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Determination of chlorophyll a and b by simultaneous multi-component spectrophotometry

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Abstract. The mathematical technique of over-determined simultaneous equations has been applied to the analysis of chlorophyll extracts from plant materials. The choice of spectral range is important – it is shown that 50 data points in the range 500–700 nm give better results than a similar number in the range 400–700 nm, as they avoid interferences from carotenoids. Results agree with those calculated using Arnon's method (1949), but have a better precision of 2% rsd compared with 4%. The computing procedure also allows testing of the correctness of the results.

Introduction

The colouring materials in green plants consist mainly of the two magnesium-containing green chlorophylls, denoted a and b, the pheophytins which result from abstraction of the magnesium, and the carotenoids. Determination of chlorophylls in plants or in algae is important as it gives a measure of the capability for photosynthesis [1]. However, their determination by solution spectrophotometry is complicated by the fact that they are always present in mixtures, and is susceptible to numerous sources of error, primarily because of the possibility of photo-induced degradation of the pigments during sample preparation [2]. Since the absorption bands of chlorophyll a and b overlap to a considerable extent, most of the spectrophotometric methods which have been developed are based on the measurement of the absorbances at the two absorption maxima in the red region [3].

Arnon [4] proposed the following equations for the determination of chlorophylls in an 80% aqueous acetone extract by measuring the absorbances at 663 nm and 645 nm in a 1-cm cell using the specific absorption coefficients given by Mackinney [5]:

$$\text{Total chlorophyll} \quad (\text{mg/L}) = 20.2A_{645} + 8.02A_{663} \qquad (1)$$

$$\text{chlorophyll a} \quad (\text{mg/L}) = 12.7A_{663} - 2.69A_{645}$$

$$\text{chlorophyll b} \quad (\text{mg/L}) = 22.9A_{645} - 4.68A_{663}$$

These equations were based on the specific absorption coefficients reported by Mackinney [5] for chlorophylls in 80% acetone (not in pure acetone as suggested by Wellburn and Lichtenthaler [6]). This solvent composition is preferred because it readily dissolves the compounds, and because small variations in water content (arising from the water in the samples) do not have too large an effect on the absorbances. Mackinney does, nevertheless, warn that changes of water content of a few percent do have a significant effect.

Other equations for a range of solvent compositions have been given by Wellburn and Lichtenthaler, who also included coefficients enabling total carotenoids to be determined by measurement of the absorbance at 470 nm [6]. Sometimes the coefficients given by Comar and Zscheile [7] are quoted, but it should be pointed out that these refer to ether as solvent, and are not comparable. However, Cresser and O'Neill [2] observed that discrepancies in the published values of molar absorptivities could also arise from the presence of concomitant impurities arising through incomplete separation in the preparation of standards, or from the presence of photochemical and chemical degradation products because of the variation in preparation techniques, or from instrumental factors of which spectrometer bandwidth was most important. Vernon suggested that it was necessary to determine the pheophytins as well as the chlorophylls, regarding the whole as a four-component system, in order to minimise errors due to overlap of spectral bands [3].

The aim of this work was to develop an alternative method for the determination of chlorophylls a and b in their naturally occurring mixtures by using simultaneous multi-component spectrophotometry with a large number of data points from a major section of the absorption

Dedicated with best wishes to Professor Dr. K. Doerffel, on the occasion of his 70th birthday, remembering in particular how he helped to open cooperation with the west before reunification

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spectrum, and the method of overdetermined simultaneous equations to find the concentrations of the two chlorophylls. This approach to the analysis of multicomponent mixtures began to be adopted when computers became available: Herschberg and Sixma demonstrated in 1962 how mixtures of four to six aromatic compounds could be analysed by using absorbance values measured at between 20 and 50 different wavelengths [8], but it has still not become widely used.

Errors arising through the use of such methods were analysed by Brown et al. who found that instrumental noise, which includes the uncertainty of the photometric measurement, presented the biggest problem [9]. Nevertheless, satisfactory performance for the analysis of two-component pharmaceutical preparations using the matrix least-squares method has been reported, with relative standard deviations of 1–2% [10]. Factors to be considered in this work were first, the time for an analysis – to be compared with that for HPLC for example [11], and second, the preferred wavelength range.

