Photometric Method for the Quantification of Chlorophylls and Their Derivatives in Complex Mixtures: Fitting with Gauss-Peak Spectra

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Accurate quantification of pigments in mixtures is essential in all cases in which separation of pigments by chromatography is impracticable for one reason or another. An example is the analysis of *in vivo* formation of heavy metal-substituted chlorophylls in heavy metal-stressed plants. We describe here a novel, accurate UV/VIS spectrophotometric method for the quantification of individual chlorophyll derivatives in complex mixtures, which has the potential for universal applicability for mixtures difficult to separate. 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 and baseline instability of the spectrometer as well as a correction of the widening of absorbance peaks in more concentrated pigment solutions. The automatic correction of peak shifts can also partially correct shifts caused by processes like allomerization. In this paper, we present the Gauss-peak spectra for Mg-chlorophyll *a*, *b*, *c*, pheophytin *a*, *b*, *c*, Cu-chlorophyll *a*, *b*, *c*, and Zn-chlorophyll *a* in acetone; Mg-chlorophyll *a*, *b*, pheophytin *a*, *b*, Cu-chlorophyll *a*, *b*, allomerized Cu-chlorophyll *a*, *b*, and Zn-chlorophyll *a*, *b* in cyclohexane; Mg-chlorophyll *a*, *b*, pheophytin *a*, *b*, and Cu-chlorophyll *a*, *b* in diethyl ether. © 2000 Academic Press

Key Words: chlorophyll *a* + *b* + *c*; copper; Gaussian peaks; nonlinear optimization; pigment quantification; pheophytin; spectroscopy; zinc.

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Quantification of pigments, especially chlorophylls, is a basic prerequisite for the study of many aspects of plant physiology. In most cases, only the main pigments (e.g., Chl *a* + *b* in higher plants)⁴ are estimated since in unstressed, healthy tissue other chlorophyll derivatives are present only in small amounts. In these cases, quantification can be done by photometry using linear equations like those published by Arnon (1).

However, the situation becomes more difficult if the number of pigments with overlapping absorbance peaks increases, and if separation by HPLC is impracticable for one or another reason. One example is the quantification of pigments in extracts from heavy metal-stressed plants. It has been found that under conditions of heavy metal stress the central ion of Chl, Mg²⁺, can be exchanged by heavy metals, which causes inhibition of photosynthesis and thus constitutes an important damage mechanism in stressed plants (2, 3). Extracts of such plants contain a mixture of Mg-chlorophylls, hms-chlorophylls, and pheophytins. Publica-

⁴ Abbreviations used: Chl, chlorophyll; buffer A, buffer used for harvesting cells and preparing thylakoids for green gels, also used for samples, which were subsequently dried and extracted with cyclohexane or acetone; GPS, Gauss peak spectrum, i.e., the series of 4–5 Gaussian peaks describing a chlorophyll spectrum in the region from 550 to 750 nm; 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; SQP, successive quadratic programming; SRCM, spectral reconstitution method (method of Naqvi *et al.* (6)); *v* = vector of parameters, which are subject to optimization (here: *Y0*, slope, different chlorophyll derivatives expected in the sample, e.g., Mg-Chl *a*, Mg-Chl *b*, Pheo *a*, and Pheo *b* in a sample of an unstressed green plant); *wldev*, wavelength deviation; parameter in the GPS method for the estimation of chlorophylls.

tions on heavy metal chlorophylls *in vivo* cite several methods that have been used to detect these compounds in stressed plants. UV/VIS spectroscopy has proved to be the most important and most reliable method for this purpose. While already *in vivo* a qualitative (and semiquantitative) detection is possible (3), for an exact quantitative determination of these pigments they must be extracted. The reason is that *in vivo* there are too many and too wide absorbance bands in the relevant spectral region.

The quantification of chlorophylls in such extracts has been facing a number of obstacles, so that the current methods for the estimation must be used with great care. Nevertheless the accuracy was not satisfactory. To date methods for calculating the concentrations of various chlorophylls (and other pigments) in the absorbance spectrum of a mixture have always used linear equations based on the absorption coefficients at the positions of the absorbance maxima of the individual compounds. The first such method was published in 1949 by Arnon (1) for the estimation of Chl *a* + *b*. This method was later adopted for the estimation of hms-Chls (4, 5). There have always been some inherent obstacles when using this traditional method, limiting its accuracy. The use of only six points for the estimation of six substances makes this method very vulnerable to any kind of noise. Also a slight drift in the baseline of the spectrometer can lead to large errors of estimation. The limited wavelength accuracy of spectrometers increases the problem: a shift in peak positions of only 1 nm causes large deviations (see Results). Finally, the acidification, which is used in this method to improve the distinction between Cu-Chl and Mg-Chl, can cause problems by pigment degradation and the occurrence of turbidity in the acidified extract.

For the quantification of Mg-Chls *a* and *b*, there has been an attempt to improve the accuracy by fitting ASCII files of absorption spectra of standard solutions to the sample spectrum (6). This method, developed by Naqvi *et al.* and called SRCM (spectral reconstitution method), improved the accuracy of the results by a reduction of noise problems, which was due to the much larger number of points used for quantification. However, these authors also found that both sample and fitting spectra had to be measured on the same photometer in order to yield meaningful results. This was again because of the limited wavelength accuracy and baseline stability of spectrometers, which could not be corrected with this method. As we also tested in the present work, the lack of such correction possibilities drastically lowers the accuracy of such a method.

In this publication, we present a novel method of estimation, which we developed during our studies of Mg substitution in hm-stressed plants (2, 3). Its most

important feature is a mathematical description of the absorption spectrum of each compound throughout the whole relevant spectral region. This enables an automatic correction of inaccuracies in the baseline and in the peak positions caused by instrument inaccuracy and sample preparation. It also partially corrects the problems caused by interactions of pigment molecules in more concentrated solutions.

