenthaler ($51.9 \text{ mM}^{-1} \text{ cm}^{-1}$).

Table 1. Extinction coefficients at selected wavelengths of chlorophyll *a*, pheophytin *a*, β -carotene and chlorophyll *b* in 80% acetone, calculated as described in the text

Pigment	Extinction coefficient ($\text{mM}^{-1} \text{cm}^{-1}$)					
	ϵ_{663}	$\epsilon_{535-551}$	ϵ_{480}	ϵ_{460}	ϵ_{431}	ϵ_{412}
Chlorophyll <i>a</i>	86.30	0.000	1.52	2.43	95.99	77.85
Pheophytin <i>a</i>	50.16	7.579	4.58	3.86	19.22	112.57
β -Carotene	0.46	1.049	120.84	135.88	100.82	64.21
Chlorophyll <i>b</i>			43.95	134.14	57.43	20.04

With the extinction coefficients of Chl *a*, Pheo *a* and β -Car being known at all wavelengths between 370 and 720 nm, Equation (1) can now be solved by using three key wavelengths (or four if the concentration of Chl *b* is to be estimated as well). The best result is obtained when at each of these wavelengths the total absorption is dominated by a different pigment. For Method 1 we chose for 431 nm (where the absorption of Chl *a* dominates the total absorbance), 412 nm (Pheo *a* absorption dominates), 480 nm (β -Car absorption dominates) and 460 nm (to get an idea on possible Chl *b* contamination), whereas for Method 2 we chose for 663 nm (Chl *a* absorption dominates), 535–551 nm (Pheo *a* absorption dominates) and again 480 nm (β -Car). The extinction coefficients of the four individual pigments at these wavelengths are listed in Table 1. With these extinction coefficients, Equation (1) gives the following equations for Method 1:

$$c_a = -1.709 A_{412} + 11.970 A_{431} - 2.998 A_{460} - 5.708 A_{480} \quad (2)$$

$$c_p = +10.280 A_{412} - 8.380 A_{431} + 2.456 A_{460} - 1.233 A_{480} \quad (3)$$

$$c_c = -0.430 A_{412} + 0.251 A_{431} - 4.376 A_{460} + 13.216 A_{480} \quad (4)$$

$$c_b = -0.171 A_{412} - 0.230 A_{431} + 11.871 A_{460} - 13.248 A_{480} \quad (5)$$

For Method 2 these equations were calculated:

$$c_a = +11.577 A_{663} - 76.994 A_{535-551} + 0.624 A_{480} \quad (6)$$

$$c_p = +0.020 A_{663} + 132.505 A_{535-551} - 1.150 A_{480} \quad (7)$$

$$c_c = -0.146 A_{663} - 4.054 A_{535-551} + 8.311 A_{480} \quad (8)$$

In these equations, c is given in μM and A_λ in cm^{-1} ; the suffices *a*, *p*, *c* and *b* stand for Chl *a*, Pheo *a*, β -Car and Chl *b*, respectively.

Results and discussion

From Equations (2)–(5) (Method 1) or (6)–(8) (Method 2) it is possible to give an estimate of the concentrations of Chl *a*, Pheo *a* and β -Car in the 80% acetone extract of a certain PS II RC preparation. Method 1 also gives an indication of the contamination with Chl *b*. Table 2 lists the results of both methods on extracts of some of our preparations and compares the results with those obtained by HPLC quantification. The ratios were normalized to 2.0 Pheo *a*, except for CP47, in which case they were normalized to 2.0 β -Car.

For the reaction center preparations the results with both spectroscopic methods are comparable to results obtained with the HPLC-method, and the contamination with Chl *b* is negligible (Table 2). This suggests that the two spectroscopic methods give reliable results for PS II RC preparations, and that the set of applied extinction coefficients must be quite good.

In the case of the CP47-containing complexes the results of the spectroscopic methods deviated in some respects from those of the HPLC method. Method 2 (the method that primarily monitors the Q_y and Q_x transitions of the pigments) gives good results for the Pheo *a*-content; the finding that this content is essentially zero in the CP47 spectrum indicates that the set of extinction coefficients used in the calculations must be very good. The Chl *a*-content calculated from the

Table 2. Ratio of chlorophyll *a*, pheophytin *a* and β -carotene in 80% acetone extracts of selected preparations. The ratios were normalized to 2.0 Pheo *a*, except for CP47, in which case it was normalized to 2.0 β -Car

