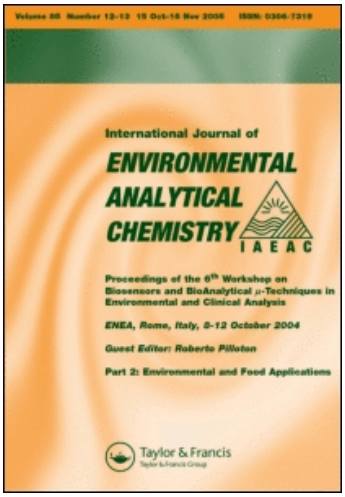

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The Use of High Pressure Liquid Chromatography with Fluorimetric Detection for Chlorophyll A Determination in Natural Extracts of Chloropigments and their Degradation Products

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The Use of High Pressure Liquid Chromatography with Fluorimetric Detection for Chlorophyll A Determination in Natural Extracts of Chloropigments and their Degradation Products

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To realize chlorophyll a analysis, a method is proposed that combines the sensitivity of fluorimetric determinations and the inherent selectivity and accuracy of chromatographic separations. A partial application of the overlapping resolution mapping (O.R.M.) method led to the use of a methanol:acetone:water (75:22:3, v/v) mixture for the isocratic separation by high pressure liquid chromatography on a reserved phase C-18 column of at least sixteen chloropigments and degradation products in a natural extract.

Ten replicate measurements of a 50 $\mu g. l^{-1}$ standard showed a reproducibility of 3%, while a detection limit of 84 pg chlorophyll a was estimated.

KEY WORDS: Chlorophyll a, phytoplankton, high pressure liquid chromatography, fluorimetry.

I. INTRODUCTION

Chlorophyll a is widely used as a parameter for living phytoplankton in natural waters. A simple and direct method for the estimation of chlorophyll concentrations depends upon measurements of the spectral absorption properties in acetone extracts. Commonly the separated

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particulate matter is extracted with 90% acetone. Then, the extract is examined spectrophotometrically at three different absorption maxima[1,2] or at one wavelength only.[2] It also is possible to perform quantitative analysis of pigments by fluorimetry,[3,4] even with greater sensitivity.

Although these methods are of common use, they have been largely criticized in the literature. The extraction efficiency increases with approximately 35% by the use of DMSO as the extraction solvent.[5,6,7,8] The use of 95% methanol also results in a better extraction efficiency.[9] But even if the difficulties with the quantitative extraction are disregarded, these methods have several drawbacks. Natural phytoplankton extracts always contain more than one pigment.

As the absorption bands of accessory pigments and even of bacteriochlorophyll overlap seriously with the chlorophyll a absorption band, estimates of chlorophyll a concentrations are likely to be inaccurate.[10–14] In addition there is a large number of described degradation products of the chlorophylls,[15] which have similar absorption spectra and interfere in the determination too.[10,11,12,16] Chlorophyll a, e.g., is increasingly overestimated by the trichromatic spectrophotometric equation as the pheophytin/chlorophyll ratio increases.[13]

Analogically, interference exists between the fluorescense of accessory pigments and pigment degradation products with that of chlorophyll a.[13,17] These difficulties are often avoided by methods involving a series of simultaneous equations and multiwavelength measurements.[18,19] It is however impossible to take into account every breakdown product and every accessory pigment, without making the calculations extremely complicated.

To overcome the majority of these problems chromatographic separation prior to the determination of concentration can be performed. Classical liquid chromatography is essentially used for preparation. The best results were obtained with a Sepharose CL. 6B column and a solvent program of 2, 3, 10 and 20% propanol in hexane.[20] For analytical purposes, thin layer chromatography,[10,21,22] and especially high pressure liquid chromatography (HPLC) are actually the most common methods.

Our aim was to develop an elution method capable to separate chlorophyll a from other pigments and a large number of degradation products present in a complex mixture and to quantify afterwards the chlorophyll a content in an accurate way. We opted for the HPLC approach with isocratic elution because it is much easier to handle and more suitable to apply in routine analyses. As there is no need for restabilization after every run with the isocratic elution, time can be saved by shorter analysis duration. The quantification itself is carried out fluorimetrically.