In principle, this method can also be adopted for the estimation of other pigments in mixtures, provided that all substances present have clearly distinguishable peaks which can be well described by Gaussian peak functions.

MATERIALS AND METHODS

Pigment Standards

Pigment standards for the calibration of the novel chlorophyll estimation procedure (described below) were prepared from extracts of algae. Chlorophylls *a*, *b*, and derivatives were isolated from the green alga *Scenedesmus quadricauda* (Turp.) Bréb (strain Greifswald 15) provided by the culture collection of the Botanical Institute, Czech Academy of Sciences, Třeboň, ČR. Chlorophylls *c*₁, *c*₂, and their derivatives were prepared from the brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye (strain Port Aransas) provided by D. G. Müller, Konstanz University, Federal Republic of Germany. For substituting Mg²⁺ by Zn²⁺ or Cu²⁺, samples dissolved in 90% methanol were acidified with 10 µl concentrated HCl/ml of extract and subsequently saturated with ZnSO₄ or CuSO₄ at about 30°C. These hms-Chl preparations were then transferred to cyclohexane, which was evaporated either in a stream of N₂ or in a vacuum rotary evaporator, and finally the pigments were redissolved in 100% acetone. For isolating derivatives of chlorophylls *a* and *b*, these hms-Chl preparations as well as untreated extracts (for preparation of Mg-chlorophylls) were separated by preparative HPLC on a Biospher C18 column (25 × 250 mm, 5 µm particle size) using an isocratic elution with 80% methanol and 20% acetone. Since Cu-Chl *a* tended to stay on the column too long using this system, the solvent was changed to a methanol:acetone ratio of 1:1 after elution of all other pigments. Purified standards were transferred as rapidly as possible to cyclohexane to prevent allomerization caused by methanol. For chlorophyll *c* derivatives, in most cases the standards were prepared by thin-layer chromatography on cellulose sheets with a mixture of petrol ether:acetone = 5:1 for elution (7). Thus, chlorophylls *c*₁ and *c*₂ eluted together, representing the natural mixture of these pigments in brown algae (e.g., 8).

Extraction of Pigments from Plants

Plants were washed in buffer A (25 mM Tris/HCl (p.a.), pH 7.5, containing 1 mM aminocaproic acid (p.a.) and 1 mM aminobenzamidine (p.a.)), lyophilized for 2 days in the dark, and ground with sand. Samples of *Chlorophyta* were then extracted 2 days (in the dark) with cyclohexane (Uvasol or LiChrosolv, Merck) plus 0.5% of isopropanol (Merck p.a.) saturated/dried with NaHCO_3 , CaCl_2 , and K_2CO_3 (all Merck p.a.). Usually about 1 ml of solvent was added per 100 mg of dry sample. This method of extraction was chosen to prevent the complexation of heavy metal ions by chlorophyll during extraction (2). Since chlorophyll *c* is too hydrophilic for extraction with this solvent mixture, for brown algae an extraction with 100% acetone (Merck p.a.) was used instead. Extracted pigments were analyzed at once. If extracts were stored for any reason, this was done at -70°C in the dark.

Test Solutions of Known Composition

To test the accuracy of the new method and to compare it to that of the traditional method (4, 5), standard solutions (see above) were combined to compose mixtures of known composition. For this purpose, concentrated stock solutions in cyclohexane were diluted about 200-fold by either diethyl ether or cyclohexane. Each test mixture was produced three times to get three independent replicates.

To yield exactly identical conditions for the comparison of methods (Table 2), the same sample in diethyl ether was analyzed by both methods. The spectrum before acidification of the sample (which is required for second step of the method of White *et al.* (4)) was used for quantification with the new method as well as the first step of the method of White *et al.*

UV/VIS Spectroscopy

All samples were dried with anhydrous CaCl_2 , centrifuged at 4000g for 10 min to sediment particles, and diluted to a maximum OD (400–700 nm) of 0.2 to 0.5. Spectra were measured with the UV/VIS spectrophotometer Shimadzu UV3000.

A slit (spectral bandwidth) of 0.5 nm was selected for all measurements. This is important because slit-widths over 1 nm lead to significant lowering of the sharp peaks of pheophytins, widths over 2 nm even change the shape of the peaks of the metallochlorophylls. Therefore, the equations presented here are valid only for measurements carried out with slits <1 nm; larger slits can result in major errors.

Calculation of Gauss-Peak Spectra

The spectrum of each pigment standard was normalized to the absorbance of a 1 μM solution in a cuvette

of 1 cm optical path. The absorption coefficients used are listed in Table 3. For Chl *c* derivatives, the natural mixture of Chl c_1 and c_2 in brown algae as extracted from *E. siliculosus* was used.

Then these normalized data were fitted with a series of 4 to 5 Gaussian peaks using the Levenberg-Marquard algorithm (see below) so as to mathematically describe the spectrum between 550 and 750 nm. This was called a Gauss-peak spectrum (GPS). For quantifying the constituents of an extract, the GPS were fitted to the measured spectrum as follows. 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* 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. Similarly, a parameter for correction of wavelength inaccuracy (the "wldv" parameter) was added (Table 1). Since the wldv 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. Finally, a parameter "peakwidth" was introduced as a factor adjusting simultaneously the width of all peaks in the GPS to the widening of peaks which is observed at higher pigment concentrations due to the interaction of pigment molecules. This peakwidth parameter was coupled to the total pigment concentration using an empirically determined quadratic equation.