This project therefore consisted of two steps: writing software which would both control the spectrophotometer and carry out the solution of the overdetermined simultaneous equations making use of the fifty or so individual absorbance readings, and then testing the performance of the method.

Theory

Absorbances are related to concentrations of coloured components through the Beer-Lambert Law:

$$A = \varepsilon \ell C \tag{2}$$

where A is the absorbance at a particular wavelength, ε is the molar absorptivity at that wavelength, ℓ is the path length (cell thickness), and C is the molar concentration of the analyte. Most papers on chlorophylls, however, use specific absorption coefficients relating absorbance to concentration in mg/L.

Because the cell thickness is usually constant for one experiment, it is simpler to write the equation as follows:

$$A = k.C \tag{3}$$

For a multi-component system with *n* components, the total absorbance of the mixture at a given wavelength is given by:

$$A = k_1.C_1 + k_2.C_2 + k_3.C_3 + \ldots k_n.C_n \tag{4}$$

To solve this equation, n measurements, are needed, at n different wavelengths, which will give the following equations:

$$A = k_{11}.C_1 + k_{12}.C_2 + k_{13}.C_3 + \ldots k_{1n}.C_n \tag{5}$$

$$A = k_{21}.C_1 + k_{22}.C_2 + k_{23}.C_3 + \ldots k_{2n}.C_n$$

etc

$$A = k_{n1}.C_1 + k_{n2}.C_2 + k_{n3}.C_3 + \ldots k_{nn}.C_n$$

These can be arranged into the matrix notation of a product:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ \text{etc} \\ A_n \end{bmatrix} = \begin{bmatrix} k_{11} & k_{12} & k_{13} & \ldots & k_{1n} \\ k_{21} & k_{22} & k_{23} & \ldots & k_{2n} \\ k_{31} & k_{32} & k_{33} & \ldots & k_{3n} \\ & & \text{etc} & & \\ k_{n1} & k_{n2} & k_{n3} & \ldots & k_{nn} \end{bmatrix} \cdot \begin{bmatrix} C_1 \\ C_2 \\ C_3 \\ \vdots \\ C_n \end{bmatrix} \tag{6}$$

The square matrix, denoted by K, has an inverse K^{-1} which we can use to solve for the set of concentrations (displayed also as a column matrix):

$$C = K^{-1}.A \tag{8}$$

If there are fewer measurements than there are components in the mixture, then no solution can be obtained for this matrix equation. However, if we have more measurements than components, and as long as we have the full set of relevant absorption coefficients, then we have more equations than we need, so we can multiply the matrix of coefficients by its transpose to obtain a square matrix with n components. This last method is called over-determination of simultaneous equations, and has the effect of giving a least squares solution fit. Gaussian elimination is then used on the same matrix to list the elements of the concentration vector C, the answer to the analytical problem known as multi-component spectrophotometry.

The use of over-determined equations has an important advantage: it permits a reduction in the errors of the final results over those obtained with the minimum number of measurements. However, a significant improvement in precision is obtained only when the number of measurements is many times the number of components, and when all the data points used contain useful information.

Experimental

Apparatus. The spectrophotometer used in these experiments was a Perkin-Elmer Lambda 15, a double-beam UV/Vis spectrophotometer with conventional monochromator in the Littrow configuration fitted with a holographic grating with 1440 lines/mm. A tungsten lamp was used as light source of the visible range, and the exit slit which provided a spectral bandpass of 0.25 nm was selected. The spectrophotometer was connected to an ATARI 1040 ST computer through a Perkin-Elmer RS 232 C interface. A custom program was written in Fast Basic to control to spectrophotometer and to carry out the mathematical procedures used in this investigation.

A unicam SP-9 atomic absorption spectrophotometer with air-acetylene flame, and a magnesium hollow-cathode lamp run at 6 mA, was used to assay solutions of individual chlorophylls in terms of their magnesium contents.

Sample preparation. A portion of 2–10 g of fresh grass or spinach was transferred into a homogeniser flask, to which were added 40 mL of acetone and some calcium carbonate to neutralise the acids of the sap. The mixture was then macerated in an MSE homogeniser with a stainless steel blade for 5 min, and filtered through a Whatman No. 41 filter paper by suction. The extraction was repeated on the residue with a further 30 mL of acetone to ensure a complete extraction. To the combined extracts was added 20 mL of distilled water and the solution was then made up to 100 mL with acetone to give an 80% v/v aqueous acetone solution. This solution was subsequently diluted as required (typically × 100) with more of the 80:20 v/v mixed solvent to obtain a concentration suitable for photometric measurement.