II. EXPERIMENTAL

a. Apparatus

Solvent was delivered at a rate of $1\,ml.min^{-1}$ or $3\,ml.min^{-1}$ by means of a DuPont three pistonpump, model 870, depending on whether an analytical Altex RP-18 column (particle size 5μ, $25 \times 0.4\,cm$ i.d. and $15 \times 0.4\,cm$ i.d.) or a semipreparative Altex RP-18 column (particle size $5\,\mu$, $25 \times 1.0\,cm$ i.d.) was used. The sample was introduced to the column by means of an automatic injector, Micromeritics model 725, fitted with a $50\,\mu l$ sampling loop. The effluent from the column was fed to the $10\,\mu l$ flowcell of the UV/VIS detector, Micromeritics model 785, operated at 428 nm (maximal absorption of chlorophyll a). In series herewith we connected a fluorimeter, Gilson-Spectra/GLO, equipped with an excitation filter 5-60 X (420–460 nm), an emission filter 2-60 M (630–680 mn) and a flow through cell of $15\,\mu l$. The detector signals were recorded and processed by a reporting integrator, Hewlett Packard 3390 A.

b. Reagents

All the organic solvents were purchased from Merck AG. They were degassed and filtered on glassfiber filters (Whatmann GF/C). The water we used was suprapure demineralised water (Millipore-Milli Q).

c. Extraction of chlorophyll from spinach leaves

As every pigment extract of natural phytoplankton contains several pigments and their degradation products and as there was no standard solution of all those components at our disposal, it was recommendable to look for the best chromatographic conditions with a natural and complex extract. This can be obtained as well from higher plants as from algae, since there exists no structural difference between chlorophyll of phytoplankton and of spinach leaves for example. The easiness of obtaining the latter plant and its high chlorophyll a content make it very suitable for our purposes. So an extraction with methanol and a precipitation of the green pigments using dioxane and water is applied on spinach leaves.[23]

To ascertain the presence of degradation products, a freshly prepared extract (that certainly contains chlorophyll a) was mixed with an aged, five months old, methanol extract. As chlorophylls show poor stability in methanol solutions,[24,25] the concentration of breakdown products in this old solution was markedly increased.

d. Standards

A chromatographically pure chlorophyll a standard was isolated from a

fresh extract of spinach leaves (see II.c) by semipreparative chromatography. To make certain about its identity, the absorption- and emissionspectra were respectively recorded with a Pye Unicam spectrophotometer, SP8-100, and a Perkin Elmer fluorimeter, model 2000, fitted with an excitation filter of 429 nm. Comparison of these spectra with those of the commercial chlorophyll a (sigma C 5753) and with literature data[26,27] showed very good agreement.

III. RESULTS AND DISCUSSION

a. Chromatographic separation

Several solvent mixtures, cited in the literature,[28–31] were tested on our complex pigment extract. None of them gave a separation as good as the separation with pure methanol. However, since chlorophylls are not stable in pure methanol,[24,25] we looked for another solvent to avoid the formation of oxidation products on the column itself. In search for an optimal resolution we partially applied the overlapping resolution mapping (O.R.M.) method, recently developed by Glajch.[32] Three solvents were selected from the solvent triangle,[33] each with fairly different properties: methanol (MeOH), acetone (Ace) and tetrahydrofuran (THF). Water always is used as fourth solvent in reversed-phase chromatography.

In the optimization of the resolution (R_s):

$$R_s = 1/4(\alpha - 1)(N)^{1/2}\ (k'/1 + k') \tag{1}$$

three factors are important. The number of theoretical plates (N) is an inherent function of the column. We found out that for the separation of chlorophyll a, the semipreparative Altex RP-18 column (25 × 1 cm) has a greater number of theoretical plates than the corresponding analytical columns (25 × 0.4 cm and 15 × 0.4 cm). Maybe this is due to the exclusion of wall-effects in the former one.

