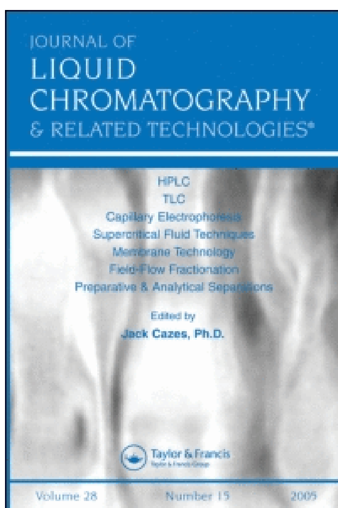
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Rapid Preparative Method for Purification of Bacteriochlorophyll A and B

G. Berger[a]; A. M. Wollenweber[b]; J. Kléol[a]; S. Andrianambinintsoa[a]; W. G. Mäntele[b]
[a] Département de Biologie C.E.N. Saclay, Service de Biophysique, France [b] Institut für Biophysik und Strahlenbiologie der Universität, Freiburg Federal, Republic of Germany

To cite this Article Berger, G. , Wollenweber, A. M. , Kléol, J. , Andrianambinintsoa, S. and Mäntele, W. G.(1987) 'A Rapid Preparative Method for Purification of Bacteriochlorophyll A and B', Journal of Liquid Chromatography & Related Technologies, 10: 7, 1519 — 1531
To link to this Article: DOI: 10.1080/01483918708066784
URL: http://dx.doi.org/10.1080/01483918708066784

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(7), 1519-1531 (1987)

A RAPID PREPARATIVE METHOD FOR PURIFICATION OF BACTERIOCHLOROPHYLL A AND B

G. Berger[1], A. M. Wollenweber[2], J. Kléo[1], S. Andrianambinintsoa[1], and W. G. Mäntele[2]

[1] *Service de Biophysique*
Département de Biologie
C.E.N. Saclay
91191 Gif-sur-Yvette cedex
France

[2] *Institut für Biophysik und*
Strahlenbiologie der Universität Freiburg
Albertstrasse 23, D-7800 Freiburg
Federal Republic of Germany

Downloaded At: 15:19 24 January 2011

ABSTRACT

For the purification of bacteriochlorophyll a and b (BChl), which are rather susceptible to e.g. oxygen, light, and polar solvents, a rapid and efficient purification method is described. After extraction of the cells with methanol-water, an octadecyl silica cartridge is used for retention of the BChl and thus polar compounds are removed from the complex mixture. Ethanol elutes the BChl, while less polar compounds remain on the cartridge. High performance liquid chromatography on reversed phase columns (rp-HPLC) proved to be a powerful tool in obtaining highly purified BChl, as controlled by analytical rp-HPLC, visible (VIS) spectroscopy, gas liquid chromatography (GLC), and SDS-polacrylamide gel electrophoresis (PAGE).

INTRODUCTION

Different techniques have been used for analytical and preparative separation of photosynthetic pigments : column, paper, counter-current distribution and thin layer chromatography (1). Improved resolution and

Copyright © 1987 by Marcel Dekker, Inc.

rapidity have been obtained in recent years with high performance liquid chromatography (2).

In the case of chlorophylls of higher plants and bacteriochlorophylls (BChl's), two types of chromatography have been applied :

a) adsorption chromatography on silica gel, with hexan-isopropanol (3,4,5,6) or hexan-acetone (7) mixtures as eluents.

b) reversed phase chromatography on C_8 or C_{18} alkyl coated silica gel as stationary phase and methanol, acetonitrile, acetic acid, tetrahydrofuran or ethyl acetate mixtures as solvents (8-24).

Polyethylene glycol has also been used as a packing material for the purification of BChl c (25).

Objections have been presented against each type of chromatography. Instability of chlorophylls has been shown to occur on polar adsorbents like silica (14), allomerization (12,20) or epimerization (3) proceed spontaneously in nucleophilic solvents like methanol or ethanol used in reversed phase chromatography. It seems, however, that the latter is better suited because only weak hydrophobic interactions come into play and epimerization or allomerization are relatively slow reactions (epimerization of chlorophyll a in ethanol : $k = 1,6 \times 10^{-6}\ s^{-1}$ (3)). Moreover, elution volumes are more reproducible and less sensitive to impurities than in adsorption chromatography.