All GPS and corresponding equations are listed in Table 1. The combinations of GPS were then fitted to the sample spectrum using the Levenberg-Marquardt method as implemented, e.g., in SigmaPlot or SQP (Successive Quadratic Programming) method, e.g., using the Optimization Toolbox of Matlab.

The region from 550 to 750 nm was selected for the quantification method for several reasons:

- In this region, spectral shifts caused by Mg substitution are particularly large.
- Carotenoids and other compounds present in plants have hardly any absorbance beyond 550 nm, so that chlorophylls can be estimated selectively.
- 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.

Description of Fitting Algorithm

In general, fitting is the process of finding the best possible solution with respect to a given objective function with given boundary conditions. If a criterion for the quality of a fit can be given this problem corresponds to an optimization problem. In many applica-

tions the task is to minimize a so-called least-squares problem like

$$\min_{v \in \mathbb{R}^{N_{\text{var}}}} f(v) = \frac{1}{2} \|F(v)\|_2^2 = \frac{1}{2} \sum_{i=1}^{N_{\text{var}}} (F_i(v))^2, \quad [1]$$

where v denotes the vector of N_{var} variables which are subject to optimization and $F_{i(v)}$ denotes the deviation between the given value at point i and the fitting function (here, the weighted sum of the Gauss-peak spectra).

In the literature (e.g., 9) gradient-based and gradient-free algorithms can be distinguished. The latter are efficient for small problems with one or two variables. For the problem under consideration which has up to 13 variables and a time-consuming calculation of the quality function (depending on the number of given values) gradient-based methods are strongly recommended and will be discussed in detail.

The efficiency of an optimization algorithm depends on a skilled determination of the direction and the stepwidth for choosing the next configuration. The gradient method requires that the objective function can be differentiated at least once. A straightforward approach is to search in the direction of the steepest descent. This algorithm is simple but often inefficient, especially if the minimum is flat. To overcome this difficulty the search direction is determined depending on the gradient and the curvature. For this, the Jacobian matrix $J_{(v)}$ is required which is usually obtained by computationally expensive calculations.

In the Gauss-Newton method the search direction d_k is determined based on

$$\min_{v \in \mathbb{R}^{N_{\text{var}}}} \|J(v_k) d_k - F(v_k)\|_2^2, \quad [2]$$

where k denotes the number of iterations. Following Coleman *et al.* (10) the Gauss-Newton method encounters problems when second order terms involving the Hessian matrix are significant. To overcome this problem, the Levenberg-Marquard method can be used. It is based on the solution of the linear set of equations

$$(J(v_k)^T J(v_k) + \lambda_k I) d_k = -J(v_k) F(v_k), \quad [3]$$

where λ_k controls the magnitude and direction of d_k . When λ_k is zero, d_k is identical to the direction obtained with the Gauss-Newton method; as d_k goes to infinity d_k points in the direction of steepest descent. By this

$$F(v_{k+1}) = F(v_k + d_k) < F(v_k) \quad [4]$$

is ensured even when second order terms are of importance. The Gauss-Newton method is faster than the Levenberg-Marquard method but as it is not sure whether second order terms are significant or not, the latter method is recommended for many practical problems due to its robustness.

For large problems with many variables it may become important to define constraints, e.g., positive concentration and maximum wavelength shift. For optimization with such constraints Lagrangian multiplier methods are used. The state of the art method is the SQP method. The basic idea is to approximate the Hessian matrix to account for second order terms using an iterative Quadratic Programming routine. Especially for problems with large numbers of variables this method shows a better convergence than Newton methods like the Levenberg-Marquard method.

RESULTS AND DISCUSSION

Equations Used in the GPS Method of Chl Estimation

In Table 1 the equations are presented in the form which was used in the curve fitting program SigmaPlot. The equations are valid for any spectrometer which has a slit (spectral bandwidth) smaller than 1 nm. The most accurate results are obtained at a total absorbance of the sample between 0.2 and 0.5. Note that once these equations are defined, only the parameters used for optimization (PUO), which are Y_0 , slope, and the different chlorophyll derivatives expected in the sample (e.g., Mg-Chl *a*, Mg-Chl *b*, Pheo *a*, and Pheo *b* in case of a sample from an unstressed green plant), are varied to fit the measured spectra.

In this paper, optimization results are presented obtained with SQP (using MatLab) and the Levenberg-Marquard (using SigmaPlot 5.0) algorithm. The performance of these two algorithms did not show striking differences: With both methods, on modern personal computers (already a Pentium 200 MHz processor and 32 MB RAM are sufficient) results were obtained within less than 30 s. Therefore the presented method is applicable independently of the available optimization algorithm, as long as the software in which it is implemented is fast enough. The latter is important, since it turned out that older versions of the same software needed up to 400 times longer on the same computer.

Accuracy of the Chl Estimation

As shown in Fig. 1, the simulation of standard spectra with Gaussian peaks yielded curves almost indistinguishable from the measured spectra and characterized by very low χ^2 values. It is obvious in Fig. 2 that

TABLE 1a
Standards of Chlorophyll Derivatives in Diethyl Ether: Parameters of Gauss-Peak Spectra (GPS)