Once the choice of the column was fixed, the R_s-value is optimised by changing the capacity factor as well as the selectivity factor. The capacity factor (k'), affected by the solvent strength (S), should amount between 1 and 10. The selectivity factor (α) is optimised by changing the solvent composition. To obtain an optimal k'-range, the solvent strength is adapted by mixing the organic solvent with an appropriate amount of water. For the first solvent the k'-values are experimentally determined, for the second and third solvent the volume fraction (Φ) in water is theoretically predicted by

$$\Phi_B = \frac{S_A \cdot \Phi_A}{S_B} \tag{2}$$

The k'-values for 100% MeOH range from 1 to 10. The theoretically predicted compositions for Ace and THF were experimentally verified and adapted in order to keep k' between 1 and 10 (Table I). This experimental observation is not unusual as water does not act as an inert carrier.[32]

TABLE I

Solvent composition for Altex RP-18 column

Solvent	% Organic in water	
	Predicted	Experimental
MeOH	100	100
Ace	76	87
THF	59	67

With these three solvents and their combinations we calculated the respective k'- and R_s-values for two adjacent peaks (Table II) in order to predict all the resolutions with the special cubic equations of Snee[34] and to perform the resolution map in the solvent triangle (Figure 1).

TABLE II

S-, k'- and R_s-values for the various solvent mixtures

Solvent mixture (% organic in water)	S	k'	R_s
MeOH (100%)	2.600	3.625	0.000
THF (67%)	2.992	5.235	0.000
Ace (87%)	2.958	5.800	0.780
MeOH/THF (50:50)	2.796	21.375	1.400
MeOH/Ace (50:50)	2.779	8.474	1.500
THF/Ace (50:50)	2.975	7.825	0.630
MeOH/THF/Ace (33:33:33)	2.850	13.425	1.170

Although the shaded area in Figure 1 is the collection of every solvent composition possibility for which the resolution is about 1.25, a value of 1.25 means a peak separation of 99.5% at the baseline,[35] the k'-value exceeds 10. This unexpected result led us to consider in an analogical way the map of every k'-value for this same solvent triangle (Figure 2). Comparison of these two maps does not reveal a common optimal solvent composition.

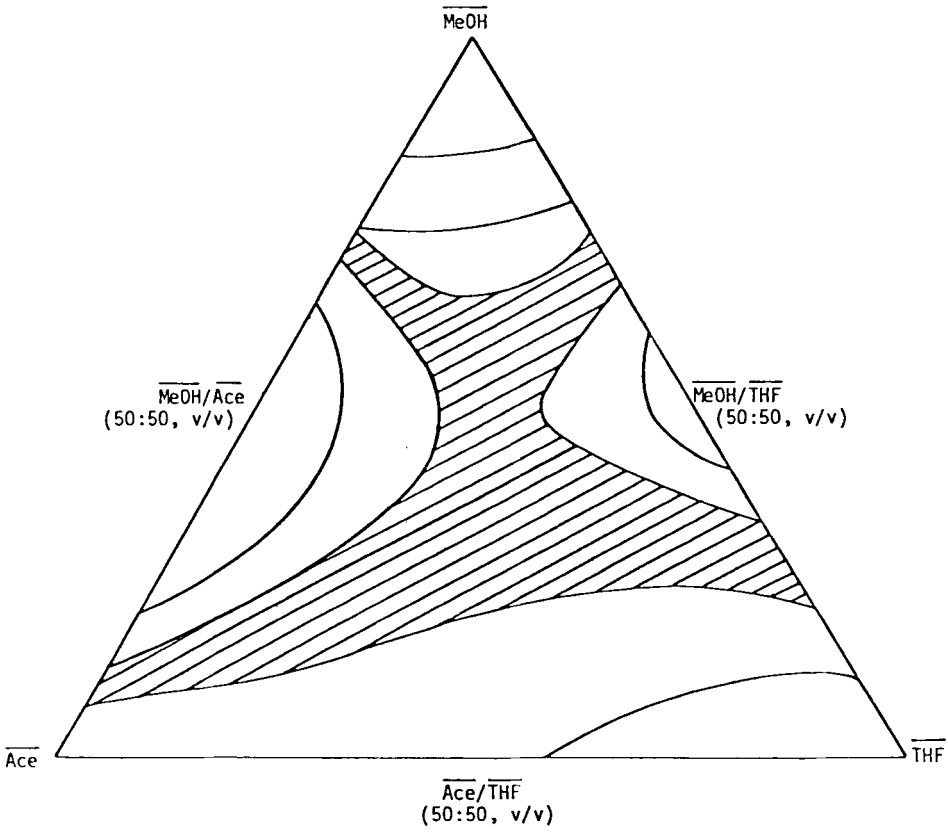

FIGURE 1 Chromatographic resolution map for the peaks of Table II. Shaded area corresponds with resolution of 1.10 to 1.25.