Preparation	Method 1	Method 2	HPLC ^a
	Chl <i>a</i> : Pheo <i>a</i> : β -Car (: Chl <i>b</i>)	Chl <i>a</i> : Pheo <i>a</i> : β -Car	Chl <i>a</i> : Pheo <i>a</i> : β -Car
RC _{DM-3} ^b	6.2: 2.0: 1.8 (: 0.07)	6.5: 2.0: 2.0	6.5: 2.0: 2.0
RC _{short} ^c	6.4: 2.0: 1.7 (: 0.09)	6.3: 2.0: 1.7	6.4: 2.0: 1.7
CP47-RC ^d	22.2: 2.0: 3.7 (: 0.9)	16.1: 2.0: 3.0	18.9: 2.0: 3.2
CP47 ^e	14.8: -0.26: 2.0 (: 0.6)	13.2: -0.02: 2.0	15.4: 0.03: 2.0

^aPerformed as described by Eijkelhoff and Dekker (1995).

^bSee Eijkelhoff et al. (1996).

^cSee Eijkelhoff and Dekker (1995).

^dSee Dekker et al. (1989).

^eSee Groot et al. (1995).

CP47-RC spectrum, however, seems somewhat low. For the CP47 and CP47-RC complexes Chl *a*-contents of 13–15 and 20, respectively, are expected (Kwa et al. 1992), which agree reasonably well with the values obtained from the HPLC analysis. Method 1 (the method that primarily monitors the Soret region of the pigments), however, seems to give a small underestimation of the Pheo *a* content, because the calculated Pheo *a* content in CP47 is negative and the calculated Chl *a* and β -Car contents in CP47-RC are somewhat high (note that in Table 2 these values were normalized to 2.0 Pheo *a*). It is possible that the underestimation of the Pheo *a* content actually arises from variations in the spectra of β -Car in the Soret region. The absorption properties of β -Car depend heavily on the direct environment (Renge et al. 1996), and small differences in pH, ionic strength or cis-trans isomerization may induce bandshifts in the region where Pheo *a* shows characteristic absorbances.

From the above described results we conclude that the quantitation method works very well for PS II RC preparations. We would like to stress, however, that both methods have to be used with caution, in particular for complexes of the reaction center with (a) core antenna(s) or individual core antenna preparations. It should also be noted that the extract needs to have a reasonable absorption, an optical density between 0.3 and 0.6 in the Q_y-absorption region, in order to calculate correct values in the regions of low absorption. This demands preparations of reasonable concentration. Furthermore, when extracting reaction center preparations obtained by the isolation method as described by Vacha et al. (1995) we encountered the problem of contamination of the extract with cytochrome *b559*, which can give a disproportioning

of the extract spectrum in the Soret region. This hampers the use of particularly our spectroscopic Method 1, because here the calculated Chl:Pheo ratio is primarily assessed by the absorption at 416 nm, where cytochrome *b559* causes very significant absorption.

The spectroscopic methods as described in this paper have, however, also many advantages. The methods are very quick, do not require acidification of the extracts (as in Montoya et al. 1991), and thus can easily be used to assess the quality of the PS II RC during purification. In addition, with these methods it is possible to directly compare the pigment content of preparations of different groups. This contrasts the comparison of spectra of intact preparations, which are often disturbed by baseline irregularities and/or scattering, and the comparison of HPLC results, which are often carried out with different types of columns and different organic solvents. It is likely that HPLC methods generally have better resolving power, but these methods are usually more laborious. In addition, it remains uncertain to which respects systematic errors occur in the determination of the pigment ratios by the different groups as long as double-blind tests are not performed.

When we compared the extinction coefficients used in this paper with those used by Montoya et al. (1991) we found a substantial difference. Based on the ratio $\epsilon_{665}/\epsilon_{535-551}$ of the spectrum of Pheo *a* in 80% acetone we calculated with our extinction coefficients a value of $51.95/7.579 = 6.85$, while Montoya et al. used a ratio of $49.3/6.504 = 7.58$. Because our extinction coefficients were carefully checked by HPLC analysis and appeared identical to the values reported by Lichtenthaler (1987), these numbers suggest that Montoya

et al. (1991) have *underestimated* their Chl/Pheo ratio by $(7.58-6.85)/6.85 \times 100\% \cong 10\%$.

We conclude that the spectroscopic method is very suitable for quick, routine analysis of the pigment content of PS II reaction center preparations. It should be stressed, however, that counting pigments alone does not give information on the quality of the preparation. In Eijkelhoff et al. (1996) we therefore presented a FPLC gel filtration/diode array detection technique that gives information on the presence of loosely bound pigments (particularly pheophytins) that in principle can be responsible for unrealistic pigment ratios.