Preparation of standard chlorophyll solutions. All the work for the preparation of the standards must be carried out in a room with a very low level of lighting, and certainly in the absence of sunlight. A 100-mL portion of extract from spinach leaves in 80% aqueous acetone was added to 100 mL of light petroleum, and the extract was washed thoroughly with a saturated aqueous potassium chloride solution (enough to give two phases), separated from the water, and dried over anhydrous sodium sulphate. It was then introduced into a glass chromatograph column (7.5 cm diameter × 35 cm length) packed with powdered sucrose (domestic sugar), to which was added 3% m/m soluble starch, and eluted with light petroleum (bp < 40°) = 0.5% v/v n-propanol until the bands were separated but still on the column. The sections of the column packing containing the chlorophyll a and the chlorophyll b were dug out of the column and transferred into separate containers to which 80% v/v acetone was added to dissolve the chlorophylls.

The concentrations of the chlorophylls in each stock solution were determined by estimating the magnesium content by flame AAS. An amount of chlorophyll solution was evaporated until dry, then 1 mL of sulphuric acid and a few drops of hydrogen peroxide were added and boiled until a clear solution was obtained. The solution was diluted with water to an appropriate volume, then aspirated into the flame. The absorbance at 285.2 nm was measured and compared with the absorbance of standard magnesium solutions. The concentration of chlorophyll in the solution can be calculated based on the empirical formulas of chlorophyll a ($C_{55}H_{72}O_5N_4Mg$, m.w. 893.5) and chlorophyll b ($C_{55}H_{70}O_6N_4Mg$, m.w. 907.5).

Results and discussion

The absorption spectra of the samples of chlorophylls a and b and also of a carotene extract, in 80% v/v aqueous acetone, are shown in Fig. 1. The close overlap of the chlorophyll bands is obvious, and also the strong absorption by all three components at shorter wavelengths, which emphasises the need for a reliable spectrophotometric method for analysing their mixtures. The specific absorption coefficients were measured in diethyl ether/light petroleum after column chromatographic separation, and were in good agreement with values reported by Wellburn and Lichtenthaler [6]. Values obtained from measurements on 80% v/v aqueous acetone solutions, recommended for the analysis of plant extracts [2], are compared with values from the literature in Table 1. Specific absorption coefficients are values of ε in Eq. (2) calculated from the Beer-Lambert law, with concentrations given in mg/L.

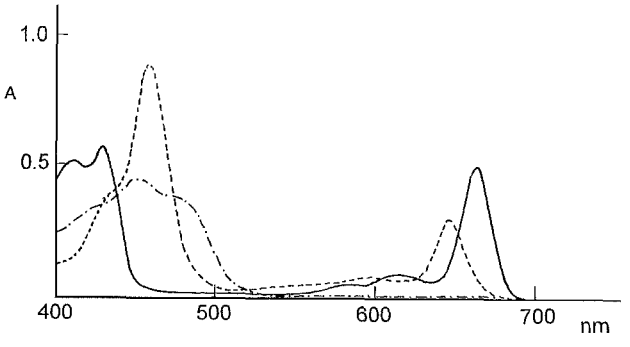

Fig. 1. Absorption spectra of chlorophyll a ———, chlorophyll b – – –, and of a crude carotene extract –·–·–. All prepared in 80% v/v aqueous acetone

Wavelength range for multi-component spectrophotometry

Two wavelength ranges were studied, the first from 400 nm to 695 nm, in 5-nm increments (Method 1) and the second from 550 nm to 697 nm, in 3-nm increments (Method 2). The second series was chosen to avoid the interferences from carotenoid pigments in the blue region, below 500 nm. Measurements of precision were based on a mixture of 6.62 ppm chlorophyll a and 7.19 ppm chlorophyll b, and the correlations were carried out for standard solutions which contained in addition also 4 ppm of carotene as an interfering substance since its spectrum overlaps in the blue region, as shown in Fig. 1. The results of measurements using both methods were compared with the results obtained by using Arnon's equation (see Eq. 1) as reference method (Table 2).