Therefore we took the solvent mixture, that is MeOH/Ace (75:25, v/v) or MeOH/Ace/H_2O (75:22:3, v/v), which is a compromise between the two optimum areas. With this solvent mixture the obtained chromatogram showed a good resolution in an acceptable separation time (Figure 3).

For the different solvents we used, we were limited in our knowledge of peak identity and peak position, due to the fact that most of the components present in our extract are unknown. Despite this limitation the O.R.M.-method allowed us to realise an optimal separation of sixteen chloropigments and their alteration products.

b. Quantitative analyses

For routine analyses a stock solution of pure chlorophyll a is very useful.

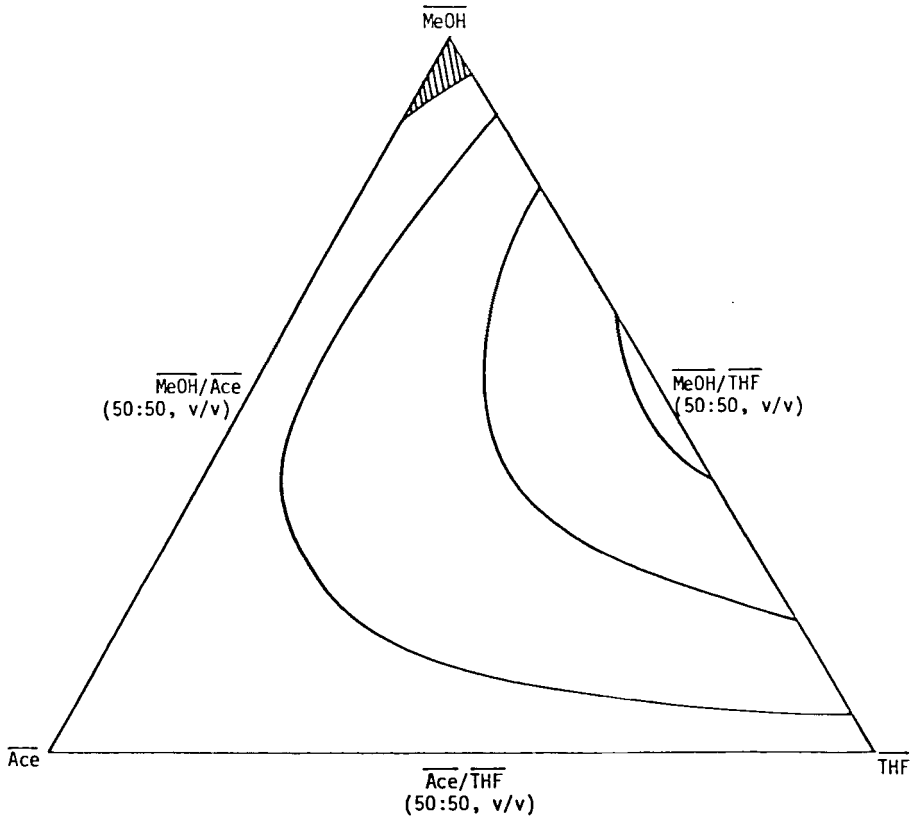

FIGURE 2 Chromatographic map of the capacity factor k' of Table II. Shaded area corresponds with a k'-value of 3.

As the absorption bands in diethylether are very sharp[27] and as this solvent can easily be evaporated to redissolve the chlorophyll a afterwards in the elution mixture for chromatography, we decided to use diethylether as the solvent for the standards. Of course the volatility of the solvent constrains us to determine the exact concentration before every run. The best way to do so, is by the use of the absorption coefficient for chlorophyll a in diethylether. The great variety of literature data, however, led us to remeasure this value.