Pigment	Parameter	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
MgChla <i>N</i> = 5	a_N	0.0624	0.0276	0.0134	0.0050	0.0036
	$X_{p,N}$	660.5	656.9	613.6	576.6	561.8
	σ_N	6.16	11.19	14.27	8.32	13.11
MgChlb <i>N</i> = 5	a_N	0.0261	0.0286	0.0105	0.0028	0.0053
	$X_{p,N}$	641.3	638.4	602.8	588.8	552.5
	σ_N	5.49	9.75	33.88	7.05	16.84
CuChla <i>N</i> = 5	a_N	0.0258	0.03383	0.0102	0.0056	0.0042
	$X_{p,N}$	648.9	648.4	628.5	597.3	547.8
	σ_N	11.12	7.32	30.56	10.62	18.08
CuChlb <i>N</i> = 5	a_N	0.025	0.0197	0.0079	0.0105	0.00384
	$X_{p,N}$	633.8	631.6	625.8	585.6	549.0
	σ_N	11.38	19.5	5.33	16.9	8.75
Pheoa <i>N</i> = 5	a_N	0.0417	0.0161	0.0046	0.0055	0.0028
	$X_{p,N}$	666.0	662.6	628.4	606.8	556.7
	σ_N	6.45	10.52	32.28	7.70	12.27
Pheob <i>N</i> = 5	a_N	0.0203	0.0151	0.0032	0.0056	0.0069
	$X_{p,N}$	653.3	652.0	609.9	597.8	552.5
	σ_N	5.31	9.89	38.27	7.91	10.98

Note. Each substance is represented by a GPS with the formula $g(x)$. For quantification of pigments in an extract, the necessary GPS are combined to the function $g(x)$ for simulating the absorbance spectrum of the extract.

$$f(x) = Y0 + x \cdot \text{slope} + \text{MgA} + \text{MgB} + \text{CuA} + \text{CuB} + \text{PheA} + \text{PheB}$$

$$\text{MgA} = \text{MgChla} \cdot g(x), \text{MgB} = \text{MgChlb} \cdot g(x), \dots$$

$$g(x) = \sum_{i=1}^N a_N \cdot \exp\left(-0.5 \cdot \left(\frac{x - \text{wlddev} - x_{p,N}}{\sigma_N \cdot \text{peakwidth}}\right)^2\right)$$

$$\text{peakwidth} = 1 + 0.0002 \cdot (\text{MgChla} + \text{MgChlb} + \dots)^2$$

Symbols for Tables 1a, 1b, and 2c:

a_N = amplitude of peak (in spectrum normalized to 1 μM , 1 cm lightpath)

$f(x)$ = function combining the GPS of the individual pigments

$g(x)$ = GPS = function describing the absorbance spectrum of a single pigment

MgA, MgB, ... = parameters representing the individual GPS in $f(x)$

MgChla, MgChlb, ... = micromolar concentrations of the components in the mixture (= parameters under optimization (PUO))

N = number of Guassian peaks used for the description of the absorbance spectrum of a pigment from 550 to 750 nm

slope = factor for linear approximation of baseline drift

wlddev = deviation from ideal peak position (nm); this parameter automatically corrects wavelength inaccuracy of the spectrometer (PUO)

x = wavelength (nm)

$x_{p,N}$ = center of peak (nm)

$Y0$ = offset for linear approximation of baseline drift (PUO)

σ_N = peak halfwidth (nm)

this is also true for the fitting of extract spectra. Ideally, i.e., if only those components which were fitted contribute to the measured spectrum, the residuals of the fitting process represent only the noise of the baseline.

The comparison of results, obtained with the GPS method and the conventional method, showed the impact of several factors on estimation accuracy (Table 2). The new method proved to be reliable under a wide range of conditions, so that errors in the estimation of six Chl derivatives (Mg-Chl *a*, Mg-Chl *b*, Cu-Chl *a*,

Cu-Chl *b*, Pheo *a*, Pheo *b*) in cyclohexane were typically (at a total absorbance between 0.2 and 0.5) below 2% of total pigment except for Pheo *b*. In mixtures containing less Chl derivatives (e.g., in tests simulating extracts of red algae), errors were even smaller. This was caused by the following major differences compared to the earlier method based on a six-point matrix (4, 5):

(a) The earlier method used only the absorption coefficients at the absorbance maxima of the individual components, i.e., a six-point matrix was used to set up

TABLE 1b
Standards of Chlorophyll Derivatives in Cyclohexane: Parameters of Gauss-Peak Spectra (GPS)

Pigment	Parameter	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
MgChla <i>N</i> = 5	<i>a_N</i>	0.0655	0.0304	0.0146	0.00544	0.00374
	<i>X_{p,N}</i>	661.8	658.2	615	577	562.9
	<i>σ_N</i>	5.9	10.9	14.8	8.3	12.2
MgChlb <i>N</i> = 5	<i>a_N</i>	0.0325	0.0274	0.00393	0.00488	0.0063
	<i>X_{p,N}</i>	642.9	641.8	613.1	594.2	568.8
	<i>σ_N</i>	5.1	9.4	8.2	7.5	40
CuChla <i>N</i> = 4	<i>a_N</i>	0.0537	0.02	0.0109	0.00392	
	<i>X_{p,N}</i>	651.5	648.2	603.1	550.7	
	<i>σ_N</i>	7.74	17.5	14	19.1	
CuChlb <i>N</i> = 4	<i>a_N</i>	0.0353	0.0152	0.00398	0.00765	
	<i>X_{p,N}</i>	627.1	615.3	578.3	551.2	
	<i>σ_N</i>	9.1	33.9	8.4	15	
ACuChla <i>N</i> = 4	<i>a_N</i>	0.0504	0.0179	0.0092	0.0032	
	<i>X_{p,N}</i>	646.9	640.9	596.5	554.4	
	<i>σ_N</i>	9.7	23.4	13.7	16.6	
ACuChlb <i>N</i> = 4	<i>a_N</i>	0.0351	0.0121	0.009	0.005	
	<i>X_{p,N}</i>	621.4	625.3	572	547.1	
	<i>σ_N</i>	9.1	30	15.5	7.2	
ZnChla <i>N</i> = 4	<i>a_N</i>	0.0632	0.0281	0.0129	0.0068	
	<i>X_{p,N}</i>	656.8	652.7	609.1	565.4	
	<i>σ_N</i>	6.13	11.8	13.5	12	
ZnChlb <i>N</i> = 4	<i>a_N</i>	0.0107	0.0369	0.0125	0.00813	0.00673
	<i>X_{p,N}</i>	638.2	637.4	636.9	589.3	555.9
	<i>σ_N</i>	3.95	6.94	25.3	11.3	14.6
Pheoa <i>N</i> = 5	<i>a_N</i>	0.0401	0.0236	0.00386	0.00783	0.00232
	<i>X_{p,N}</i>	669.52	666.3	636.7	611.1	561.4
	<i>σ_N</i>	5.35	10.2	8.36	10	18.7
Pheob <i>N</i> = 5	<i>a_N</i>	0.0233	0.0156	0.00205	0.00733	0.00701
	<i>X_{p,N}</i>	656	653.5	625.5	600.4	554.7
	<i>σ_N</i>	4.53	10.3	6.31	10.1	11.8