Downloaded At: 15:19 24 January 2011

High performance liquid chromatography is a relatively rapid technique (from ten minutes to an hour), but extraction and preliminary partial purification steps (26) are often much longer (several hours).

In order to avoid degradation by prolonged contact of chlorophylls with polar solvents we developed a preparative method that is both more rapid and easier than the procedures previously described.

It involves the prepurification with Sep Pak cartridges that are usually used for prepurification of complex mixtures. With C_{18} alkyl coated silica cartridges, for instance, high polar compounds are eliminated with polar solvents chosen so that the compounds of interest remain on the cartridge. The latters are then removed by washing with a less polar solvent, while non polar compounds remain on the cartridge and are separated. This procedure prevents the accumulation of hydrophobic substances on HPLC columns, which causes high back pressure and reduced column life. It has been already described for analytical preparations of chlorophylls (21,27).

MATERIALS and METHODS

Pigment preparation : Rhodospirillum (Rs.) rubrum (BChl a, fig. 1a) and Rhodopseudomonas (Rp.) viridis (BChl b, fig. 1b) were grown according to

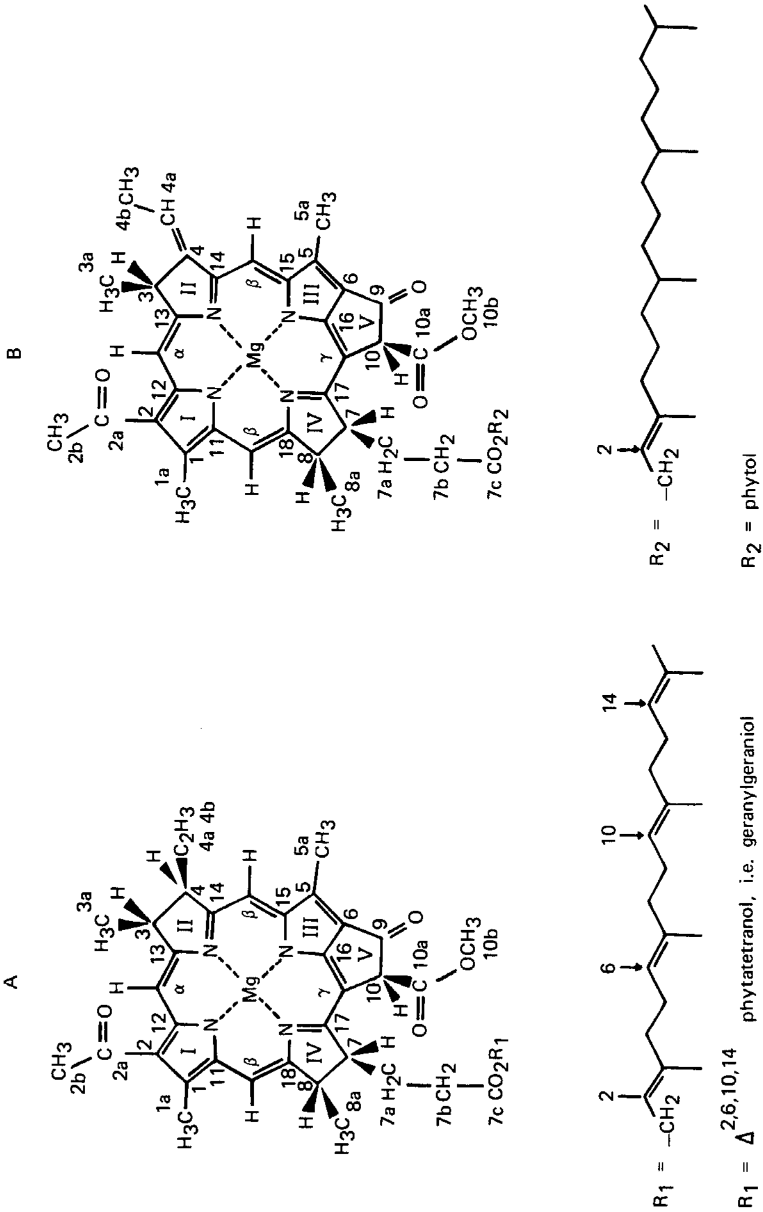


Figure 1 : Structures of Bacteriochlorophyll a and b

Hutner (28), modified by Cohen Bazire (29). Pelleted cells (1 g wet weight) were resuspended in cold (4°C) methanol (4 ml), stirred vigorously in dim light for 1 min, and filtered through a glass filter (Schott GS 4). Extraction was performed three times at the same conditions for quantitative extraction (methanol-water 80:20, v/v). The pigment solution was passed through an octadecyl silica cartridge (Sep Pak C_{18}, Waters Assoc.) up to saturation in bacteriochlorophyll (about 2 mg per cartridge under these conditions). In the case of BChl a the water concentration was brought to 30 % (v/v) for a better retention capacity at the resin. The cartridge was washed with 5 ml of a cold methanol-water mixture (70:30, v/v for BChl a, 80:20, v/v for BChl b). Under these conditions, polar substances were separated from bacteriochlorophylls and carotenoids, which remained on the stationary phase. Bacteriochlorophyll was eluted with about 5 ml of pure ethanol, a small fraction of carotenoids being coeluted in the case of Rp. viridis. The solvent was then evaporated under vacuum and the BChl's stored at - 20°C. The cartridges are reusable several times after washing with acetone.

Downloaded At: 15:19 24 January 2011