We prepared two standard series of pure chlorophyll a from which the absorbances were read at 428 nm and at 662 nm. Every measurement was carried out in a thermostatised cell at 20°C. For the considered concentration range (0–1 mg chlorophyll a.l^{-1}), the least squares method showed us that the best correlation between concentration and absorbance

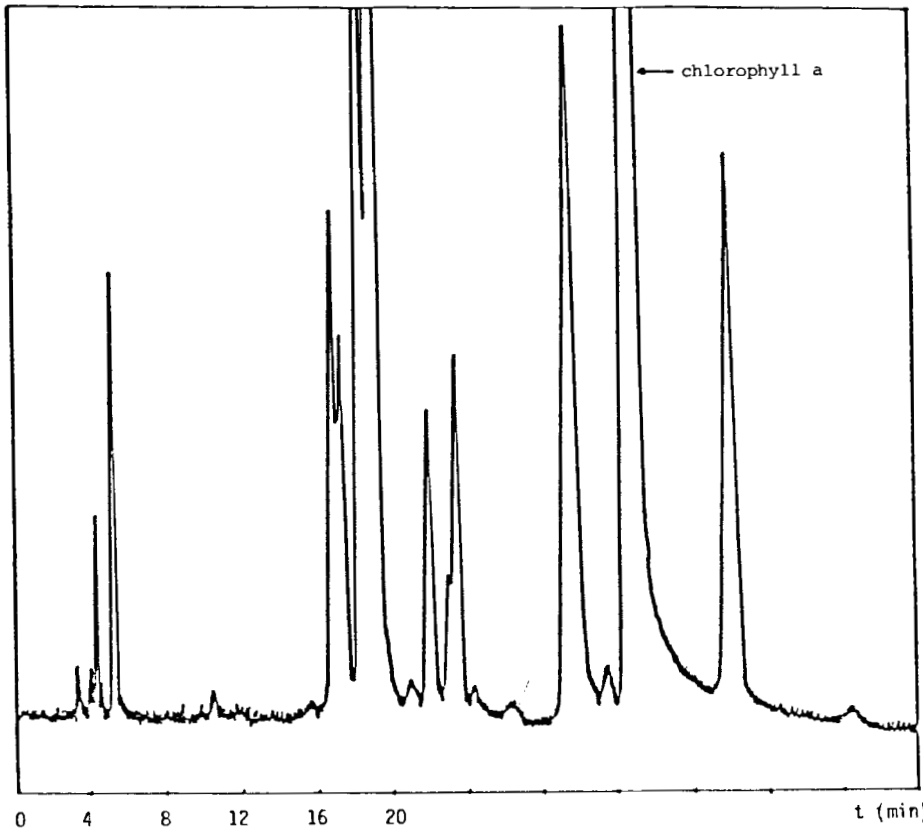

FIGURE 3 Chromatogram of chloropigment extract with optimum solvent-mixture: MeOH/Ace/H_2O (75:22:3, v/v); flow-rate 3 ml/min (pressure: 100 ± 1 bar).

TABLE III

Comparison of the absorption coefficients of chlorophyll a with literature data[26]

Wavelength (nm)	Found (l/g cm)	Strain *et al.*	Zscheile and Comar	Smith and Benitez
662	93.69	96.6	102.1	100.9
428	117.94	125.1	135.0	131.0