Both Method 1 and Method 2 gave a much better agreement between expected and found concentrations than did Arnon's method. The precisions of the two methods were not significantly different, but they were both better than that which could be achieved by Arnon's method. A number of extracts of plant materials were analysed by the three methods, giving the results shown in Table 3.

Testing the quality of the results

The concentrations of the chlorophylls in the samples were calculated by overdetermined simultaneous equations,

Table 1. Specific absorption coefficients for chlorophylls in 80% v/v aqueous acetone, relating to concentrations, C in mg/L in a 1-cm path length cell

	Absorption coefficient (wavelength, nm)	
Chlorophyll a		
Mackinney, 1941 [5]	82 (663)	91 (430)
Vernon, 1960 [3]	91 (665)	101 (433)
Wellburn and Lichtenthaler, 1983 [6]	87 (663)	—
This work	85 (663)	86 (432)
Chlorophyll b		
Mackinney, 1941 [5]	46 (645)	130 (460)
Vernon, 1960 [3]	53 (648)	148 (460)
Wellburn and Lichtenthaler, 1983 [6]	58 (646)	104 (470)
This work	49 (647)	123 (460)

Table 2. Correlation of results for mixtures of chlorophylls and carotenes in 80% v/v aqueous acetone using two different wavelength ranges, and of those using Arnon's method at two wavelengths

		Slope	Intercept	r^2	rsd.%
Chlorophyll a					
Method 1	400–700 nm	1.038	0.02	0.999	1.8
Method 2	550–700 nm	1.009	0.02	1.000	1.9
Arnon's method	663/645 nm	0.87	0.256	1.000	2.4
Chlorophyll b					
Method 1	400–700 nm	1.058	0.06	0.996	0.7
Method 2	550–700 nm	1.029	0.05	0.998	1.4
Arnon's method	663/645 nm	0.959	0.44	1.000	6.5

Table 3. Chlorophyll contents of some plant extracts, % w/w of dry matter

Sample	Chlorophyll a			Chlorophyll b		
	Method 1	Method 2	Arnon	Method 1	Method 2	Arnon
Spinach	1.81	1.77	1.87	0.62	0.83	0.67
Cabbage	1.66	1.72	1.70	0.59	0.76	0.65
Grass-1	0.74	0.71	0.78	0.40	0.31	0.38
Grass-2	0.57	0.59	0.64	0.35	0.25	0.32
Lettuce	0.51	0.55	0.52	0.27	0.17	0.24

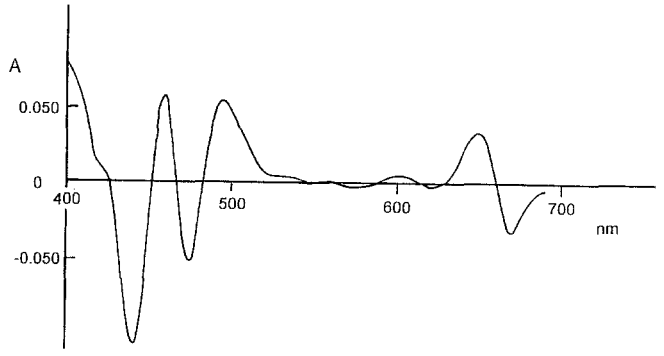

Fig. 2. Residual spectrum obtained by subtracting the predicted sample spectrum (calculated from the library spectra and the concentrations determined experimentally using Method 1 for the wide wavelength range 400–700 nm) from the original experimental sample spectrum. Data for a mixture containing 6.6 mg/L chlorophyll a and 7.2 mg/L chlorophyll b in 80% v/v aqueous acetone