Note. Each substance is represented by a GPS with the formula $g(x)$. For quantification of pigments in an extract, the necessary GPS are combined to the function $g(x)$ for simulating the absorbance spectrum of the extract.

$f(x) = Y0 + x \cdot \text{slope} + \text{MgA} + \text{MgB} + \text{CuA} + \text{CuB} + \text{ACuA} + \text{ACuB} + \text{ZnA} + \text{ZnB} + \text{PheA} + \text{PheB}$

$\text{MgA} = \text{MgChla} \cdot g(x), \text{MgB} = \text{MgChlb} \cdot g(x), \dots$

$$g(x) = \sum_{i=1}^N a_N \cdot \exp\left(-0.5 \cdot \left(\frac{x - \text{wlddev} - x_{p,N}}{\sigma_N \cdot \text{peakwidth}}\right)^2\right)$$

$\text{peakwidth} = 1 + 0.000034 \cdot (\text{MgChla} + \text{MgChlb} + \dots)^2$

linear equations for the estimation of six components. The SRCM method ((6), only Mg-Chl *a* and Mg-Chl *b*) uses a larger number of data points, but is still limited to a fixed sample interval and does not allow inclusion of any correction parameters. In contrast, the new method is based on a continuous function, so that the number of points which can be used for the estimation is limited only by the sampling interval of the spectrometer (and the performance of the computer used for the fitting process). Because of this feature, the new method works with different sampling intervals and, more importantly, noise in the baseline of the spectrometer has less influence on the accuracy of the es-

timation. In addition, a drift in the baseline is automatically corrected by linear approximation (the parameters “Y0” and “slope”). As a result, it is possible to estimate low concentrations of pigments with much increased accuracy.

(b) The traditional method of quantification using linear equations is strongly influenced by the wavelength accuracy of the spectrometer: since peaks of chlorophyll absorbance spectra are quite sharp (half-width around 7 nm), even small deviations in the peak position cause large errors in the quantification using the traditional method (Table 2). The same is true for the SRCM method (6), which is in this respect compa-

TABLE 1c
Standards of Chlorophyll Derivatives in Acetone: Parameters of Gauss-Peak Spectra (GPS)

Pigment	Parameter	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
MgChla $N = 5$	a_N	0.0585	0.0241	0.0147	0.0067	0.00294
	$X_{p,N}$	661.7	657.9	614.8	576.9	545.4
	σ_N	6.94	12.6	14.2	11.2	24
MgChlb $N = 5$	a_N	0.0343	0.01301	0.00204	0.00486	0.00702
	$X_{p,N}$	644.74	641.9	614.6	595.9	572.6
	σ_N	7.2	12.42	6.09	9.6	46.2
MgChlc $N = 4$	a_N	0.014	0.008	0.00778	0.011	
	$X_{p,N}$	629.4	622.6	581	568.5	
	σ_N	6.7	17.2	7.9	22.3	
CuChla $N = 4$	a_N	0.046	0.0142	0.0094	0.005	
	$X_{p,N}$	650	644.6	600.6	544	
	σ_N	8.9	20.3	13.1	25.5	
CuChlb $N = 5$	a_N	0.0243	0.0128	0.0127	0.005	0.0069
	$X_{p,N}$	627.8	617.3	616.6	575.5	545.8
	σ_N	8.4	33.9	7.5	11.6	14.6
CuChlc $N = 4$	a_N	0.0011	0.00649	0.00712	0.0080	
	$X_{p,N}$	651.9	610.8	603	552	
	σ_N	12.4	37.88	9.08	21.2	
ZnChla $N = 4$	a_N	0.0658	0.0248	0.0152	0.0084	
	$X_{p,N}$	655.5	651.5	607.6	564.6	
	σ_N	7.17	13.8	12.9	14.5	
Pheoa $N = 5$	a_N	0.0322	0.0151	0.0024	0.008	0.003
	$X_{p,N}$	665.18	659.9	630.9	608	554.7
	σ_N	7.28	13	7.37	10.95	22.3
Pheob $N = 5$	a_N	0.0216	0.0096	0.0017	0.0078	0.0074
	$X_{p,N}$	653.05	650	618.9	598	551.6
	σ_N	6.33	13.5	7	9.7	15.8
Pheoc $N = 4$	a_N	0.00185	0.00881	0.00684	0.00712	
	$X_{p,N}$	652	595.5	573	554	
	σ_N	20.3	11	6.7	49	

Note. Each substance is represented by a GPS with the formula $g(x)$. For quantification of pigments in an extract, the necessary GPS are combined to the function $g(x)$ for simulating the absorbance spectrum of the extract.

