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Fast, Sensitive, and Inexpensive Alternative to Analytical Pigment HPLC: Quantification of Chlorophylls and Carotenoids in Crude Extracts by Fitting with Gauss Peak Spectra

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Quantification of pigments in complex mixtures is an important task in the physiology of photosynthetic organisms, because pigment composition differs depending on the species, tissue, and physiological state. Currently available methods, however, are either limited to very few pigments (classical UV/vis spectroscopic methods), or they are time-consuming, labor intensive, or costly (e.g., HPLC). Here we describe a UV/vis spectrophotometric method that is capable of a rapid (∼1 min/sample) and inexpensive (<1 euro/sample) quantification of more than a dozen pigments in a crude extract, which means it is suitable for high-throughput screening applications. A detection limit of <1 pmol for each pigment allows for determining the pigment composition in only 0.5 μg of lyophilized leaves or algae. The method is based on the description of each pigment spectrum by a series of Gaussian peaks. A sample spectrum is then fitted by a linear combination of these "Gauss peak spectra" including an automatic correction of wavelength inaccuracy, baseline instability, sample turbidity, and effects of temperature/water content. Here we present the Gauss peak spectra from 350 to 750 nm for acetone solutions of all chlorophyll and carotenoid derivatives that are abundant (including conditions of Cd, Cu, or Zn stress) in leaves of higher plants, *Euglena*, brown algae, and various cyanobacteria like *Anabaena* and *Trichodesmium*: [Mg]-Chl a, b, c_1, c_2; pheophytin a, b; [Cd]-Chl a, b; [Cu]-Chl a, b; [Zn]-Chl a, b; antheraxanthin, aurochrome, β-carotene, β-cryptoxanthin, *cis*- and *trans*-canthaxanthin, diadinochrome (=diadinoxanthin 5,6-epoxide), *cis*- and *trans*-diadinoxanthin, diatoxanthin, *cis*- and *trans*-echinenone, fucoxanthin, lutein, myxoxanthophyll, neoxanthin, violaxanthin, and all three stereoisomers of zeaxanthin in acetone. We present extensive tests of our new quantification method for determining optimal and limiting conditions of its performance and for comparison with previous methods. Finally, we show application examples for *Thlaspi fendleri* (Chlorophyta), *Euglena gracilis*c (Euglenophyta), *Ectocarpus siliculosus* (Phaeophyta), and *Trichodesmium erythraeum* IMS101 (cyanobacteria).

Quantitative analysis of pigments, mainly carotenoids and chlorophylls, is a basic prerequisite for the study of many aspects of the physiology of plants and other photosynthetic organisms. Pigment composition of a tissue/cell changes in the course of ontogenesis, as a response to varying environmental conditions, and can be a decisive difference between species, ecotypes, and mutants. A detailed analysis of all physiologically relevant carotenoids and chlorophylls, however, is still a time-consuming and costly procedure, because it requires not only the extraction of the pigments from the cells but usually also a separation by HPLC with simultaneous or subsequent analysis (identification and quantification) of the resolved peaks.

Therefore, in most cases where rapid quantification is needed, compromises are made in a way that only the main pigments (e.g., Chl a, Chl b, and some rough estimate of "total carotenoids" in higher plants) are given. In these cases, quantification can be done by photometry using linear equations like those from the classical publication by Arnon[3] or various later publications with more accurate extinction coefficients but the same basic principle (e.g., White et al.,[4] Jones et al.,[5] Lichtenthaler and Wellburn,[6] Ritchie,[7]

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and various further methods recently summarized in the review of Porra[8]). These simplistic estimation methods, however, often yield very inaccurate results, for several reasons. First, they only function correctly when few pigments with well-separated peaks are present in the sample. This may work for a simple estimation of Chl a, Chl b, and total carotenoid content in a healthy plant. A more detailed quantification, e.g., of individual carotenoids, is impossible with such methods. Further, applying such methods to stressed plants can yield dramatically wrong results. Under conditions of heavy metal stress, for example, the central ion of Chl (Mg^{2+}) can be exchanged by heavy metals (e.g., Küpper et al.[9−13]). Such heavy metal chlorophylls (hms-Chls) usually have a blue-shifted Q_y-band. Extracts of such plants contain hms-Chls, and pheophytins in addition to the regular [Mg]-Chls and carotenoids. Therefore, a traditional Chl a+b estimation would "detect" a reduced Chl a/b ratio and at the same time would not detect the formation of the hms-Chls. This would lead to completely wrong conclusions about the mechanism of heavy metal-induced damage.

For the task of determining [Mg]-Chls, hms-Chls, and pheophytin (Pheo), a set of six linear equations designed for this task (e.g., White et al.,[4] Jones et al.[5]) may be sufficient for a rough estimation of these pigments. An accurate quantification with this traditional method, however, is not possible because of the overlap of the absorbance peaks combined with the unavoidable noise and wavelength inaccuracy as analyzed in detail by Küpper et al.[14] So far, there have been mainly two attempts to overcome these difficulties in photometric quantification. The first was an attempt to improve the accuracy by fitting ASCII files of absorption spectra of standard solutions to the sample spectrum (Naqvi et al.[1]). This method, called the spectral reconstitution (SRC) method, improved the accuracy of the results by using a much larger number of wavelengths for quantification. In its original published version, no correction parameters for inaccuracy of the spectrometer were included. Further, due to the limited wavelength accuracy of spectrometers, both sample and fitting spectra had to be measured on the same photometer in order to yield usable results, or the wavelength axis of the ASCII files had to be shifted manually to match a difference in wavelength calibration, which is impracticable in everyday use. Therefore, already slight baseline and wavelength deviations led to large errors as analyzed by Küpper et al.[14] In the publication by Küpper et al.,[14] a second novel method of estimation was presented, the most important feature of which was a mathematical description of the absorption spectrum of each compound throughout the whole relevant spectral region. This enabled an automatic correction of inaccuracies in the baseline and in the peak positions caused by instrument inaccuracy and sample preparation. It also partially corrected the problems of varying peak widths caused by interactions of pigment molecules in more concentrated solutions. That method, however, had other problems. First, at the time of publication, the required computing power severely limited the number of peaks per compound that could be fitted. In practice, this meant its application to plant extracts was still limited to the quantification of chlorophylls, using the spectral range of 550−750 nm where carotenoids do not significantly contribute to absorption. This small spectral range also limited accuracy; the quantification of pheophytin b and [Cu]-Chl b was not very reliable.[14] A later update of the SRC method[2] improved the SRC method by adding a baseline correction as in the Gauss peak spectrum (GPS) method,[14] but the wavelength shift and peak width variation problems still could not be corrected due to the principle of the method (i.e., directly using measured values of standard spectra in a linear array to simulate the sample spectrum).

In the current paper, we describe an extension of our earlier GPS method of pigment quantification.[14] This extension makes the GPS method much more versatile by allowing for an accurate estimation of all physiologically important carotenoids along with all major chlorophyll derivatives. In this way, the method described here will be in many cases a much easier, less expensive, and faster alternative to analytical high-performance liquid chromatography (HPLC). Further, by including a large library of data sets for various pigments (all major chlorophyll derivatives and carotenoids from higher plants, brown algae, *Euglena*, *Trichodesmium*, and *Anabaena*), for all plant scientists working with those organisms there will not usually be any necessity to isolate further pigment standards for defining further GPS. Finally, we enhanced the automatic correction of measuring problems by adding a simulation of light scattering to counteract artifacts caused by turbidity (e.g., dust in the samples) and making the automatic peak width correction an independent fitting parameter to correct effects of temperature differences and water contamination.

MATERIALS AND METHODS

Pigment Standards. Pigment standards for the calibration of the procedure (described below) were prepared from extracts of the following organisms. Chlorophylls a and b were isolated from the higher plants *Thlaspi caerulescens* J.&C. PRESL and *Ceratophyllum demersum* L., and the alga *Euglena gracilis* (strain UTEX753). Chl c_1 and Chl c_2 were isolated from *Ectocarpus siliculosus* (DILLWYN) LYNGBYE (strain Port Aransas). Due to the much lower yield of Chl b compared to Chl a, further Chl b required for the synthesis of the hms-Chls (see below) was purchased from Sigma-Aldrich (www.sigmaaldrich.com), from where we also purchased β-carotene. Antheraxanthin and lutein were isolated from *C. demersum* and *T. caerulescens*. Diadinoxanthin, diadinochrome (=diadinoxanthin-5,6-epoxide), diatoxanthin, and echinenone were isolated from *E. gracilis*. All three zeaxanthin stereoisomers were isolated from *Trichodesmium erythraeum* ISM101. Myxoxanthophyll and canthaxanthin were isolated from *Anabaena variabilis*. β-Cryptoxanthin was purchased from CaroteNature (Lupsingen, Switzerland, www.carotenature.com). Neoxanthin and violaxanthin were purchased from DHI Lab Products (Hoersholm, Denmark, www.c14.dhi.dk).

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All pigments to be isolated were purified to homogeneity by HPLC on a semipreparative (250 × 25 mm inner diameter) column filled with biosphere C-18 (5-μm particle size) and purchased from Watrex (Praha, Czech Republic, www.watrex.com). This column was always run with a water−methanol−acetone gradient. All samples were loaded onto the column in a solvent mixture equal or similar to the starting conditions of the elution gradient. For the primary separation of crude extracts, a gradient of 87.5% methanol + 12.5% water to 100% methanol (1 h) followed by a second gradient of 100% methanol to 100% acetone (2 h), both with 4 mL.min^{-1} flow rate, was applied. For further purification steps of isolated pigments, only the relevant sections of this gradient were rerun or an isocratic solvent mixture slightly more hydrophilic than the previous elution conditions of the respective pigment was applied. Purified standards were transferred (via a vacuum rotary evaporator, Savant SpeedVac SVC100, Thermo Fisher Scientific, Waltham, MA, www.thermo.com) as rapidly as possible to acetone to prevent degradation (for Chls allomerization, for carotenoids epoxidation, both caused by alcohols). This well-known degradation of pigments in alcoholic solvents and the possible artifactual formation of hms-Chls in alcohols and aqueous (80%) acetone (both problems reviewed, e.g., by Küpper et al.[13]) were the main reasons for choosing 100% acetone for the GPS method as presented here. Tests of extracting lyophilized leaves ground to powder were 100% quantitatively up to 50 mg of plant dry weight/mL of acetone (data not shown).

The purity and identity of the pigments were checked by comparison of their elution behavior and by their UV/vis absorption spectra (330−750 nm).

For preparing hms-Chls, first Mg^{2+} was removed from pure [Mg]-Chl a or [Mg]-Chl b to yield the corresponding pheophytins by addition of 0.1−37% HCl (v/v) to a solution of the pigment in acetone. Subsequently, cyclohexane was added to this solution (usually 20% of the volume), and the pheophytin was driven into the cyclohexane phase by adding half-saturated NaCl solution. After evaporating the cyclohexane in a vacuum centrifuge (see above), the pheophytin was redissolved in methanol to yield a saturated solution. To this solution, acetates of the desired central ion (here Cd^{2+}, Cu^{2+}, Zn^{2+}) dissolved in 90% methanol (saturated solution of each salt) were added at room temperature (25 °C). The amount of the methanolic heavy metal acetate solution to be added was adjusted to yield a high efficiency of insertion of the central ion with minimal degradation of the pigment. Good results were usually obtained by adding heavy metal solution of ∼10−20% of the volume of the pheophytin solution, although less is required for those ions that insert easiest in Chl, here Cu^{2+}.[9] After the color of the solution changed from the brown-gray of the pheophytin to the green of the hms-Chl (seconds for [Cd]-Chl and [Cu]-Chl, minutes for [Zn]-Chl), the reaction was stopped by transferring the hms-Chl to cyclohexane as described above for Pheo. After evaporating the cyclohexane in the vacuum centrifuge, the crude hms-Chl was redissolved in 100% acetone for purification by HPLC (see above).