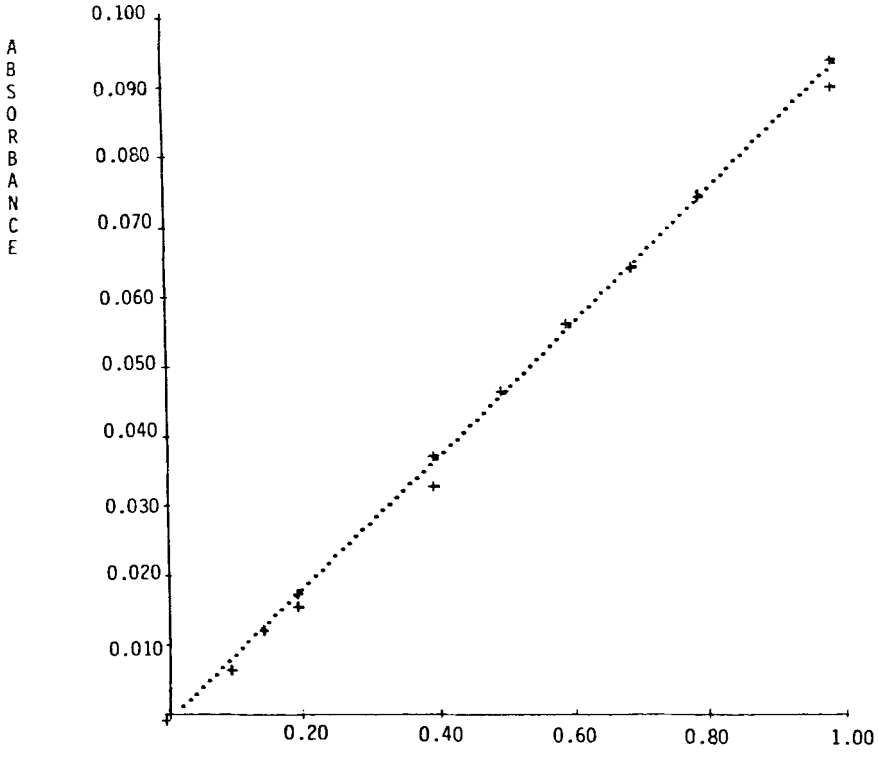

FIGURE 4A. Absorption curve of chlorophyll a, at 662 nm. Equation: $y = -0.0001 + 0.00937x$; $R = 0.9980$.

is given by the linear equation (Figure 4A and 4B). From the slopes of these lines we inferred the absorption coefficients at the respective wavelengths and compared them to the literature data[26] (Table III). In comparing our values with those of Strain *et al.* one must take into account that the literature data go with a temperature of 25°C. In further work we will use our values for the absorption coefficients.

In a second step we drew the response curve. The same standards as for the determination of the absorption coefficient were used here. The ether solutions were evaporated by gently blowing N_2 over the surface, and the chlorophyll was redissolved in a same volume of eluens for chromatographic analyses.

This manipulation did in no way induce the formation of denaturation products. For every concentration the measurement was repeated three times. At the same occasion a comparison was made between the UV/VIS

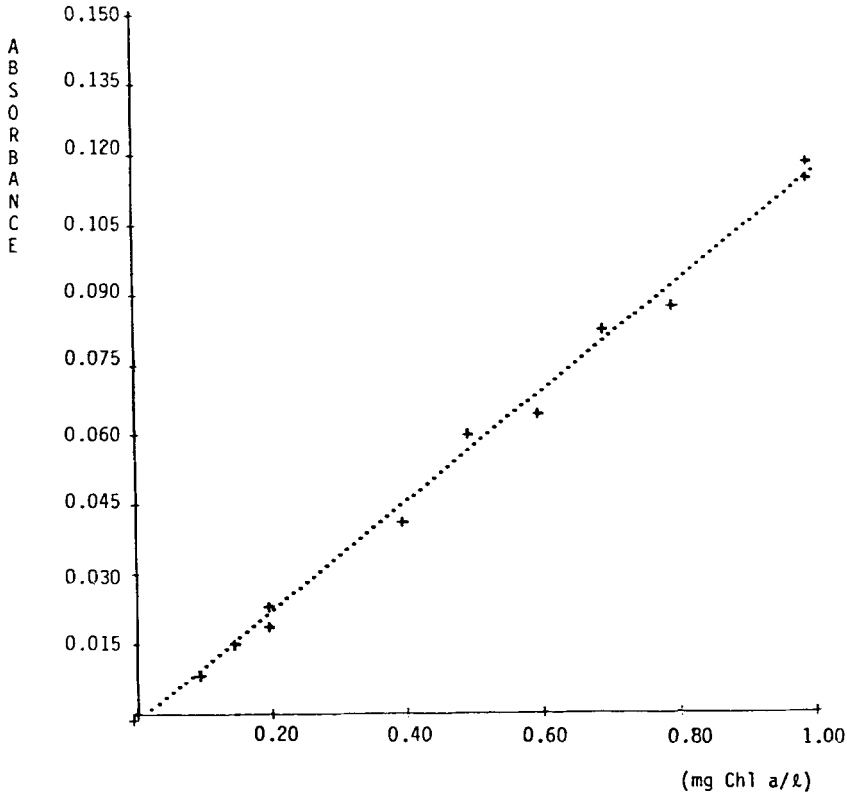

FIGURE 4B Absorption curve of chlorophyll a, at 428 nm. Equation: $y = -0.00017 + 0.0118x$; $R = 0.9956$.