$$f(x) = Y0 + x \cdot \text{slope} + \text{MgA} + \text{MgB} + \text{MgC} + \text{CuA} + \text{CuB} + \text{CuC} + \text{ZnA} + \text{PheA} + \text{PheB} + \text{PheC}$$

$$\text{MgA} = \text{MgChla} \cdot g(x), \text{MgB} = \text{MgChlb} \cdot g(x), \dots$$

$$g(x) = \sum_{i=1}^N a_N \cdot \exp\left(-0.5 \cdot \left(\frac{x - \text{wldev} - x_{p,N}}{\sigma_N \cdot \text{peakwidth}}\right)^2\right)$$

$$\text{peakwidth} = 1 + 0.0002 \cdot (\text{MgChla} + \text{MgChlb} + \dots)$$

able to the results of the new method without any correction parameters (Table 2). The shifts in the peak position may not only be caused by inaccuracy of the spectrometer, but also by aggregation and allomerization of chlorophylls, salinity, and pH of the sample. The use of the wldev parameter in the GPS corrects these errors automatically in such a way that the wavelength inaccuracy of the spectrometer is compensated completely and the other types of peak shifts partially. Peak shifts caused by aggregation, allomerization, salinity, and pH cannot be completely compensated, because in these cases not only the position but also the shape of the peaks are changed. But even in these

cases, the new method has a great advantage over the traditional one: the residuals of the fitting process and the standard deviations of the parameters are a reliable indication for the accuracy of the results. In contrast, the traditional method does not include any measure of estimation accuracy except the standard deviation between replicates.

(c) If the wavelength accuracy of the spectrometer is high enough and a standardized extraction buffer (e.g., buffer A) is used for plant extraction, the results of the parameter wldev can be used to detect processes related to allomerization. This is because allomerization and other processes which involve an opening of the

FIG. 1. Accuracy of GPS fitted to standards. UV/VIS spectra of isolated chlorophyll standards were fitted with a series of Gaussian peaks—Gauss-peak spectra (GPS). (Top) Mg-Chl *a* in cyclohexane; (bottom) Mg-Chl *b* in cyclohexane.

cyclopentanone ring of Chl lead to a slight (Mg-Chl, Zn-Chl, Pheo) to strong (Cu-Chl) blue shift of absorbance maxima accompanied by only slight changes in the shape of the peaks (e.g., 11). The introduction of the peakwidth parameter in the new method enables a partial correction of problems occurring at high pigment concentrations. Under these conditions, a slight widening of the peaks is observed, which could be caused by interactions between the pigment molecules. Without correction, this widening leads to severe errors in the quantification already at total pigment concentrations around 10 μM , where linearity of the spectrometer is usually not yet a problem (Tables 2 and 3). Such a correction is impossible using the methods of White *et al.* and Jones *et al.* (4, 5) or Naqvi *et al.* (6).

(d) The new method is much faster than the traditional one (4, 5), because no chemical treatment (e.g., acidification) of the sample is required for the analysis.

This is important also for quantification accuracy for several reasons. The acidification may lead to the occurrence of turbidity in the plant extracts, and if the whole process of analysis is not carried out fast enough, pigment degradation may also occur. Additionally, using diethyl ether as a solvent, some percentage error due to solvent evaporation is almost inevitable.

The new method is certainly much faster than that of Naqvi *et al.* (6), since the latter requires the standard spectra to be recorded on the same spectrometer as the sample spectrum. That is not necessary with the new method as far as the spectrometer has a slit (spectral bandwidth) smaller than 1 nm, so that the equations listed in Table 1 can be applied in any lab which has a high-quality spectrometer. This is important for a universal application of the method, since it is not possible

FIG. 2. Accuracy of GPS estimation of extracts. UV/VIS spectra of *Scenedesmus quadricauda* samples from cell cycle experiments, extracted with cyclohexane after lyophilization. The measured spectra were fitted with a combination of six Gauss-peak spectra (Mg-Chl *a*, Mg-Chl *b*, Cu-Chl *a*, Cu-Chl *b*, Pheo *a*, Pheo *b*). (Top) Stressed with 100 μM Cu^{2+} for 5 h; (bottom) control: straight line, measured spectrum; dashed line, simulated spectrum; dotted line, residuals.

TABLE 2

Analysis of Text Mixtures of Known Composition (Prepared from Standards)