The function for correction of light scattering was determined by taking an acetone extract of a clean GF/F filter (Whatman, www.whatman.com), as it is commonly used for phytoplankton analysis (also here, see below). According to our experience, handling of such filters (e.g., inserting them into vials for extraction of the algae on them) will usually lead to increased light scattering due to fine particles of glass. Such particles can be sedimented by centrifugation, but are again easily resuspended when taking out the supernatant afterward and therefore often represent a problem when analyzing phytoplankton samples with little biomass per filter.

Test Solutions of Known Composition. To test the accuracy of the new method and to compare it to that of previous methods, standard solutions (see above) in acetone were combined to compose mixtures of known composition. Each test mixture was produced three times to get three independent replicates. For the tests of pure [Mg]-Chl a and the unstressed higher plant leaf, dilution series were prepared to check which is the optimal concentration range for the application of our method and what are the artifacts when this concentration range is left. The recorded spectra were analyzed by all methods to be compared: The method of Lichtenthaler and Wellburn[6] for quantifying [Mg]-Chls a and b and total carotenoids, the methods of Naqvi et al.[1,2] as well as the current method for all pigments were used. All quantifications were done in 100% acetone (see above).

Tests of Effects Caused by Variations in Temperature and Water Content. To check on how far the parameters "peakwidth" and "wldev" in our GPS equation are capable of correcting effects caused by changes in measuring temperature and residual water content of the samples, systematic tests were performed. Pure Chl a was used as the main example, because it has about equally high and sharp peaks in the short- and long-wavelength range of visible light, so that it allows for testing whether peakwidth and wldev change differently in these spectral ranges. For these tests, independent wldev and peakwidth parameters were used for the two parts of the Chl a spectrum represented by peaks 1−6 in Table 1 for the long-wavelength range and peaks 7−10 for the short-wavelength range. Temperature effects were tested using a temperature-controlled cuvette holder in the spectrometer and increasing the temperature from 15 to 35 °C in 5 °C steps. Water content was varied by adding a defined percentage of water to fresh dried (<0.0005% H_2O) acetone (Uvasol, Merck KgA, Darmstadt, Germany), in which the sample was prepared by diluting a highly concentrated (30 mM) solution of pure [Mg]-Chl a in acetone. Each test was performed at least three times with independently prepared samples.

Examples of Crude Pigment Extracts from Photosynthetic Organisms. To further evaluate the performance of the method, we applied it to the analysis of crude acetone extracts from photosynthetic organisms. *Thlaspi fendleri* (NELS.) HITCHC was grown hydroponically as described by Küpper et al.,[15] *T. erythraeum* IMS101 was kept in batch cultures as described by Küpper et al.,[16] *E. gracilis* (strain UTEX753) was grown as described by Rocchetta et al.,[17] *E. siliculosus* (DILLWYN) LYNGBYE (strain Port Aransas) was grown as described in Küpper et al.[11] *Ectocarpus*, *Euglena*, and *Trichodesmium* were harvested by filtration on GF/F filters (Whatman), *Thlaspi* leaves were cut off. After harvesting, all samples were frozen in liquid nitrogen, lyophilized

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Table 1. Absorption Coefficients Used for Normalizing Spectra of Pigment Standards[a]

pigment	absorption coefficient ($cm \cdot mM)^{-1}$)	source
[Mg]-Chl a	82	Ziegler and Egle[20]
[Mg]-Chl b	49	Ziegler and Egle[20]
[Cd]-Chl a	90	estimated value due to similarity to [Zn]-Chl
[Cd]-Chl b	60	estimated value due to similarity to [Zn]-Chl
[Cu]-Chl a	60	Küpper et al. [12]
[Cu]–Chl b	43	Küpper et al. [12]
Pheo a	46.5	Küpper et al. [12]
Pheo b	31	Küpper et al. [12]
[Zn]–Chl a	90	Küpper et al. [12]
[Zn]–Chl b	60	Küpper et al. [12]
antheraxanthin	137	Britton[21]
aurochrome	143	ACEMOGLU M.; EUGSTER C. H.;, 1984, Diediastereomeren Aurochrome: Synthese, Analytik and chiroptische Eigenschaften, *Helv. Chim. Acta* **1984**, *67*, 471−487
cis-canthaxanthin	80	Nelis et al.[22] in acetonitrile/methanol/dichloromethane 5:3:2.
trans-canthaxanthin	125	Nelis et al.[22] in acetonitrile/methanol/dichloromethane 5:3:2.
β-carotene	134	Davies et al.[23]
β-cryptoxanthin	134	estimated value due to similarity to β-carotene
diadinochrome (= ox. diadinoxanth)	130	estimated value due to similarity to diadinoxanthin
diadinoxanthin	130	Britton (1995) (cis isomer: estimated by similarity) Britton[21]
diatoxanthin	119	Johansen et al.[24]
echinenone	116	Britton[21] in cyclohexane cis-isomer: estimated by similarity
fucoxanthin	69.7	Britton[21]
lutein	145	Britton[21] in ethanol
myxoxanthophyll	158	Britton[21]
neoxanthin	135	Britton[21] in ethanol
violaxanthin	144	Britton[21]
all-trans-zeaxanthin	133	Britton[21]
9-*cis*-zeaxanthin	88.4	Englert et al.[25] in Et_20/isopentane/EtOH 5:5:2
13-*cis*-zeaxanthin	113	Englert et al.[25] in Et_20/isopentane/EtOH 5:5:2

[a] If no further information is given, the all-trans isomer is meant. For Chl derivatives, the value of the red absorption maximum is given; for carotenoids, the value of the maximum absorption in the spectral range 350−750 nm is listed.

for 1 or 2 days, and subsequently extracted in 100% spectroscopy-grade acetone (Uvasol, Merck KgA).

For comparison of the GPS results with HPLC, the same sample preparation, HPLC column, and elution gradient was used as for the preparation of standards (see above). For quantification, each fraction was evaporated in the vacuum centrifuge, redissolved in 100% spectroscopy-grade acetone, and analyzed by spectroscopy (see below). The slightly complicated latter procedure was used instead of the usual online integration of HPLC peak areas in order to reduce the error that is introduced by changes in absorption peak position and amplitude in HPLC solvent mixtures compared to published values in pure solvents.

UV/Vis Spectroscopy. All samples were centrifuged at 16000*g* (4 °C) for >10 min to sediment particles. For recording standards, a maximum OD (400−700 nm) between 0.5 and 1.5 was chosen; samples of the extraction efficiency test were diluted, when necessary, to a maximum OD of 1.5. Almost all spectra were measured with the double-beam UV/vis spectrophotometer Lambda 16 (Perkin-Elmer, Wellesley, MA, las.perkinelmer.com). As the only exceptions, the Chl c and fucoxanthin standard spectra and the application example of *Ectocarpus* were measured with the double-beam UV/vis spectrophotometer UV3000 (Shimadzu, www.shimadzu.com). All spectra were measured at 22 ± 2 °C with a scanning speed of 240 $nm \cdot min^{-1}$. A spectral bandwidth (slit setting, not recording interval) of 1 nm was selected for all measurements.

Calculation of Gauss Peak Spectra. The spectrum of each pigment standard was normalized to its absorbance maximum in the range of 330−750 nm. Then these normalized data were fitted with a series of up to 13 Gaussian peaks using the Levenberg−Marquard algorithm to mathematically describe the spectrum between 330 and 750 nm, as described in detail in our earlier publication establishing the principles of this method.[14] The resulting equation was called a Gauss peak spectrum. For quantifying the constituents of an extract, the GPS were fitted to the measured spectrum as described earlier.[14] Several GPS were combined to simulate the constituents, which possibly could be present in the sample, e.g., [Cu]-Chl a, [Cu]-Chl b, [Mg]-Chl a, [Mg]-Chl b, Pheo a, Pheo b, antheraxanthin, β-carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin for a sample of Cu-stressed green algae or higher plants. Additionally, for an automatic correction of baseline drift of spectrometers, a linear function was added to this simulation. Further, we introduced an exponential function in the baseline fit to reduce the influence of light scattering. This problem often occurs in crude extracts due to contaminations by dust from the ground tissue, since filtering may not remove the smallest particles and often some particles are inadvertedly resuspended after centrifugation. Similarly, a parameter for correction of wavelength inaccuracy (the wldev parameter) was added (Table 1). Since the wldev parameter is additive to the wavelength and identical for all GPS in the simulation, it is not influenced by the shape of the measured spectrum, but only by wavelength shifts. The parameter peakwidth that was introduced in our earlier publication as a factor adjusting the widening of peaks due the interaction of pigment molecules

was now made free to optimization for automatic correction of temperature effects and water contaminations.

The region from 330 to 750 nm was selected for the quantification method for several reasons: (a) All chlorophylls and major carotenoids of higher plants, algae and cyanobacteria have characteristic absorbance spectra within this spectral region. These spectra are unique for each of the chromophores involved. (b) None of the quantitatively relevant pigments of the plants analyzed here absorbs significantly above 700 nm, making the region 700−750 nm ideal for the automatic baseline correction.

All absorptivities (millimolar extinction coefficients) used for our GPS are listed in Table 1; the GPS themselves are given in supplement 1 (Supporting Information) and the spectra of the standards used for their calibration are provided in supplement 2. The combinations of GPS were then fitted to the sample spectrum using the Levenberg−Marquardt Method as implemented, e.g., in SigmaPlot, and described earlier.[14]

RESULTS AND DISCUSSION

Equations Used in the GPS Method of Chl Estimation. Figure 1A shows [Mg]-Chl a as an example of how the measured spectra were described by Gaussian peaks and how well this fit matched with the measured data. The description of spectra of isolated pigments with Gaussian peaks yielded curves almost indistinguishable from the measured spectra and characterized by very low X^2 values. This was also true for the fitting of extract spectra, as demonstrated in Figure 1B. Usually the residuals of the fitting process represented only the noise of the baseline. All other GPS fits for the pigments presented in this publication are in supplement 1 (Supporting Information). The complete GPS equations are provided in supplement 2 in text format and in supplement 3 as a ready-to-use fitting library for the data analysis program SigmaPlot (version 5 and above). The final equation used for fitting has the form of

$$f(x) = y0 + x \times \text{Slope} + \text{Scattering} \times \left(57100000000 \exp\left(\frac{-x}{12.5}\right)\right) + \exp\left(\frac{-x}{84}\right) + \sum_{i=1}^{N} \text{Pigment}_n$$

with

$$\text{Pigment} = \text{Concentration} \times \text{Absorptivity} \sum_{i=1}^{M} a_M \exp\left(-0.5\left(\frac{x - \text{wldev} - xp_M}{\sigma_M \times \text{Peakwidth}}\right)^2\right)$$

Symbols in order of their occurrence in the equations (VPO = variable parameter of optimization when analyzing an unknown sample): x, wavelength (nm); $f(x)$, function combining the GPS of N individual pigments; N, number of pigments used for analyzing the sample; Y0, Slope, offset and factor for linear approximation of baseline drift (VPO); Scattering, factor for exponential approximation of light scattering (VPO); Pigment_n, GPS = function describing the absorbance spectrum of a single pigment (N); concentration, micromolar Concentration of each of the N components in the mixture (VPO); Absorptivity, micromolar extinction coefficient at the absorption maximum between 350 and 750 nm; M, number of Gaussian peaks used for the description of the absorbance spectrum of each pigment from 350 to 750 nm; a_M, amplitude of peak M (in spectrum normalized to maximum OD = 1 between 350 and 750 nm with 1-cm light path); xp_M, center of peak M (nm); wldev. deviation from ideal peak position (nm) as determined in the standards (VPO); σ_M, peak half-width (nm); Peakwidth, deviation from ideal peakwidth as determined in the standards (VPO).