detector and the fluorimeter. Treating the peak areas as function of the concentration with a least squares method the linear correlation appeared to be the best for both detectors. The sensitivity of the fluorimeter is however at least 4 times greater than the sensitivity of the UV/VIS detector (Figure 5). This led us to consider only the fluorimetric detection for the further work. A ten times repeated 50 μl injection of a 1 mg Chlor. a.l^{-1} solution yields with this method a reproduceability of 3%. The detection limit is estimated by the concentration equivalent to a signal due to the analyte which is equal to three times the standard deviation of three replicate measurements of a 50 μg.l^{-1} standard solution. The injection volume, chromatographic conditions and detector sensitivity were kept constant for every run. With this procedure the obtained value for the detection limit is 84 pg chlorophyll a.

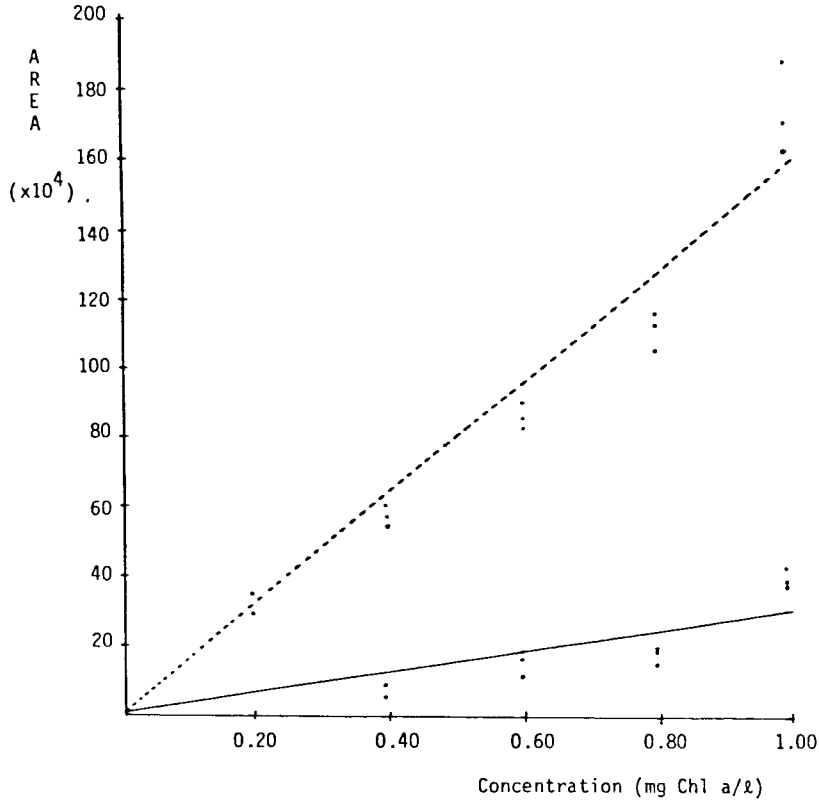

FIGURE 5 Calibration curves for fluorimetric detection (----) (equation: $y = -3.7975 + 162.4999x$; $R = 0.9642$), and absorbance detection (———) (equation: $y = -3.3060 + 36.4216x$; $R = 0.8467$).

IV. CONCLUSIONS

The use of HPLC with fluorimetric detection is very suitable for the determination of chlorophyll a concentrations. In the chromatogram the peak of chlorophyll a is clearly separated from other pigments and/or alteration products. This has the important advantage that interference-free measurements are guaranteed. Moreover the high accuracy and the sensitivity of the method make it very recommendable. With the presented isocratic elution method the analysis time is 30 minutes for chlorophyll a. In order to reduce it the more sophisticated but expensive gradient elution will probably be the only alternative solution.

The possibility of measuring many different green pigments and also their breakdown products in one single run is another principal

property of this approach. In ecosystem studies the knowledge of the evolution of a phytoplankton crop demands more than only the chlorophyll a concentration.

Pheophytins, pheophorbides and chlorophyllides can offer information about mortality by nutrient limitation, grazing, age, etc.[36,37,38] On the other hand presence and quantity of accessory pigments can inform about the differentiation of the phytoplankton population.

Evidently a knowledge of the concentration of different green pigments and their degradation products asks for pure standards of those different products. Until now most of them are not commercially available, which makes preparative chromatography a very useful instrument in this kind of research.

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