Test type	Pigment		Method of White <i>et al.</i> (4): calcd concn/ μM ($\pm\text{SD}$)		Method of Küpper <i>et al.</i> (this work): calculated concentrations/ μM ($\pm\text{SD}$)				
	Name (PUO)	Concn. added/ μM	Correct wavelength	1 nm red shift ^a	Full equations	Without wldev correction	Without Y0/slope correction	Without peakwidth correction	Without any correction
Control green plant	Mg-Chl <i>a</i>	3.30	2.87 (0.36)	2.61 (0.36)	3.37 (0.15)	3.27 (0.01)	3.39 (0.15)	3.35 (0.14)	3.24 (0.15)
	Mg-Chl <i>b</i>	1.05	0.94 (0.12)	1.00 (0.12)	1.03 (0.07)	1.04 (0.05)	0.99 (0.05)	1.02 (0.07)	1.03 (0.05)
	Cu-Chl <i>a</i>	0	0.22 (0.04)	0.22 (0.04)	0.06 (0.04)	0.00 (0.00)	0.10 (0.00)	0.07 (0.03)	0.00 (0.00)
	Cu-Chl <i>b</i>	0	0.14 (0.06)	0.14 (0.06)	0.01 (0.01)	0.01 (0.01)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
	Pheo <i>a</i>	0.33	0.90 (0.52)	0.98 (0.52)	0.23 (0.02)	0.60 (0.03)	0.21 (0.02)	0.27 (0.02)	0.60 (0.03)
Cu-stressed green plant	Pheo <i>b</i>	0	0.22 (0.11)	0.49 (0.13)	0.11 (0.11)	0.00 (0.00)	0.02 (0.03)	0.12 (0.11)	0.00 (0.00)
	MgChl <i>a</i>	4.00	4.23 (0.45)	3.87 (0.45)	4.49 (0.08)	4.37 (0.10)	4.49 (0.08)	4.43 (0.08)	4.30 (0.09)
	Mg-Chl <i>b</i>	1.27	1.47 (0.20)	1.54 (0.20)	1.51 (0.05)	1.52 (0.05)	1.49 (0.03)	1.51 (0.05)	1.49 (0.03)
	Cu-Chl <i>a</i>	1.47	2.06 (0.08)	2.18 (0.11)	1.74 (0.03)	1.58 (0.04)	1.76 (0.05)	1.79 (0.04)	1.67 (0.05)
	Cu-Chl <i>b</i>	0.36	0.36 (0.05)	0.38 (0.05)	0.24 (0.02)	0.23 (0.02)	0.22 (0.02)	0.25 (0.02)	0.22 (0.02)
Cu-stressed green plant	Pheo <i>a</i>	0.40	0.45 (0.58)	0.52 (0.57)	0.44 (0.03)	0.89 (0.02)	0.41 (0.04)	0.60 (0.04)	1.00 (0.02)
	Pheo <i>b</i>	0	-0.05 (0.22)	-0.04 (0.17)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Mg-Chl <i>a</i>	5.30	5.85 (0.07)	5.42 (0.08)	5.91 (0.01)	5.74 (0.01)	5.90 (0.00)	5.78 (0.01)	5.61 (0.00)
	Mg-Chl <i>b</i>	1.70	1.96 (0.05)	2.06 (0.04)	1.94 (0.02)	2.00 (0.02)	1.90 (0.02)	1.92 (0.02)	1.93 (0.02)
	Ch-Chl <i>a</i>	0.98	1.45 (0.04)	1.57 (0.06)	1.14 (0.04)	0.92 (0.04)	1.18 (0.04)	1.24 (0.04)	1.10 (0.04)
Cu-stressed green plant	Cu-Chl <i>b</i>	0.24	0.27 (0.01)	0.26 (0.004)	0.14 (0.01)	0.12 (0.01)	0.11 (0.00)	0.15 (0.01)	0.10 (0.00)
	Pheo <i>a</i>	0.53	0.06 (0.12)	0.16 (0.12)	0.54 (0.00)	1.08 (0.01)	0.49 (0.02)	0.80 (0.01)	1.26 (0.03)
	Pheo <i>b</i>	0	-0.11 (0.03)	0.05 (0.06)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Mg-Chl <i>a</i>	5.96	6.32 (0.42)	5.86 (0.44)	6.61 (0.12)	6.36 (0.11)	6.63 (0.12)	6.35 (0.11)	6.14 (0.09)
	Mg-Chl <i>b</i>	1.91	2.10 (0.04)	2.21 (0.03)	2.09 (0.06)	2.15 (0.07)	1.99 (0.05)	2.07 (0.06)	2.01 (0.05)
Cu-stress green plant	Cu-Chl <i>a</i>	0.49	0.98 (0.13)	1.09 (0.11)	0.57 (0.01)	0.39 (0.02)	0.68 (0.04)	0.69 (0.01)	0.70 (0.01)
	Cu-Chl <i>b</i>	0.12	0.15 (0.02)	0.14 (0.03)	0.04 (0.00)	0.04 (0.00)	0.00 (0.00)	0.08 (0.00)	0.01 (0.02)
	Pheo <i>a</i>	1.86	1.85 (0.83)	1.88 (0.83)	2.10 (0.06)	2.70 (0.03)	1.94 (0.08)	2.52 (0.04)	2.99 (0.03)
	Pheo <i>b</i>	0.32	0.38 (0.08)	0.79 (0.14)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.23 (0.05)	0.00 (0.00)
	Mg-Chl <i>a</i>	12.6	12.12 (0.70)	11.26 (0.69)	14.08 (0.59)	12.83 (0.51)	14.08 (0.60)	12.73 (0.45)	12.10 (0.45)
Control red alga	Mg-Chl <i>b</i>	4.02	3.93 (0.10)	4.16 (0.13)	3.60 (0.07)	3.52 (0.05)	3.66 (0.13)	3.51 (0.08)	3.72 (0.10)
	Cu-Chl <i>a</i>	0.49	1.17 (0.11)	1.23 (0.12)	0.50 (0.01)	0.00 (0.00)	0.41 (0.09)	1.35 (0.06)	0.83 (0.06)
	Cu-Chl <i>b</i>	0.12	0.27 (0.06)	0.25 (0.06)	0.12 (0.00)	0.23 (0.01)	0.15 (0.02)	0.22 (0.01)	0.24 (0.04)
	Pheo <i>a</i>	2.53	3.35 (1.18)	3.50 (1.22)	0.55 (0.06)	3.21 (0.08)	0.61 (0.08)	3.08 (0.20)	4.74 (0.23)
	Pheo <i>b</i>	0.32	0.93 (0.38)	1.97 (0.35)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.06 (0.08) ^b	0.07 (0.08) ^b	0.00 (0.00) ^b
Cu-stressed red alga	Mg-Chl <i>a</i>	3.3	2.70 (0.06)	2.36 (0.08)	3.37 (0.07) ^b	3.37 (0.10) ^b	3.37 (0.07) ^b	3.36 (0.07) ^b	3.35 (0.10) ^b
	Mg-Chl <i>b</i>	0	0.003 (0.04)	0.16 (0.04)	0.00 (0.00) ^b	0.01 (0.01) ^b	0.01 (0.01) ^b	0.00 (0.00) ^b	0.02 (0.01) ^b
	Cu-Chl <i>a</i>	0	0.05 (0.02)	-0.15 (0.02)	0.00 (0.00) ^b	0.01 (0.01) ^b	0.01 (0.01) ^b	0.00 (0.00) ^b	0.02 (0.01) ^b
	Cu-Chl <i>b</i>	0	0.12 (0.06)	0.13 (0.05)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b
	Pheo <i>a</i>	0.33	1.21 (0.18)	1.39 (0.20)	0.25 (0.01) ^b	0.25 (0.06) ^b	0.22 (0.03) ^b	0.28 (0.01) ^b	0.26 (0.05) ^b
Cu-stressed red alga	Pheo <i>b</i>	0	0.36 (0.15)	0.91 (0.08)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b
	Mg-Chl <i>a</i>	6.6	6.21 (0.28)	5.65 (0.27)	7.19 (0.17) ^b	6.98 (0.19) ^b	7.20 (0.17) ^b	6.97 (0.25) ^b	6.81 (0.28) ^b
	Mg-Chl <i>b</i>	0	0.15 (0.04)	0.45 (0.03)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b
	Cu-Chl <i>a</i>	0.24	0.32 (0.04)	0.02 (0.03)	0.23 (0.05) ^b	0.09 (0.07) ^b	0.22 (0.07) ^b	0.37 (0.08) ^b	0.27 (0.09) ^b
	Cu-Chl <i>b</i>	0	0.07 (0.04)	0.12 (0.04)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b
	Pheo <i>a</i>	0.66	1.67 (0.43)	1.91 (0.42)	0.79 (0.24) ^b	1.28 (0.12) ^b	0.78 (0.23) ^b	1.21 (0.04) ^b	1.56 (0.08) ^b
	Pheo <i>b</i>	0	0.61 (0.14)	1.52 (0.11)	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b	0.00 (0.00) ^b