The equations are valid for any spectrometer that has a spectral bandwidth smaller than or equal to 1 nm and linear wavelength dispersion. The spectral bandwidth is important because widths over 1 nm lead to significant lowering of the sharp peaks of pheophytins and carotenoids with pronounced fine structure (e.g., violaxanthin); widths over 2 nm even change the shape of the peaks of the metallochlorophylls and other carotenoids. Therefore, the equations presented here are only valid for measurements carried out with spectral bandwidths of ≤1 nm; larger bandwiths (as they are usually found in diode array detectors) can result in major errors. The grating monochromator is important because it results in an approximately linear wavelength dispersion. Without linear wavelength dispersion of the monochromator, a constant spectral bandwidth over the whole required range (350−750 nm) is almost impossible. Further, the wldev parameter can only correct fluctuations in spectrometer calibration if a shift in calibration leads, via linear wavelength dispersion, to a constant offset of all wavelengths in the measured range. However, grating monochromators with linear wavelength dispersion are used in almost all modern UV/vis absorption spectrometers.

Note that once these equations are defined, only the variable parameters used for optimization (VPO), which are Y0, slope, scattering, wldev, peakwidth and the concentrations of pigments expected in the sample (e.g., [Mg]-Chl a, Mg- Chl b, Phaeo a and Phaeo b, antheraxanthin, β-carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin in the case of a sample from an unstressed green plant) are varied to fit the measured spectra (Figure 1B). The VPOs "Y0" and "Slope" automatically correct baseline drift by a linear approximation. The VPO "Scattering" was introduced to correct effects of turbidity of the sample (caused, e.g., by small particles from extracting lyophilized plant powder passing through the filter or being inadvertently resuspended after centrifugation); the parameters of the exponential function were determined empirically. The VPO wldev automatically corrects wavelength inaccuracy of the spectrometer. The VPO peakwidth automatically corrects, for example, effects of slight (±10 °C) temperature differences between calibration of the standards and measurement of the sample, slight (<2%) water contaminations, and partially the aggregation of Chls at high concentrations.

Some molar coefficients used in the GPS method are not determined in 100% acetone. If future authors find more accurate values for the absorptivity of a pigment in 100% acetone, all parameters of the GPS spectrum (Table 2) remain valid and only the parameter absorptivity in the final GPS equation of that pigment has to be updated.

In this paper, fitting results are presented obtained with the Levenberg−Marquard algorithm that is commonly implemented in data analysis programs such as SigmaPlot (versions 2000 and 9.0 tested for this publication). On modern personal computers (already a Pentium IV processor with 3 GHz and 1 GB RAM is

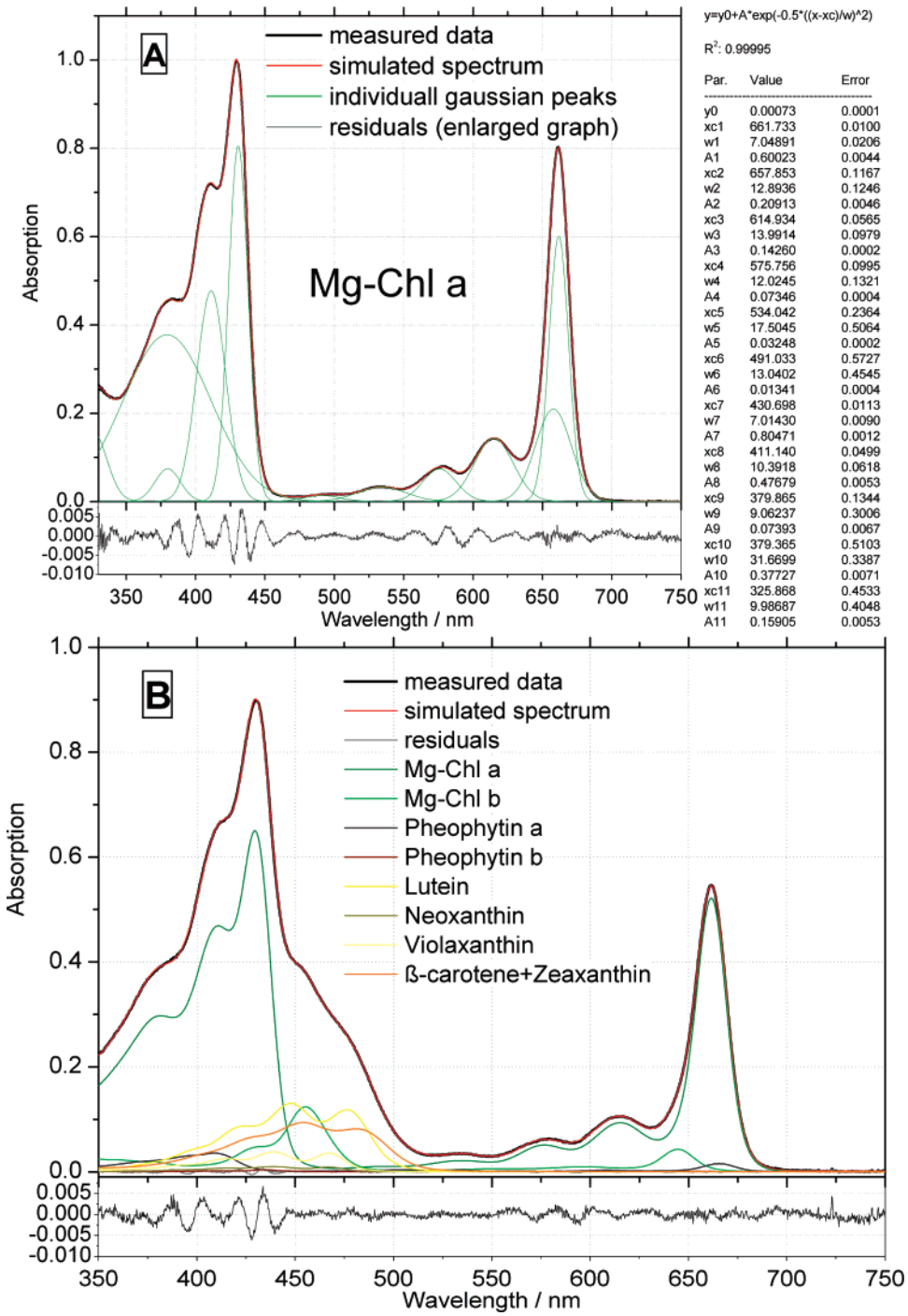


Figure 1. Principle of fitting GPS to measured UV/vis spectra. (A) Determining a GPS of an isolated pigment standard, here [Mg]-Chl a. (all other GPS presented in this article are available in the Supporting Information). The measured spectrum, normalized to OD = 1 at its absorption maximum in the measured range, was fitted with a series of Gaussian peaks. To make the residuals of the fit visible, they are displayed in a strongly stretched scale in the graph below the main graph. (B) Using GPS fitting to quantify the concentrations of pigments in a mixture (shown here: crude extract in 100% acetone from a lyophilised *T. fendleri* leaf). The fitting directly yields the μM concentrations of the individual pigments. Errors in the baseline (offset, slope, light scattering), in the wavelength calibration of the spectrometer and effects affecting the exact peakwidth (slight differences in temperature or residual water in the sample) are automatically corrected, so that the residuals (deviations between measured sample spectrum and GPS fit) are almost invisibly small. To make them visible, they are again displayed in a strongly stretched scale in the graph below the main graph.

sufficient), results for typical cyanobacterial extracts were obtained within less than 60 s and for higher plant extracts within ~2 min.

Accuracy of the Quantification: Tests of Performance of the Method. Comprehensive test series were performed to investigate the performance and reliability of the new method and to find conditions for its optimal performance. We tested the performance of our new method by comprehensive tests on solutions of known composition. These test solutions were composed to simulate natural samples, i.e., extracts of photosynthetic organisms. These test solutions were assembled from pure standards of the individual pigments. Compared to tests on "real samples", i.e. extracts of photosynthetic organisms, such simulated

Table 2. Parameters of the GPS Published Here[a]

(A) Chlorophylls

pigment	param	peak (M) 1	2	3	4	5	6	7	8	9	10	11	12	13
[Cd]-Chl a	*a*	0.4828	0.2295	0.1359	0.0609	0.0322	0.0111	0.0201	0.2515	0.7387	0.3399	0.3867		
	xp	660.10	657.07	614.38	576.65	534.94	494.00	459.72	432.75	428.83	408.15	387.38		
	σ	7.25	13.3	14.9	12.2	22.0	10.1	18.9	5.52	10.8	7.75	18.0		
[Cd]-Chl b	*a*	0.2221	0.0933	0.0454	0.0663	0.4366	0.2003	0.6517	0.0782	0.0094	0.2953			
	xp	637.87	633.51	588.57	553.01	459.32	445.72	437.17	422.44	372.72	342.66			
	σ	10.6	24.4	12.9	38.6	9.07	5.94	20.9	5.22	5.81	52.0			
[Cu]-Chl a	*a*	0.5720	0.1851	0.1259	0.0601	0.0420	0.0301	0.5942	0.6563	0.0619	0.4385			
	xp	650.84	645.29	600.99	550.45	505.43	469.61	423.21	405.91	393.96	365.65			
	σ	08.86	19.93	13.65	18.38	11.24	18.45	04.97	16.07	06.77	21.98			
[Cu]-Chl b	*a*	0.0099	0.2024	0.0700	0.0060	0.0191	0.7485	0.1501	0.2552	0.0931	0.0843			
	xp	631.48	624.91	579.89	578.15	518.24	432.63	428.75	408.76	391.32	351.61			
	σ	04.25	11.67	44.52	07.23	08.84	11.57	05.05	11.33	59.90	13.06			
[Mg]-Chl a	*a*	0.6002	0.2091	0.1426	0.0735	0.0324	0.0134	0.8047	0.4768	0.0739	0.3772	0.1591		
	xp	661.73	657.85	614.93	575.76	534.04	491.03	430.70	411.14	379.86	379.37	325.87		
	σ	07.05	12.89	13.99	12.02	17.50	13.04	07.01	10.39	09.06	31.67	09.99		
[Mg]-Chl b	*a*	0.2447	0.1018	0.0617	0.0453	0.7076	0.2126	0.2196	0.1267	0.0619	0.0551	0.2271		
	xp	644.65	641.98	598.72	556.09	456.81	453.48	428.81	427.33	379.62	364.69	334.30		
	σ	07.25	14.92	14.48	28.24	11.05	06.55	08.30	35.19	06.73	07.63	22.22		
[Mg]-Chl c1	*a*	0.0131	0.0845	0.0675	0.0246	0.0736	0.0058	0.0348	0.8818	0.0819	0.1358	0.3185		
	xp	678.38	629.25	627.90	578.52	571.14	502.50	452.41	440.71	413.93	396.30	277.23		
	σ	12.72	07.61	19.10	07.20	29.89	07.67	04.51	13.46	06.46	18.78	112.9		
[Mg]-Chl c2	*a*	0.0217	0.0894	0.0244	0.0669	0.0378	0.6127	0.0668	0.2803	0.3581	0.0747	0.1904		
	xp	658.24	628.75	611.16	581.38	562.34	454.43	452.66	442.91	432.89	396.50	344.94		
	σ	19.82	07.18	08.18	10.45	28.01	08.42	17.12	07.06	13.24	15.06	75.25		
Pheo a	*a*	0.3767	0.0707	0.0728	0.0274	0.0791	0.1051	0.0319	0.8503	0.1259	0.2072	0.3278	0.2511	
	xp	665.58	654.99	607.96	558.63	533.60	503.82	471.61	409.94	395.96	389.67	376.96	359.67	
	σ	07.69	18.56	10.99	18.46	05.90	10.57	10.55	09.83	04.84	06.39	13.88	41.28	
Pheo b	*a*	0.1664	0.0380	0.0508	0.0346	0.0748	0.0319	0.5993	0.3204	0.1795	0.2035	0.0272	0.1660	
	xp	653.75	646.92	598.42	560.45	524.96	481.54	436.23	433.79	411.69	410.67	369.66	356.21	
	σ	07.16	18.53	10.84	10.82	16.94	14.56	10.98	04.00	05.98	17.88	05.43	31.11	
[Zn]-Chl a	*a*	0.5510	0.2201	0.1194	0.0628	0.0449	0.0078	0.0120	0.1005	0.8192	0.4649	0.2068	0.2149	
	xp	654.61	649.86	605.94	561.30	518.46	490.26	479.37	429.12	423.23	404.19	378.62	367.62	
	σ	07.16	13.49	13.19	12.67	13.04	06.77	08.98	04.20	08.38	09.01	20.84	37.57	
[Zn]-Chl b	*a*	0.1268	0.2925	0.0971	0.0920	0.0216	0.0500	0.3414	0.5896	0.1274	0.5323	0.1384	0.0394	0.2241
	xp	649.69	639.19	636.25	593.91	562.73	540.23	456.99	450.11	432.43	428.51	405.76	377.28	351.82
	σ	06.86	07.30	15.98	14.98	08.95	21.77	09.58	07.26	32.78	09.11	08.63	07.46	25.10