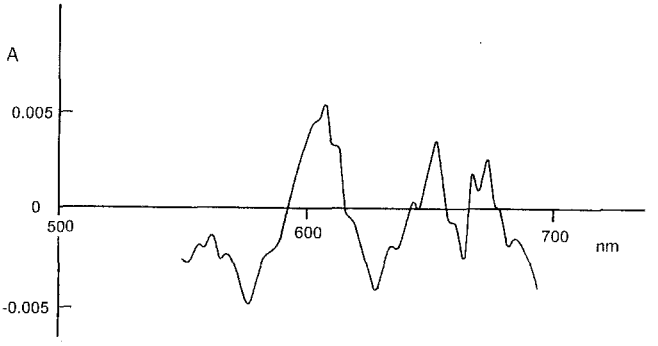

Fig. 3. Residual spectrum obtained by subtracting the predicted sample spectrum (calculated from the library spectra and the concentrations determined experimentally using method 2 for the narrow wavelength range 550–700 nm) from the original experimental sample spectrum. Data for a mixture containing 6.6 mg/L chlorophyll a and 7.2 mg/L chlorophyll b in 80% v/v aqueous acetone. Note the very small absorbance scale $\pm$ 0.005 absorbance units, compared to that in Fig. 2

using the mathematical technique described earlier, and from these calculated concentrations, the computer then went on to synthesise a new spectral set by combining in proportion our library data for the standards at the calculated concentrations. Comparison of the data was carried out by subtracting the synthesised spectrum from the original experimental spectrum. For a perfect fit, the residual absorbances should be negligible. This would indicate that all compounds in the sample affecting the measured absorbances have been accounted for, and that the results can be taken as correct.

The results are shown in Fig. 2 for Method 1 (400–700 nm) and Fig. 3 for Method 2 (550–700 nm). It should be noted that the scales for the axes in these two figures are different – the range of absorbance residuals is $\pm$ 0.03 using the wider wavelength range in Method 1 (for a sample spectrum with peak absorbances of around 0.5 and 0.3 at the chlorophyll a and b maxima wavelengths, respectively) and $\pm$ 0.005 absorbance units for Method 2. Figure 2 also shows that there are seriously unmatched areas between the two spectra in the blue region, with differences between -0.100 and 0.050 absorbance. It seems that these were due to the presence of the carotenoid pigments which gave complex spectra in the region below 500 nm, and caused interferences in the calculation. Figure 3 shows that Method 2 gave a much better match between the two spectra with differences between experimental and calculated spectra smaller by a factor of about ten (between $+0.005$ and -0.005 absorbance).

The working range for the determination of chlorophylls by simultaneous multicomponent spectrophotometry was estimated by measuring the absorbances of peak maxima in the red region for both chlorophylls. The calibration graphs for chlorophylls a and b were linear up to absorbances 2.5, equivalent to 33 ppm chlorophyll a or 50 ppm chlorophyll b. The lowest concentrations which could be measured were 0.15 and 0.6 ppm, respectively.

Conclusion

In order to get pure chlorophylls a and b for standard solutions, the separation of the chlorophylls is a very important step for simultaneous multicomponent spectrophotometry. Errors will arise in the calculation if standard solutions of pure chlorophylls cannot be obtained, but when pure standard solutions have been obtained, their spectra can be stored and used again for future analyses.

The performance of simultaneous multicomponent spectrophotometry for the determination of chlorophylls is encouraging. This approach, especially using the restricted wavelength range, gave good results and better precision than was obtained using Arnon's method. In terms of time and ease, the proposed methods offer better performance – it took only 6–7 min for a complete series of measurements and calculations, fast compared with the time of typically 20–30 min for an analysis by HPLC [11]. Herschberg and Sixma reported in 1962 that multi-component analysis reduces the amount of experimental time, materials and man power by an order of magnitude [8], which is substantiated by our experience.

References

1. Bruinsma J (1963) Photochem Photobiol 2:241–249
2. Cresser MS, O'Neill EJ (1980) Talanta 27:305–308
3. Vernon LP (1960) Anal Chem 32:1144–1150
4. Arnon DI (1949) Plant Physiol 24:1–15
5. Mackinney G (1941) J Biol Chem 140:315–322
6. Wellburn AR, Lichtenthaler H (1983) Photosynthesis Research – Proc Intern Congr 6(2):9–11
7. Comar CL, Zscheile FP (1942) Plant Physiol 16:198–209
8. Herschberg IS, Sixma FLJ (1962) Koninkl Nederl Akad Wetenskaap Proc Ser 13, 65:244–265
9. Brown CW, Lynch PF, Obremski RJ, Lavery DS (1982) Anal Chem 54:1472–1479
10. Mahalanabis KK, Basu D (1989) Analyst 114:1311–1313
11. Braumann T, Horst Grimme L (1981) Biochim Biophys Acta 637:8–17