Note added in proof

After submission of this manuscript it became clear to us that in Lichtenthaler (1987) specific extinction coefficients were reported instead of molar extinction coefficients, as assumed in this paper and in our previous papers. Therefore the calculated concentrations of Chl *a*, Pheo *a*, β -Car and Chl *b* have to be multiplied by 0.893, 0.893, 0.957 and 0.907, respectively. These changes have no significant effects on the ratios presented in Table 2 and on the general conclusions of our work. We thank Dr. E. Schlodder (TU Berlin) for this suggestion.

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References

- Chang H-C, Jankowiak R, Reddy NRS, Yocum CF, Picorel R, Seibert M and Small GJ (1994) On the question of the chlorophyll *a* content of the Photosystem II reaction center. *J Phys Chem* 98: 7725-7735
- Dekker JP, Bowlby NR and Yocum CF (1989) Chlorophyll and cytochrome *b*-559 content of the photochemical reaction center of Photosystem II. *FEBS Lett* 254: 150-154
- Eijkelhoff C and Dekker JP (1995) Determination of the pigment stoichiometry of the photochemical reaction center of Photosystem II. *Biochim. Biophys Acta* 1231: 21-28
- Eijkelhoff C, van Roon H, Groot M-L, van Grondelle R and Dekker JP (1996) Purification and spectroscopic characterization of Photosystem II reaction center complexes isolated with or without Triton X-100. *Biochemistry* 35: 12864-12872
- Gounaris K, Chapman DJ, Booth P, Crystall B, Giorgi LB, Klug DR, Porter G and Barber J (1990) Comparison of the D1/D2/cytochrome *b*-559 reaction centre complex of photosystem two isolated by two different methods. *FEBS Lett* 265: 88-92
- Groot M-L, Peterman EJG, van Stokkum IHM, Dekker JP and van Grondelle R (1995) Triplet and fluorescing states of the CP47 antenna complex of Photosystem II studied as a function of temperature. *Biophys J* 68: 281-290
- Kobayashi M, Maeda H, Watanabe T, Nakane H and Satoh K (1990) Chlorophyll *a* and β -carotene content in the D1/D2/cytochrome *b*-559 reaction center complex from spinach. *FEBS Lett* 260: 138-140
- Kurreck J, Liu B, Napiwotzki A, Sellin S, Eckert H-J, Eichler H-J and Renger G (1997) Stoichiometry of pigments and radical pair formation under saturating pulse excitation in D1/D2/cytb559 preparations. *Biochim Biophys Acta* 1318: 307-315
- Kwa SLS, van Kan PJM, Groot ML, van Grondelle R, Yocum CF and Dekker JP (1992) Spectroscopic comparison of D1-D2-cytochrome *b*-559 and CP47 complexes of Photosystem II. In: Murata N (ed) *Research in Photosynthesis*, Vol I, pp 263-266. Kluwer Academic Publishers, Dordrecht, the Netherlands
- Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. *Methods Enzymol* 148: 350-382
- Montoya G, Yruela I and Picorel R (1991) Pigment stoichiometry of a newly isolated D1-D2-Cyt.*b*559 complex from the higher plant *Beta vulgaris* L. *FEBS Lett* 283: 255-258
- Nanba O and Satoh K (1987) Isolation of a Photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559. *Proc Natl Acad Sci USA* 84: 109-112
- Pueyo JJ, Moliner E, Seibert M and Picorel R (1995) Pigment content of D1-D2-cytochrome *b*559 reaction center preparations after removal of CP47 contamination: An immunological study. *Biochemistry* 34: 15214-15218
- Renge I, van Grondelle R and Dekker JP (1996) Matrix and temperature effects on absorption spectra of β -carotene and pheophytin *a* in solution and in green plant Photosystem II. *J Photochem Photobiol A* 96: 109-122
- Vacha F, Joseph DM, Durrant JR, Telfer A, Klug DR, Porter G and Barber J (1995) Photochemistry and spectroscopy of a five-chlorophyll reaction center of Photosystem II isolated by using a Cu affinity column. *Proc Natl Acad Sci USA* 92: 2929-2933
- Van Gorkom HJ (1995) Current questions on optical properties and energy transfer in the reaction center of Photosystem II. In: Mathis P, (ed) *Photosynthesis: From Light to Biosphere*, Vol I, pp 437-442. Kluwer Academic Publishers, Dordrecht, the Netherlands
- Zheleva D, Hankamer B and Barber J (1996) Heterogeneity and pigment composition of isolated Photosystem II reaction centers. *Biochemistry* 35: 15074-15079