(B) Carotenoids (All-Trans Isomer Unless Otherwise Noted)

pigment	param	peak (M) 1	2	3	4	5	6	7	8
antheraxanthin	*a*	0.4882	0.2609	0.7795	0.2435	0.3701	0.1611	0.0213	0.4867
	xp	474.76	469.51	442.97	417.47	416.80	396.47	372.69	289.40
	σ	11.71	6.985	11.71	43.28	8.465	10.37	6.340	20.07
aurochrome	*a*	0.3176	0.2814	0.5161	0.4213	0.6366	0.2219	0.1021	
	xp	479.64	453.76	434.32	410.48	385.66	382.22	359.69	
	σ	09.04	10.60	08.38	09.26	50.81	07.77	05.74	
β-carotene	*a*	0.0053	0.5858	0.4309	0.5904	0.1226	0.0148	0.1036	
	xp	575.54	484.53	454.11	438.68	427.02	400.88	322.99	
	σ	81.60	12.54	10.67	35.38	08.66	04.90	29.37	
β-cryptoxanthin	*a*	0.0031	0.6123	0.4611	0.5532	0.1430	0.0198	0.0619	
	xp	583.73	484.64	454.21	439.61	427.07	400.77	317.35	
	σ	51.22	12.33	10.52	35.26	09.01	05.52	29.01	
cis-canthaxanthin	*a*	0.1705	0.9787	0.1084	0.2837	0.2740			
	xp	496.68	461.75	414.87	362.88	288.38			
	σ	15.40	32.62	16.62	25.50	22.50			
trans-canthaxanthin	*a*	0.1961	0.8845	0.1347	0.1305	0.1056			
	xp	505.15	471.26	423.27	407.40	311.24			
	σ	15.67	30.39	20.65	65.85	11.63			
diadinochrome	*a*	0.5721	0.6636	0.3103	0.2383	0.1888	0.0533	0.0577	
	xp	460.29	431.39	430.95	404.58	383.90	363.08	346.26	
	σ	07.35	23.47	06.91	07.75	09.87	08.33	65.65	
cis-diadinoxanthin	*a*	0.5361	0.5156	0.4738	0.3032	0.2086	0.0775	0.1068	0.2748
	xp	474.15	443.99	441.83	417.82	394.95	371.60	340.47	328.53
	σ	9.684	10.53	31.59	8.949	11.66	9.023	4.316	17.80
trans-diadinoxanthin	*a*	0.5634	0.6924	0.3058	0.3057	0.1402	0.1444	0.0520	
	xp	479.94	450.72	447.71	420.06	399.62	386.05	337.60	
	σ	7.884	24.09	8.052	8.962	8.748	18.69	10.84	
diatoxanthin	*a*	0.3800	0.2687	0.7661	0.1838	0.1811	0.0586	0.1688	
	xp	480.15	450.72	440.50	422.28	399.92	379.23	367.90	
	σ	07.89	06.67	28.53	06.87	09.57	06.90	25.61	

Table 2 (Continued)

(B) Carotenoids (All-Trans Isomer unless Otherwise Noted)

		peak (M)							
pigment	param	1	2	3	4	5	6	7	8
cis-echinenone	a	0.7203	0.1471	0.3701	0.5254	0.0807	0.2367		
	xp	476.87	476.16	449.48	424.49	396.71	355.07		
	σ	20.91	37.31	12.80	16.99	10.09	28.94		
trans-echinenone	a	0.3562	0.5011	0.3839	0.3104	0.1532	0.1359		
	xp	489.03	476.48	456.04	431.82	409.30	403.64		
	σ	16.84	28.46	12.66	12.82	14.34	70.20		
fucoxanthin	a	0.2254	0.1994	0.2042	0.4430	0.4408	0.2292	0.0614	
	xp	485.92	479.70	469.91	449.40	427.55	421.99	418.48	
	σ	17.92	09.13	06.95	13.44	25.90	50.69	06.94	
lutein	a	0.7121	0.1587	0.8912	0.6053	0.1846	0.1347	0.0605	
	xp	476.87	475.29	447.37	420.57	398.01	383.01	329.21	
	σ	10.29	20.98	10.94	11.10	8.409	13.79	26.36	
myxoxanthophyll	a	0.6003	0.2493	0.8691	0.6179	0.2992	0.1005	0.0840	
	xp	508.56	507.10	476.09	448.14	422.35	399.73	366.60	
	σ	10.95	21.58	11.31	11.37	11.06	09.81	19.45	
neoxanthin	a	0.4336	0.4521	0.8741	0.5047	0.1219	0.2001	0.0398	
	xp	467.73	466.62	438.80	413.77	410.22	391.53	369.70	
	σ	6.06	10.74	9.25	8.47	46.85	9.06	6.72	
violaxanthin	a	0.3326	0.5912	0.7958	0.2081	0.5803	0.076	0.1848	
	xp	472.04	471.68	445.43	437.28	417.59	408.04	393.5	
	σ	10.76	6.55	9.17	5.42	10.69	54.73	7.08	
all-trans-zeaxanthin	a	0.2165	0.5710	0.8665	0.5054	0.0839	0.2065	0.0425	
	xp	488.21	483.13	453.57	426.23	403.60	402.18	381.10	
	σ	15.54	11.63	12.47	11.38	64.40	10.80	08.49	
9-*cis*-zeaxanthin	a	0.6518	0.1899	0.8192	0.6042	0.1774	0.1375	0.1140	
	xp	477.94	473.39	448.40	421.71	398.91	383.62	337.59	
	σ	11.55	22.57	11.33	12.05	08.93	13.09	19.46	
13-*cis*-zeaxanthin	a	0.5947	0.2227	0.7022	0.5934	0.1732	0.1657	0.3742	
	xp	477.51	463.58	447.90	421.18	398.23	382.09	340.70	
	σ	12.79	27.30	11.33	12.59	09.10	14.15	13.81	

[a] General equation: $f(x) = y0 + x\text{Slope} + \text{Scattering}(57100000000\exp(-x/12.5) + \exp(-x/84) + \sum_{i=1}^{N}\text{Pigment}_N$. Pigment = Concentration × Absorptivity $\sum_{i=1}^{M} a_M \exp(-0.5\cdot((x - \text{wldev} - xp_M)/(\sigma_M \times Peakwidth))^2)$. For a detailed explanation of the symbols, see the text.

extracts have the big advantage that their composition is really known, so that the performance of our method could be evaluated without the need of another, potentially artifact-prone "reference" method (e.g., HPLC). Nevertheless, we also compared the GPS method with HPLC and tested its performance on crude extracts from various photosynthetic organisms (see below). Applying the method to a sample that contains significant amounts of a pigment not included in the database of fitted GPS spectra will certainly lead to errors. Unless this unknown compound has a very similar spectrum as a fitted compound (in which case it could be indistinguishable anyway), this will lead to visible residuals and deviations between the fitted curve and the measured spectrum (not shown). Therefore, to judge about the reliability of the fit, it is important to check for such deviations and amplitudes in the residuals of the fit. Further, when such residuals occur or when an organism is analyzed for the first time, a comparison of the GPS with an analytical HPLC might help in finding undetected pigments. As discussed in more detail below, however, HPLC (including sample preparation) can cause artifactual alterations of pigments, so that not all peaks detected in the HPLC are necessarily pigments that are present in the crude extract.

Comparing the GPS fitting results with the true concentrations of pigments in mixtures that were assembled from known standards showed that the GPS method yields reliable results under a wide range of conditions (Table 3). The major components of the mixtures were always quantified with good accuracy, and even for most minor components, a reasonable estimate of their abundance was possible. For compounds with clearly different absorption spectra (e.g., Chl a vs Chl b, lutein vs violaxanthin, carotenoids vs chlorophylls, etc.), the quantification of an individual pigment was not affected by other pigments in the mixture. This is demonstrated by the quantification results of a particularly complex mixture, the simulations of *Euglena*, which were generally not less accurate than those in a more simple mixture (e.g., the simulation of unstressed *Trichodesmium*); see Table 3. The only exceptions were pigments with identical chromophore and therefore (almost, since steric effects etc. cause minor differences) identical absorption spectra in the measured range (350−750 nm). In our tests, the all-trans isomers of β-carotene, β-cryptoxanthin, and zeaxanthin could not be distinguished from each other when present together in mixtures. Also distinguishing between *trans*-echinenone versus *trans*-canthaxanthin, lutein versus antheraxanthin, [Mg]-Chl c_1 versus [Mg]-Chl c_2 and [Cd]-Chl a versus [Mg]-Chl a was often not accurate. Therefore, for the quantitative data shown in Table 3, only the sum of each chromophore is presented. All tests with mixtures assembled by pipetting known standards together are affected by unavoidable errors in pipetting and solvent evaporation due to handling, etc. This type of error would not lead to any changes in the proportions of the pigments in a crude extract, while it will certainly affect the reproducibility of assembling mixtures from standard solutions. Therefore, these

Table 3. Results of GPS Analysis of Pigment Mixtures Simulating Extracts from Various Photosynthetic Organisms[a]

(A) Higher Plants
Pigment concentration in μM, mean (±SE)

	[Mg]-Chl a		[Mg]-Chl b		Pheo a		Pheo b		[Cu]-Chl a		[Cu]-Chl b		[Zn]-Chl a		[Zn]-Chl b		β-carotene + zeaxanthin		lutein		neoxanthin		violaxanthin	
test name	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc
control plant	6.51	6.36 (0.06)	0.89	0.89 (0.01)	0.28	0.33 (0.00)	0.02	0.02 (0.00)	0	–	0	–	0	–	0	–	0.71	0.69 (.001)	0.6	0.89 3 (0.01)	0.09	0.04 (0.02)	0.26	0.29 (0.02)
Cu-stressed plant	4.68	4.34 (0.04)	0.68	0.77 (0.01)	0.57	0.66 (0.01)	0.02	0.02 (0.00)	0.61	0.77 (0.03)	0.03	0.04 (0.01)	0	–	0	–	3.5	2.88 (0.14)	3.12	3.32 (0.02)	0.47	0.62 (0.05)	1.32	0.95 (0.02)
Zn-stressed plant	4.68	4.34 (0.05)	0.68	0.80 (0.03)	0.57	0.64 (0.00)	0.02	0.04 (0.01)	0	–	0	–	0.40	0.39 (0.00)	0.05	0.01 (0.00)	0.80	0.69 (0.00)	0.63	0.72 (0.02)	0.09	0.15 (0.01)	0.26	0.2 (0.01)

(B) *Euglena*
pigment concentration in μM, mean (±SE)

	[Mg]-Chl a		[Mg]-Chl b		Pheo a		Pheo b		[Cu]-Chl a		[Cu]-Chl b		β-carotene		canthaxanthin + echinenone		diadino-xanthin		diadino-chrome		diatoxanthin		neoxanthin	
test name	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc
Euglena control	5.21	4.21 (0.05)	0.72	0.76 (0.01)	0.23	0.20 (0.01)	0.02	0.00 ((0.00)	0	0.03 (0.01)	0	0.00 (0.01)	2.61	2.28 (0.04)	0.96	0.76 (0.03)	2.73	2.65 (0.14)	0.23	0.26 (0.01)	0.52	0.73 (0.01)	0.23	0.00 (0.00)
*Euglen*a Cu-stessed	4.68	4.09 (0.08)	0.68	0.76 (0.01)	0.57	0.52 (0.01)	0.02	0.00 (0.00)	0.61	0.72 (0.04)	0.03	0.01 (0.01)	2.61	2.36 (0.04)	0.96	0.80 (0.03)	2.73	2.72 (0.1)	0.23	0.31 (0.04)	0.52	0.92 (0.04)	0.23	0.00 (0.00)

(C) Cyanobacteria
pigment concentration in μM, mean (±SE)

	[Mg]-Chl a		Pheo a		[Cu]-Chl a		β-carotene + zeaxanthin		canthaxanthin+ echinenone		myxoxanthophyll	
test name	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc	add.	calc
Trichodesmium control	5.21	4.98 (0.10)	0.23	0.30 (0.01)	0	0.01 (0.01)	5.48	4.93 (0.13)	0.48	0.22 (0.04)	0.95	1.07 (0.06)
Trichodesmium Cu-stressed	4.68	4.52 (0.03)	0.57	0.86 (0.01)	0.20	0.16 (0.01)	5.51	5.80 (0.29)	0.7	0.25 (0.08)	0.95	1.31 (0.03)
Anabaena control	5.21	4.77 (0.07)	0.23	0.35 (0.00)	0	0.00 (0.01)	5.48	4.93 (0.13)	0.48	0.22 (0.04)	1.90	2.28 (0.06)

[a] Each pigment mixture was assembled from standards in 100% acetone. Each data point represents the average and standard errors of three independent GPS fits of three samples assembled separately from standard solutions.SE, standard error or the mean; –, not analyzed; add., concentration added; calc, concentration found by GPS analysis of the measured spectrum.