Note. Each test was replicated three times, and each of these samples was analyzed both by the new method and the method of White *et al.* (4)

^a If applied to the new method, this does not result in any change of calculated concentrations, but only in the “wldev” parameter being increased by 1 nm.

^b Fit only with Mg-Chl *a*, Cu-Chl *a*, and Pheo *a*. Selecting the pigments which are to be included in the fit is only possible with the new method.

in all labs and under all circumstances to isolate pure standards of all pigments which might be present in the sample.

However, even with this new method some general problems of the spectrophotometric estimation of chlorophyll derivatives remain. Substances with a low absorption coefficient in the relevant spectral region cannot be determined with a high accuracy. In samples from green plants, these are mainly Pheo *b* and Cu-Chl *b*. Since Chl *b* is a minor component of these samples

anyway, the estimation of Cu-Chl *b* content in samples from Cu-stressed plants is never very accurate. With Pheo *b* there is an additional problem. Since it has a maximum between Cu-Chl *a* and Mg-Chl *a*, the blue-shifted spectrum of allomerized Mg-Chl *a* almost coincides with that of Pheo *b*, which may cause an overestimation of Pheo *b*. The vicinity to Cu-Chl *a* may cause similar interferences, leading sometimes to over- or underestimation of Pheo *b*, especially at high total pigment concentrations.

TABLE 3
Micromolar Extinction Coefficients Used for Normalizing Spectra of Pigment Standards

Pigment	Diethyl ether		Cyclohexane		Acetone	
	Absorption coefficient/ (cm ⁻¹ /μmol)	Source	Absorption coefficient/ (cm ⁻¹ /μmol)	Source	Absorption coefficient/ (cm ⁻¹ /μmol)	Source
Mg-Chl <i>a</i>	89	White <i>et al.</i> (4)	95	This work ^b	82	Ziegler and Egle (12)
Mg-Chl <i>b</i>	59	White <i>et al.</i> (4)	61	This work ^b	49	Ziegler and Egle (12)
Mg-Chl <i>c</i>	—	—	—	—	23	Jeffrey (13)
Cu-Chl <i>a</i>	68	White <i>et al.</i> (4)	74	This work ^b	60	This work ^a
all.Cu-Chl <i>a</i>	—	—	68	This work ^a	—	—
Cu-Chl <i>b</i>	50	White <i>et al.</i> (4)	50	Küpper <i>et al.</i> (1)	43	This work ^b
all. Cu-Chl <i>b</i>	—	—	49	This work ^a	—	—
Cu-Chl <i>c</i>	—	—	—	—	14	This work ^a
Zn-Chl <i>a</i>	—	—	90	Küpper <i>et al.</i> (1)	90	This work ^c
Zn-Chl <i>b</i>	—	—	60	Küpper <i>et al.</i> (1)	60	This work ^c
Pheo <i>a</i>	56	White <i>et al.</i> (4)	64	This work ^b	46.5	This work ^b
Pheo <i>b</i>	37	White <i>et al.</i> (4)	41	This work ^b	31	This work ^b
Pheo <i>c</i>	—	—	—	—	15	This work ^a

Note. Absorption coefficient:

^a Obtained by quantitative conversion of Mg-Chl, of which the absorption coefficient is known.

^{b,c} Obtained by measuring the concentration of a standard in diethyl ether (in which the absorption coefficient is known; ^b White *et al.* (4); ^c Jones *et al.* (5)) and quantitatively transferring it into acetone.

In summary, this new method can be easily and reliably used for the quantification of those pigments presented in this paper, as long as the inevitable limitations of the method are kept in mind. Additionally, the method could also be applied to the spectrophotometric analysis of other pigment mixtures and probably completely different analytical problems too. To do so, the spectra of all pigments which might be present in the sample and absorb in the selected spectral region would have to be converted to GPS, combined, and fitted to the spectrum of the mixture as described in this work.

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