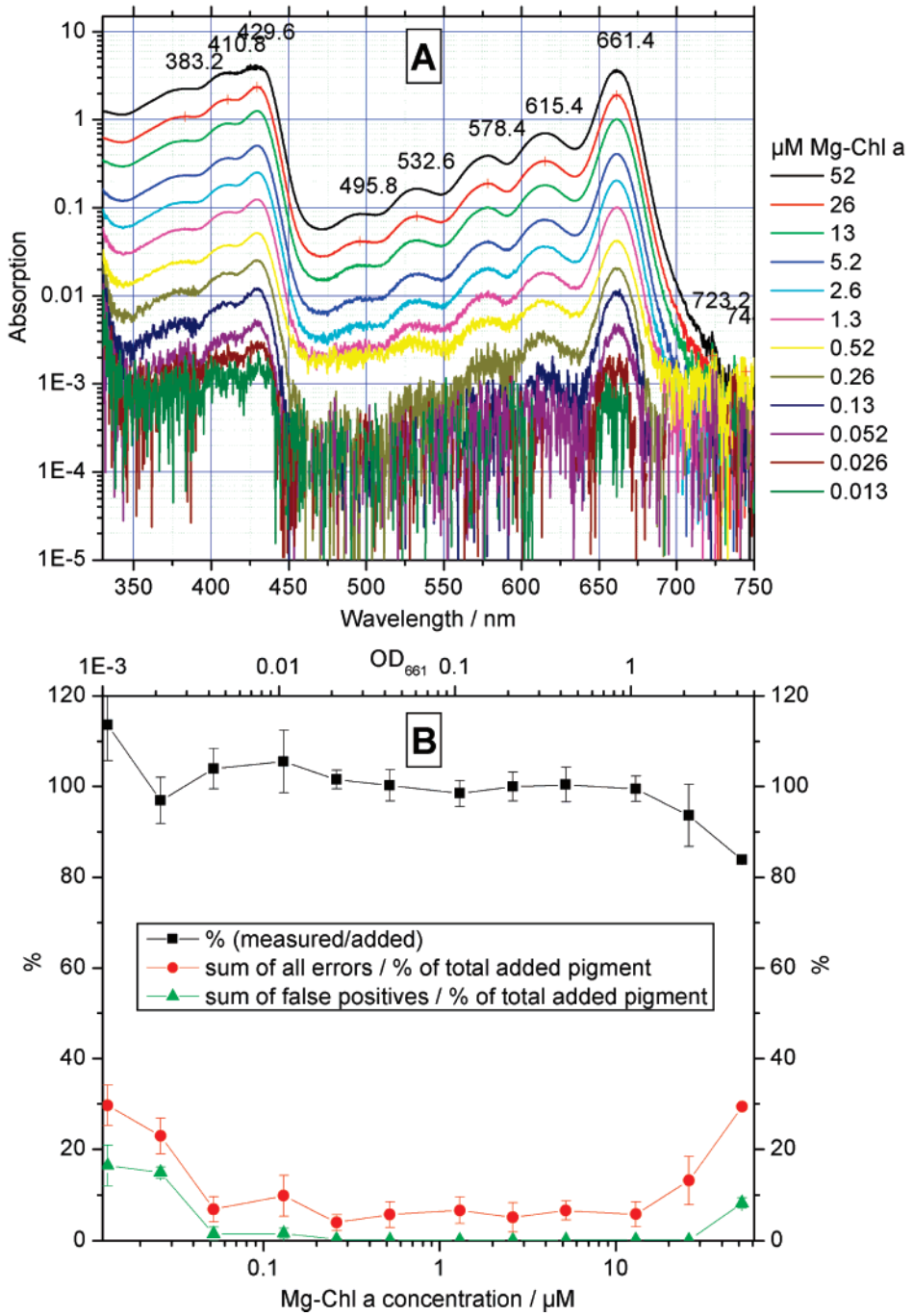


Figure 2. Dilution series of pure [Mg]-Chl a in 100% acetone for determining the performance optimum and detection limit of the GPS method for isolated pigments. (A) The measured spectra to be analyzed, ranging from the noise level of the spectrometer to its upper OD limit. Each curve represents an average of 3 separately diluted standards. (B) The results of the analysis. Each data point represents the average and standard error of three independent GPS fits of three separately diluted standards. The graphs show the concentration of detected pigment in % of the added pigment (black line), the % error of that quantification (red line), and the concentrations of all other pigments that were not added to the mixture (i.e., false positives), in % of the concentration of added pigment (green line).

errors together add additional noise to the measurement, superimposed on the noise caused by the GPS analysis method itself. Thus, the accuracy and precision of the GPS method applied to a real extract from a photosynthetic organism may be better than the values indicated by the tests. This view was supported by the high quality of the fits when applying the GPS method to crude extracts of photosynthetic organisms, as discussed in detail below.

Detection Limits and Conditions of Optimal Performance. As shown in Figure 2, the GPS method is applicable in a wide range of pigment concentrations from the upper OD limit of the spectrometer down to a level where the signal drowns in noise. Even a signal where the peaks were hardly recognizable (0.05 μM [Mg]-Chl a, maximum OD 0.0045) to the naked eye was found to be sufficient for accurate quantification. Using a microcuvette with 10-mm optical path and 10-μL volume (e.g., number 105.211 from Hellma GmbH&Co KG, Müllheim, Germany), this detection limit is equal to 0.4 ng or 0.5 pmol of Chl a. Below this limit, our tests showed a drastic decrease of the accuracy in estimation. This

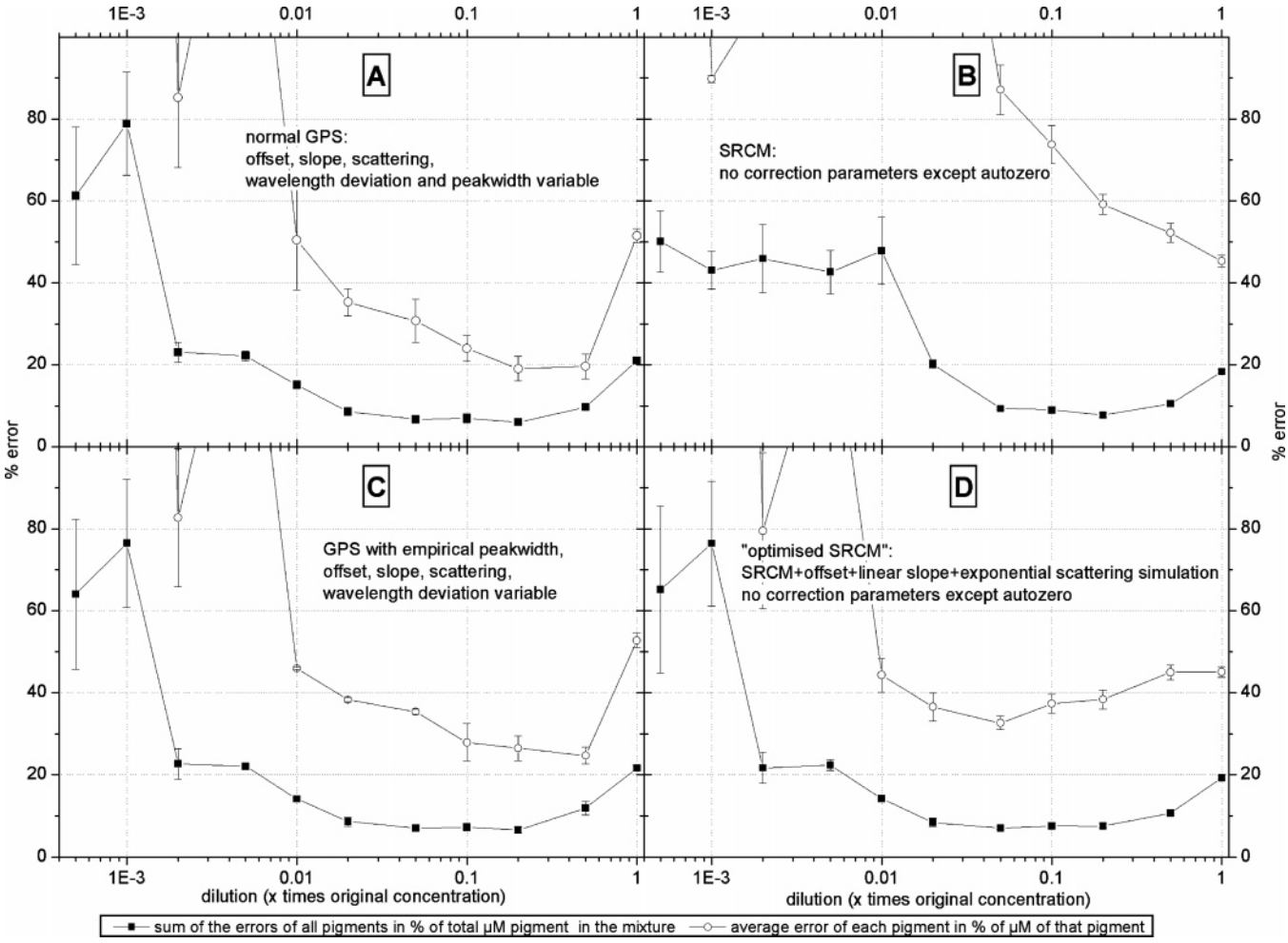


Figure 3. Comparison of accuracy of the new GPS method, the SRC method[1] and variations of both methods. These tests were done to determine the importance of the error correction parameters (offset, slope, scattering, peakwidth, and wavelength deviation) in the GPS method and to check the overall performance of the different methods in quantifying the components of a complex pigment mixture like a crude extract of a green plant. Each data point represents the average and standard error of three independent calculations of three samples assembled separately from standard solutions. The original concentrations (added from standards) in the undiluted test sample (= 1 on the *x*-axis) were chosen to simulate a crude extract from a green plant: 32.5 μM [Mg]-Chl a, 4.47 μM [Mg]-Chl b, 1.42 μM Pheo a, 0.083 μM Pheo b, 1.79 μM β-carotene, 3.13 μM lutein, 0.471 μM neoxanthin, 1.33 μM violaxanthin, and 1.74 μM zeaxanthin. (A) New GPS method, (B) SRC method of Naqvi et al.[1] (C) Simplified version of the GPS method. (D) Optimized version of the SRC method.

was even more obvious when pigment mixtures instead of individual pigments were analyzed (Figures 3A). Here the accuracy of the analysis was limited to a smaller range, because the detection limit for minor constituents of the mixture (e.g., Pheo b and minor carotenoids like neoxanthin) was certainly reached long before the concentration of the major pigments (e.g., [Mg]-Chl a or lutein) became limiting. This is illustrated by the big discrepancy between the "sum of all errors in % of the total pigment" and the "average error of each pigment in % of the added concentration of that pigment" (Figure 3). It is logical because in the same mixture at similar extinction coefficients for all pigments (all photosynthetic pigments have molar extinction coefficients in the same order of magnitude; see Table 1), the absorption of a less abundant pigment is less above the background noise of the spectrometer compared to the absorption of a more abundant pigment in the same mixture.

The measurement of error by "average error of each pigment in % of μM of that pigment" means that a detection of 0.1 μM pigment A when 0.2 μM of this pigment were present (50% error)

$$50\%\ \text{error for pigment A} \times \frac{0.2\ \mu\text{M pigment A}}{2\ \mu\text{M total pigment}} + 10\%\ \text{error for pigment B} \times \frac{1.8\ \mu\text{M pigment B}}{2\ \mu\text{M total pigment}} = 14\%\ \text{error}$$

and detection of 1.62 μM pigment B where 1.8 μM was present (10% error) would lead to an average error of 30%. The "sum of all errors of all pigments in % of total pigment in the mixture" weighs the errors of each pigment by its abundance in the mixture. For the example above, it would be

This way of error calculation was chosen to adequately take into account that less abundant pigments are usually less precisely quantified, and usually less important for the total performance of the method, compared to more abundant pigments.

The assessment of errors as the "sum of all errors of all pigments in % of total pigment in the mixture" in complex solutions (Figure 3A) followed a similar course as the error of the estimation of individual pigments (Figure 2B), with errors dramatically

increasing only below 0.002× the original concentration (equivalent to 0.064 μM [Mg]-Chl a). In contrast, the average relative (%) error per pigment drastically increased already below 0.01× the original concentration (equivalent to 0.33 μM [Mg]-Chl a and 0.031 μM lutein, but only 0.0008 μM Pheo b and 0.005 μM neoxanthin). According to tests with leaves of *Thlaspi* species, this detection limit corresponds to ~0.05 mg of lyophilized plant material/mL of acetone, i.e., ~0.5 μg of plant material is required for a complete pigment analysis using a 10-μL microcuvette. Modern high-performance spectrophotometers could extend the performance of the GPS method to even lower concentration due to their better signal/noise ratio compared to the instrument used in the current study. At the upper end of the tested concentration range, our tests showed a decrease of accuracy due to the OD limit of the spectrometer and fluorescence of Chl, both leading to the flattening of the peaks in the black curve in Figure 2A. The OD limit of the spectrometer is usually due to stray light, and fluorescence of a sample behaves like stray light as well. The former would be less a problem with a more modern high-performance spectrometer, some of which have a linear range up to OD 6. This does not influence the fluorescence problem, but in the spectrometer used here, this was not the main problem as the sample holder is far away from the detector. With the performance of our instrument, the specifications of which are typical for average laboratory-grade spectrometers, the most accurate results were obtained at a total maximal absorbance of the sample between 0.5 and 1.0.

Comparison of the Performance of the new GPS Method with Previous Methods. The systematic comparison of results, obtained with the new GPS method and previous or simplified methods, showed the impact of several factors on estimation accuracy, as shown in Figures 3−6. All methods to be compared were provided with exactly the same measured data.

All early methods for photometric quantifications used only the absorption coefficients at the absorbance maximums of the individual components, i.e., an *n*-point matrix was used to set up n linear equations for the estimation of *n* components (e.g., Arnon,[3] Lichtenthaler and Wellburn,[6] Jones et al.,[5] White et al.,[4] various further methods reviewed by Porra[8]). The SRC method of Naqvi et al.[1,2] used a larger number of data points, but was still limited to a fixed sample interval, and in its original published version[1] did not include any parameters for automatic correction of baseline inaccuracy or variations in measuring conditions such as temperature, residual water, or turbidity in the sample. This lack of correction parameters makes it unreliable for regular use, as shown in Figure 3B. As described in a later update by Naqvi et al.,[2] the linear correction of the baseline (i.e., offset and slope) can be included in the SRC method, and in our test of an optimized SRC method, we included the automatic scattering correction as well (Figure 3D). However, the performance of this "optimized SRC method" still did not come close to the performance of the new GPS method; large errors in the estimation of all minor constituents of the mixtures resulted in a large average error (Figure 3A). This is because the principle of the SRC method, i.e., directly taking a measured standard spectrum as a basis for fitting the spectrum of the mixture, does not allow us to include correction parameters like "Peakwidth" and wldev that modify the recorded standard spectra to correct unavoidable (at least in usual laboratory practice) variations in the measuring conditions. The importance of these two GPS-specific correction parameters was, therefore, investigated in a further test shown in Figure 4. This showed that already very small deviations from the optimal Peakwidth or wavelength position lead to a large increase of the errors in the quantification of individual pigments; again the minor components of the pigment mixture were affected most. Noteworthy, the optimal peak position was not exactly the one determined by the isolated/purchased standards (that would have been wldev = 0), but slightly (0.2 nm) different, due to the day-to-day and scan-to-scan deviations in wavelength calibration of the spectrometer. Similarly, the optimal peakwidth was slightly different (0.9960 ± 0.0003) from that of the standards (1.0), which

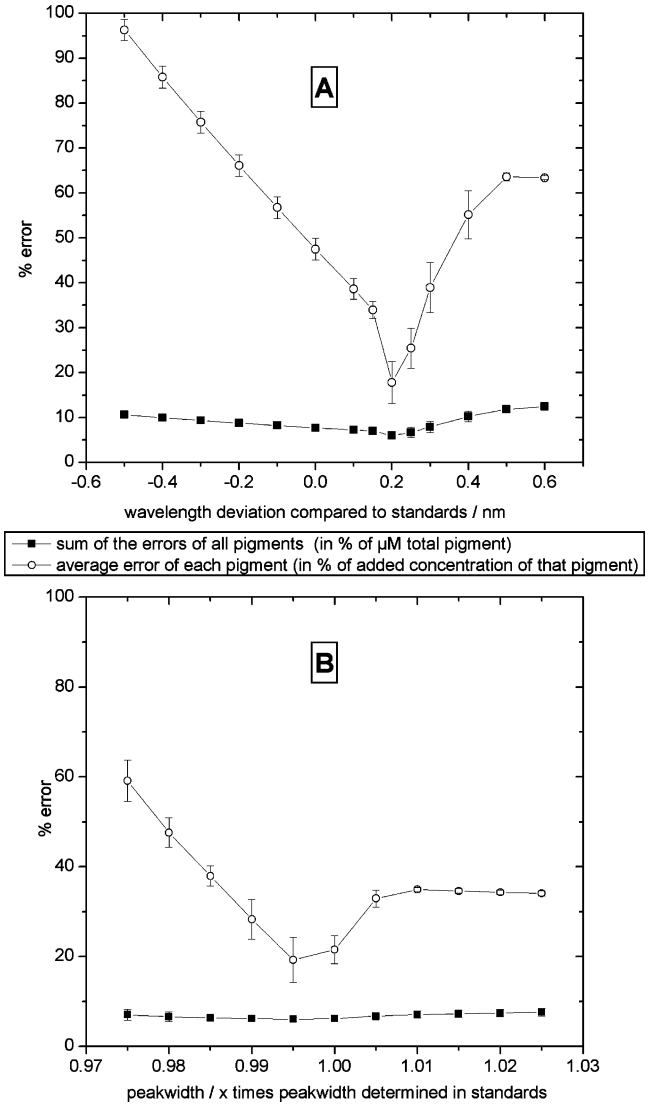


Figure 4. Test of the importance of the automatic peakwidth and wavelength deviation correction parameters in the GPS method. Each data point represents the average and standard error of three independent GPS fits of three samples assembled separately from standard solutions. The pigment mixture used for these test was the 0.2× dilution of the mixture applied for the tests shown in Figure 3. (A) GPS fitting test with fixing the wavelength deviation (wldev) parameter of the GPS equation to certain values. All other parameters of the GPS fit were kept free as usual. (B) GPS fitting test with fixing the peakwidth parameter of the GPS equation to certain values. Again, all other parameters of the GPS fit were kept free.

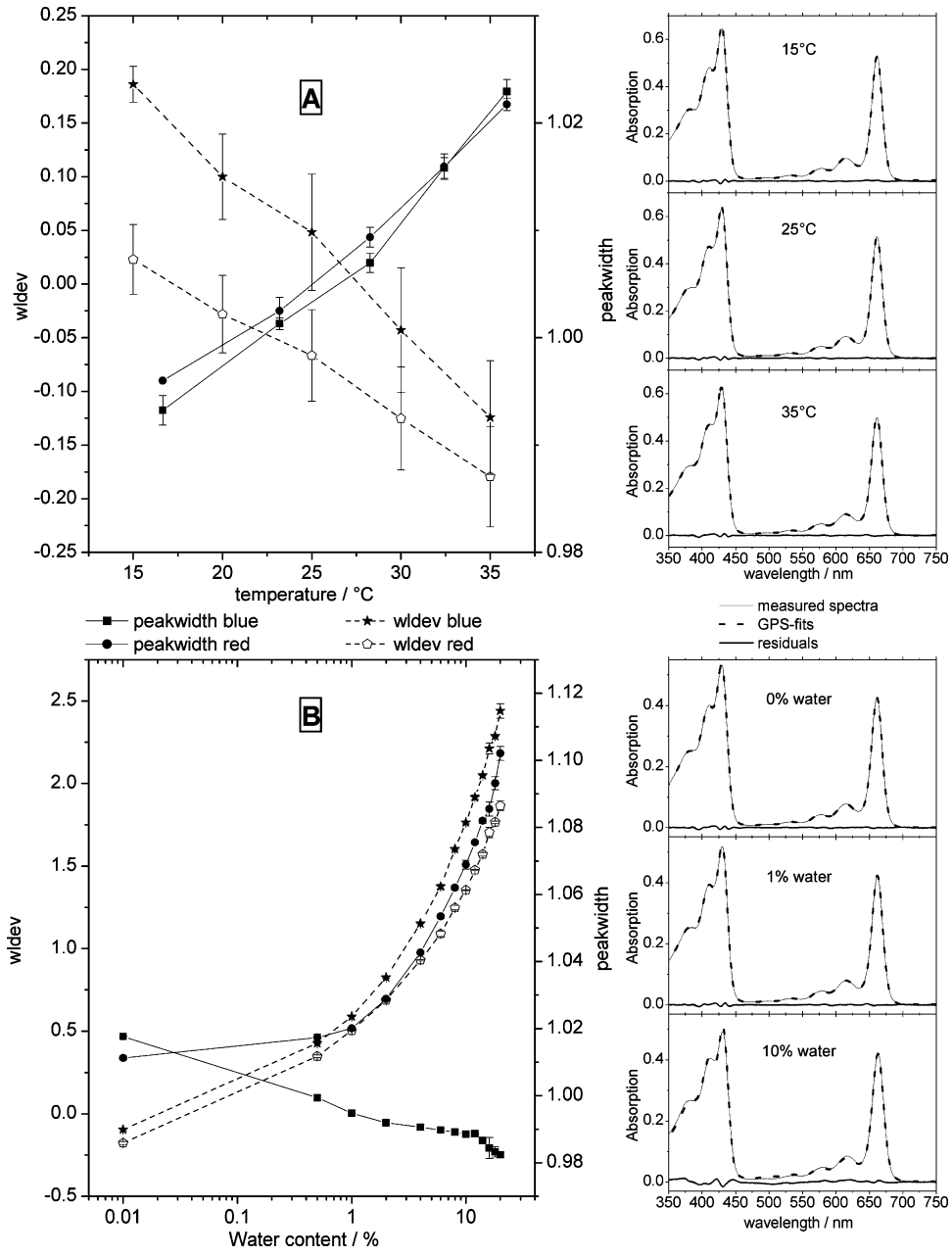


Figure 5. Tests of effects of caused by deviations in temperature and residual water content of the samples. Each point in the graphs represents the average and standard error of three or four samples of Chl a independently prepared from a concentrated stock solution. For these tests, the red and blue regions of the spectrum were fitted with independent wldev and peakwidth parameters. Left, parameters wldev and peakwidth of the GPS fits; right, selected spectra. Effects of (A) changes in temperature and (B) increasing water content.

might be due to slight differences in measuring temperature or residual water in the sample. Since such small but obviously decisive variations cannot be taken care of by measuring good standards, the lack of their correction in the SRC method is most likely the main reason why that method does not quantify the minor components of a pigment mixture in a reliable way (Figure 3B). This problem may, however, also still be limiting to the accuracy of the GPS method. There may be slight differences between these values already between the individual standards, even if they were measured on the same day. Since wldev and peakwidth are common parameters in the GPS of all pigments of the mixtures, deviations in the values of these parameters between the pigments cannot be corrected. Instead, in the fitting, the values of those parameters will mainly reflect their optimization for the main pigment(s) in the mixture, while they will be suboptimal for the minor pigments. Further, under some circumstances (e.g., water contaminations), these parameters may shift differently for different types of pigments. For these reasons, an independent fitting of these parameters for each pigment in the mixture was attempted (data not shown). This only worked, however, for the most abundant pigments with the most feature-rich spectra (mainly [Mg]-Chl a and [Mg]-Chl b), but did not work at all for minor pigments in the mixture (due to their bad signal/noise ratio) and worked badly for pigments with few distinct features in their spectra (i.e., carotenoids with only one broad main peak like canthaxanthin or not very pronounced side peaks like β-carotene). Therefore, as the most widely applicable and reliable

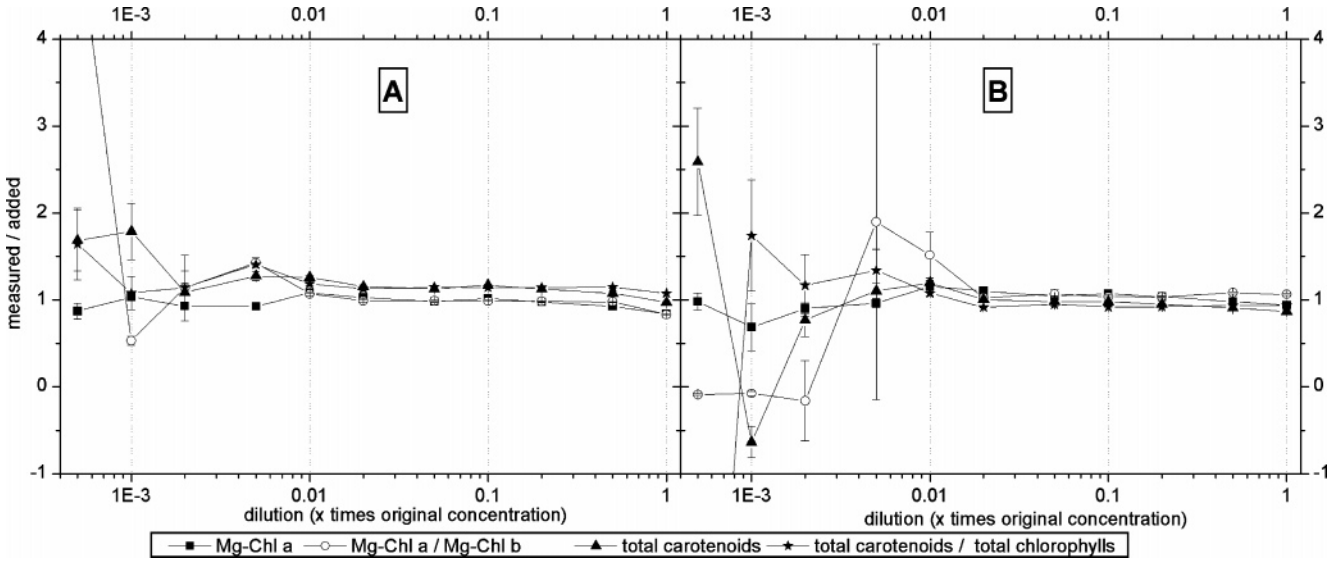


Figure 6. Comparison of the performance of the GPS method and traditional combinations of linear equations in determining commonly used parameters of pigment composition of plant samples. Each data point represents the average and standard error of three independent calculations of three samples assembled separately from standard solutions. The pigment mixture used for these test was the same mixture as applied for the tests shown in Figure 3. (A) New GPS method, (B) Method of Lichtenthaler and Wellburn.[6] Other methods of that type (i.e., n spectral points incorporated into n linear equations for *n* pigments) would lead to similar results.

form, we present the GPS method here with common wldev and peakwidth for all pigments in the mixture.

The efficiency of the wldev and peakwidth parameters was further evaluated by a test with varying temperatures and varying water content in the sample (Figure 5). Increasing temperature is well-known to cause a widening of UV/vis absorption peaks. The current test (Figure 5A) showed that in the spectral and temperature range analyzed this widening is about proportional to the temperature and is by the same factor for the blue and red region of the spectrum. The latter means that the peakwidth parameter can completely compensate such temperature effects. In addition to the widening, increasing temperature causes a slight blue-shift of the absorption peaks, as shown by the wldev parameter in Figure 5A. Since the temperature dependency of this shift was again very similar for the blue and red region of the spectrum, it can be efficiently corrected by the wldev parameter. The successful automatic correction of temperature effects was demonstrated by the almost undetectable deviations between measured spectrum and the GPS fits shown in Figure 5A. Without correction, these spectral changes would lead to dramatic errors in pigment quantification, as revealed by a comparison of the wldev and peakwidth values in Figure 5A with the test of importance of these parameters in Figure 4. Addition of water to the sample caused spectral changes that could only partially be compensated by the peakwidth and wldev parameters. The rather strong (~2.7 nm from 0 to 20% water content) red shifts in the peak positions that were caused by water addition were identical for the blue and red regions of the spectrum and therefore could be corrected by the wldev parameter (Figure 5B). In contrast to temperature effects, however, the peak widening was strongly different for the blue compared to the red region of the spectrum. Peak width slightly decreased in the blue region, but strongly increased in the red region. This discrepancy cannot, in principle, be corrected by the peakwidth parameter in its current form, so that the GPS fits showed rather severe deviations from the measured spectrum when more than ~1% water was added to the sample (Figure 5B).

Compared to our earlier method of GPS analysis,[14] the current method not only extended the applicability from chlorophylls to all pigments in a crude extract and to less ideal measuring conditions (e.g., turbid samples and temperature changes) but also made the quantification of Chl derivatives generally more accurate. For example, the problem that [Cu]-Chl a and Phe b could not be accurately distinguished in the old method was overcome now; in none of our tests was an interference between these pigments found (Table 3).

In many laboratory applications, the separate quantification of all pigments in a plant extract is not required, and parameters describing a ratio between key pigment classes or the sum of a pigment class may be more important for the physiological question to be analyzed. Typical examples are the Chl a/Chl b ratio, the Chl/carotenoid ratio, or the total carotenoid content. Therefore, the performance of the new GPS method compared to a typical traditional photometric method[6] was tested for these applications as well, as shown in Figure 6. At high pigment concentrations, both methods accomplished the task with very similar accuracy; the errors in this range were most likely dominated by the limited accuracy of pipetting the pigment standards. At lower pigment concentrations, however, the GPS method proved to be clearly more reliable. While this method yielded meaningful results down to 0.002 times the original concentration of the mixture (corresponding to 0.077 μM total chlorophylls and 0.017 μM total carotenoids), the traditional method yielded nonsense results (e.g., negative [Mg]-Chl b) at this pigment concentration. To achieve the same accuracy as with the GPS method at its limiting concentration, five times higher pigment concentrations were required for the traditional method using the same pigment mixture.

Application of GPS Analysis to Extracts of Photosynthetic Organisms, Comparison with HPLC. As a final test of the performance of the method, it was applied to crude extracts of examples of all phyla of photosynthetic organisms for which it was designed: *T. fendleri* (Chlorophyta), *E. gracilis* (Eugleno-

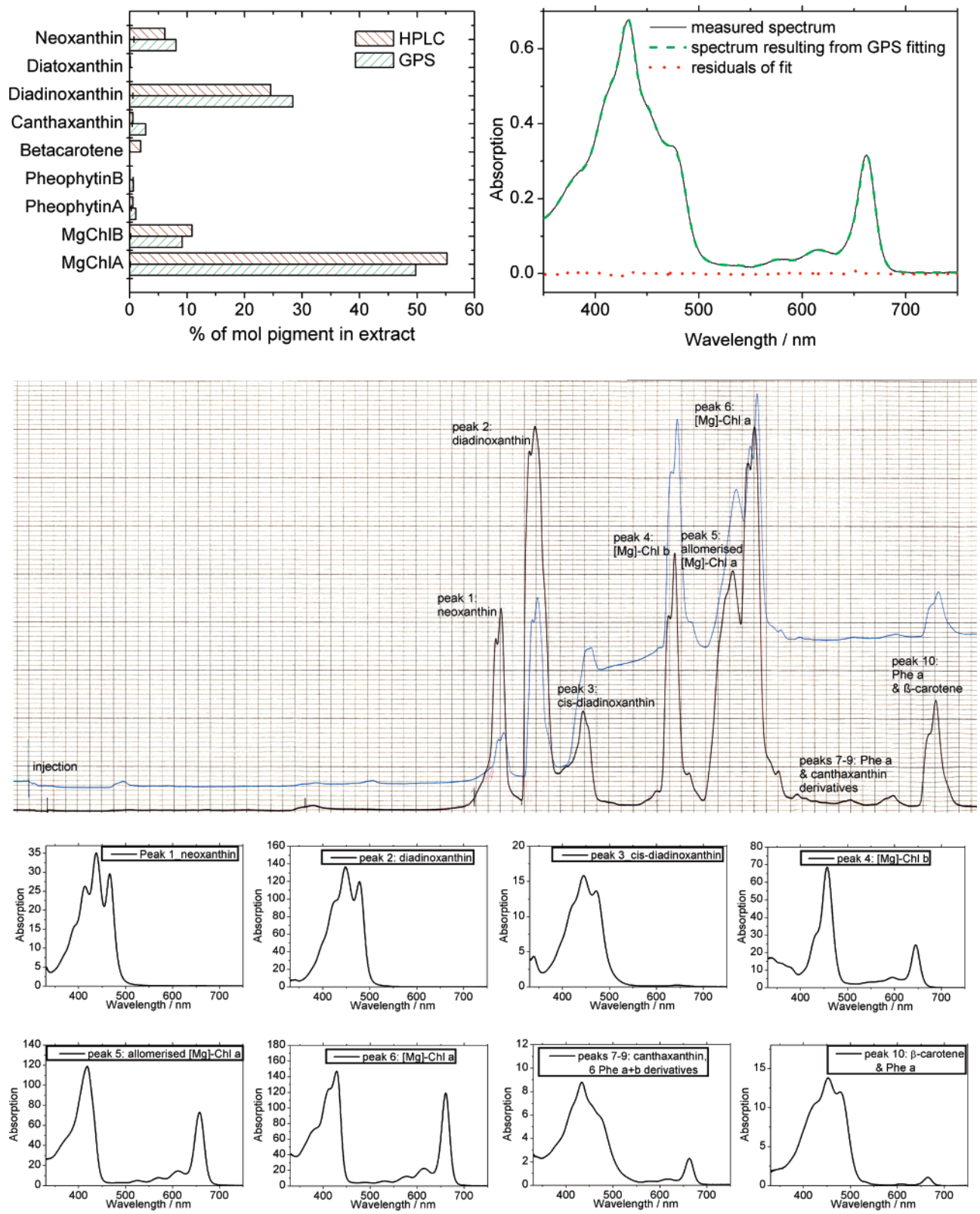
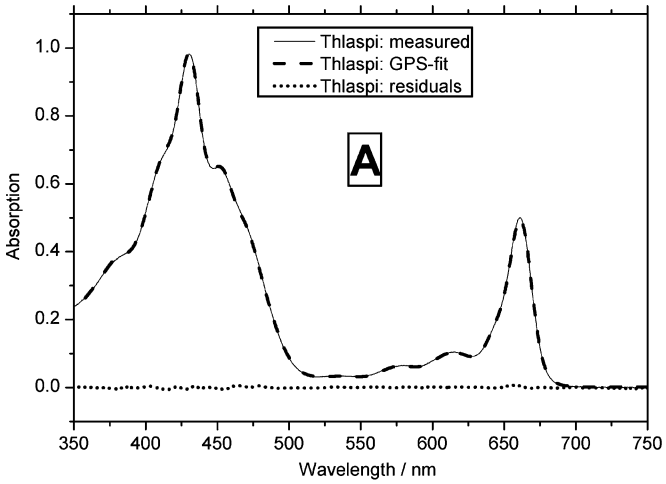
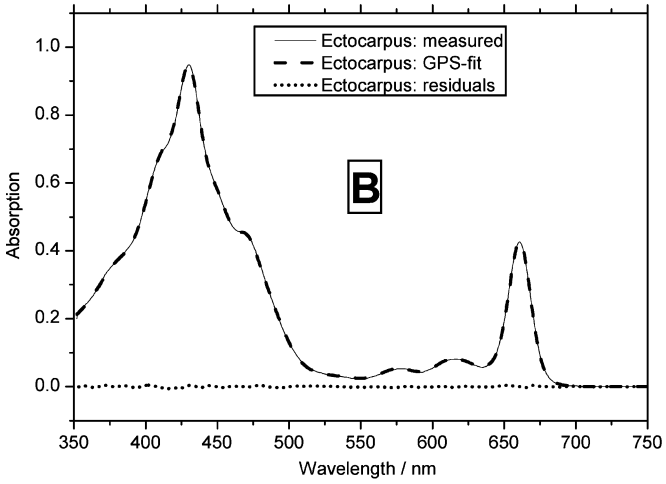


Figure 7. Comparison of analysis results obtained by GPS fitting and HPLC of a crude extract in 100% acetone (transferred to 100% methanol for HPLC) of *E. gracilis* (Euglenophyta). The blue (upper) trace in the HPLC chart shows absorption at 280 nm; the brown (lower) trace depicts the absorption at 444 nm. The spectra of the HPLC fractions were measured after dilution to an optical density of 0.5−1.5 and are shown after recalculation to their original height.

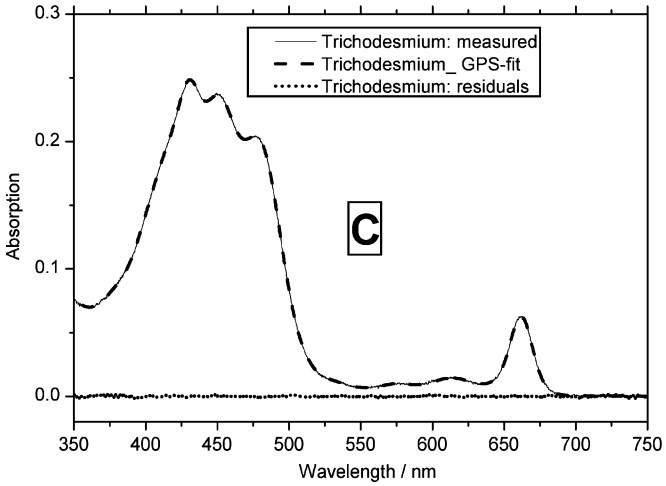
phyta), *E. siliculosus* (Phaeophyta), and *T. erythraeum* IMS101 (cyanobacteria). For all of them, the results of the GPS analysis are shown as a comparison of the measured absorption spectrum with the fitted GPS spectrum and the amounts of the pigments detected (Figures 7, 8). For *E. gracilis*, a detailed comparison with an analysis by HPLC is shown in addition (Figure 7). In all cases,



Parameter	value	error
[Mg]-Chl a	5.789	0.005
[Mg]-Chl b	2.024	0.003
Phe a	0.000	0.006
Phe b	0.000	0.006
Antheraxanthin 0	0.062	
β-Car. & Zeaxanthin	0.847	0.01
transLutein	1.127	0.038
Neoxanthin	0.390	0.027
Violaxanthin	0.545	0.015
Y0	-0.003	6E-4
slope	0.000	0.000
scattering	0.108	0.010
wldev	0.141	0.006
peakwidth	0.996	6E-4



Parameter	value	error
[Mg]-Chl a	5.082	0.006
[Mg]-Chl c1	0.000	0.011
[Mg]-Chl c2	0.756	0.009
Phe a	0.106	0.006
Antheraxanthin	0.288	0.039
Betacarotene	1.214	0.017
Fucoxanthin	2.651	0.065
Violaxanthin	0.646	0.023
Y0	0.007	1E-3
slope	0.000	0.000
scattering	0.102	0.015
wldev	-0.569	0.009
peakwidth	0.998	1E-3



Parameter	value	error
[Mg]-Chl a	0.673	0.001
Phe a	0.127	0.001
cis-Canthaxanthin	0.082	0.024
trans-Canthaxanthin	0.000	0.017
cis-Echinenone	0.203	0.026
trans-Echinenone	0.000	0.031
Myxoxanthophyll	0.000	0.004
ß-Carotene, ß-Cryptoxanthin, trans-Zeaxanthin	0.718	0.013
9-cis-Zeaxanthin	0.408	0.027
13-cis-Zeaxanthin	0.633	0.026
YBCHK$_{medium}$	0.0085	3E-4
Y0	0.0015	6E-4
slope	0.000	0.000
scattering	0.124	0.014
wldev	0.169	0.011
peakwidth	1.008	0.001

Figure 8. Application of GPS analysis to crude extracts (in 100% acetone) from diverse photosynthetic organisms. (A) *T. fendleri* (Chlorophyta); (B) *E. siliculosus* (Phaeophyta); (C) *T. erythraeum IMS101* (cyanobacteria). Note the variations in the error correction parameters (Y0, slope, scattering, wldev, peakwidth) between samples in comparison to the errors that would be caused if these parameters were not present (test in Figure 4).

the fitted GPS spectrum was virtually indistinguishable from the measured spectrum, and correspondingly, the residuals did not exceed the baseline noise of the spectrometer (Figures 7 and 8). Further, the detected pigment composition matched the usual pigment composition of these classes of organisms as it is known from the literature (e.g., for brown algae, Colombo-Pallotta et al.;[18] for *E. gracilis*, Doege[19]). The comparison with HPLC showed a good correlation, but also reconfirmed the principal problems with

(18) Colombo-Pallotta, M. F.; Garcia-Mendoza, E.; Ladah, L. B. *J. Phycol.* **2006**, *42*, 1225−1234.

(19) Doege, M. Photoprotektive Mechanismen der Alge Euglena gracilis in Abhängigkeit vom Carotinoidgehalt: Untersuchungen zur nichtphotchemischen Löschung der Chlorophyll a-Fluoreszenz. Ph.D. thesis. Electronic Document of the ULB Sachsen-Anhalt, 1999.

HPLC analysis of pigments. First, the preparation of the samples for HPLC, involving vacuum evaporation of the crude extract and redissolving in a methanol−water mixture, usually caused some pigment degradation. A typical example of this problem is the formation of allomerized Chl; see peak 5 in the elution diagram of Figure 7, which occurs when the pH of crude extract is neutral to slightly alkaline.[13] If the pH is adjusted to a lower value, usually some (and sometimes a large proportion) of the [Mg]-Chl a is converted to Phe a. In addition, at slightly lower pH in *Euglena*, usually a partial conversion of diadinoxanthin to diadinochrome (diadinoxanthin-5,6-epoxide) or in higher plants a conversion of violaxanthin to auroxanthin is observed (not shown). Such chemical changes of pigments during or before HPLC cause additional peaks, shoulders, and tails in the HPLC elution diagram, limiting accuracy of the quantification (Figure 7). Further, as in the GPS method and also in HPLC analysis, some pigments show similar behavior, making them hard to separate. But while in the GPS method these are pigments with the same (or a very similar) chromophore (e.g., β-carotene and zeaxanthin), i.e. with similar light harvesting or exciton quenching properties in photosynthesis, in HPLC, these overlapping peaks are often chemically and spectroscopically very different pigments, such as Pheo a and β-carotene in peak 10 of the elution diagram shown in Figure 7. The latter two pigments are completely unrelated to each other in their photobiophysics and have a completely different physiological relevance. Therefore, the type of errors occurring in HPLC analysis may lead to more serious errors in the biochemical/biophysical conclusions derived from such an analysis of pigments of a photosynthetic organism than the errors occurring in the GPS analyses.

CONCLUSIONS

While we described the basic principle behind this method earlier, only now it became possible to fit a spectral region large enough and include enough automatic error correction parameters for reliably quantifying all physiologically relevant chlorophyll derivatives along with all major carotenoids that may occur in the same organism/tissue under physiological conditions. The new pigment quantification method has proven to provide a reliable estimate of the concentrations of various carotenoids and chlorophyll derivatives in complex mixtures, for which so far analytical HPLC was the only choice. Compared to earlier spectrophotometric methods, the new method is more versatile by providing a simultaneous quantification of a much larger number of pigments, more accurate at low pigment concentrations, and much less prone to quantification artifacts due to baseline instability and wavelength deviations of the spectrometer, temperature, turbidity, and water contaminations of the sample. Compared to analytical HPLC, the new method is easier (no physical/chemical separation of pigments, so no risk of artifacts in such procedures), less costly (only some microliters of acetone required per sample, instead of a column and large amounts of solvents), and faster (~1−2 min analysis time compared to ~30 min for a typical analytical HPLC run of similar accuracy). However, even with the new method, some general problems of the spectrophotometric estimation of pigments remain. First, substances with a low absorption coefficient in the relevant spectral region or which are only minor components of the mixture to be analyzed cannot be determined with a high accuracy. This could be, in principle, somewhat improved by using a high-performance spectrometer, since this problem is mainly dependent on the signal/noise ratio that can be achieved at the necessary spectral bandwidth (1 nm or smaller) below the upper OD limit of the detector. Second, the method cannot, due to its principle of function, distinguish between pigments with (almost) identical UV/vis absorption spectra, e.g., the all-trans isomers of β-carotene, β-cryptoxanthin, and zeaxanthin. If the ratios between such pigments need to be measured, either classical analytical HPLC has to be used or GPS of another spectral region, where these compounds differ in their absorption (in the mentioned cases the infrared region), have to be set up. The latter would be like adding an additional detector to an HPLC system to resolve overlapping peaks.

Abbreviations: Chl, chlorophyll; GPS, Gauss peak spectrum, i.e., the series of Gaussian peaks describing a pigment spectrum in the UV/vis region; hm, heavy metal; hms, heavy metal-substituted; HPLC, high-performance liquid chromatography; Mg substitution, substitution of the natural central ion of Chl, Mg, by heavy metals; Pheo, pheophytin; PUO, parameter used for optimization; SRC method, spectral reconstitution method (method of Naqvi et al.[1,2]); VPO, variable parameter of optimization when analyzing an unknown sample by the GPS method; wldev, wavelength deviation; parameter in the GPS method for the estimation of chlorophylls

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SUPPORTING INFORMATION AVAILABLE

Supplement 1: GPS fits of all pigments presented in this publication. Supplement 2: The complete GPS equations in text format, including instructions for their use in data analysis programs. Supplement 3: The complete GPS equations as a ready-to-use fitting library for the data analysis program SigmaPlot (version 5 and above). The instructions for use provided in supplement 2 apply to this fitting library as well. This material is available free of charge via the Internet at http://pubs.acs.org.

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