High performance liquid chromatography was performed on a Waters Assoc. liquid chromatograph consisting of a M510 pump, an U6K injector, and a M 481 detector system. Analytical runs were carried out on μ Bondapak C_{18} Waters columns, 3.9 mm x 30 cm, with aqueous ethanol (12 % H_2O v/v) at a flow rate of 1 ml/mn. Preparative runs were performed on Partisil 10 ODS_2 Whatman columns, 9 mm x 50 cm with aqueous ethanol (respectively 12 % H_2O for BChl a and 8 % H_2O for BChl b) at a flow rate of 6 ml/mn. In these conditions, they were eluted both in approximately 10 min. Absorbance was monitored at 580 nm.

Lipid analysis : Samples of 1 mg Bchl were taken after every purification steps. The solvent was evaporated and the samples were heated with 2 N HCl in methanol for 3 h at 85°C, the fatty acid methyl esters were extracted with petroleum ether (PE) 40°-60°C. Analysis was performed on a gas liquid chromatograph (GLC model Varian 3700) equipped with a non polar 25 m WCOT glass capillary column SE 54 (Weeke, Mülheim, FRG) and a flame ionization detector (FID). Oven temperature was set at 170°C isothermal, injector temperature at 230°C, and detector temperature at 280°C. Quantitative analysis was performed in the presence of the internal standard heptadecanoic acid (17:0). Peak integration was done by means of an automated integrator (Hewlett-Packard model 3380 A).

Gas liquid chromatography/mass spectrometric (GC/MS) analysis was performed on a Finnigan mass spectrometer, model 1020 A (injector temperature 280°C, separator oven 300°C, Helium pressure set at 14 psi, FS-CB column, 25 m). The ion source temperature was set at 60°C ; scan rate M 43-400 s^{-1}).

Protein analysis : Samples of about 20 μg Bchl were taken after every purification steps, the solvent was evaporated and the samples were solved again in buffer (30). SDS-PAGE was performed on a discontinuous slab gel with a 5 % stacking and 7,5 % - 15 % gradient gel. Proteins were visualized with Coomassie Brilliant Blue G (Sigma).

Reagents : All solvents used were analytical grade. The standard proteins were purchased from Pharmacia and contained phosphorylase b (94 KD), bovine serum albumin (67 KD), ovalbumin (43 KD), carbonic anhydrase (30 KD), soybean trypsin inhibitor (20 KD), and lactalbumin (14 KD).

RESULTS and DISCUSSION

Downloaded At: 15:19 24 January 2011

Using the combination of techniques described above, bacteriochlorophyll a or b can be prepared at the milligram scale from cell pellets of Rs. rubrum or Rp. viridis in a very short time (Fig. 2).

As measured by the optical density at the maximum of absorption, about 85 % of the Bchl's were extracted in the conditions described (13 % additional Bchl a and 17 % Bchl b were obtained by further extensive washing with acetone).

The yield of the prepurification step on Sep Pak cartridge is 88 % for Bchl a and 82 % for Bchl b.

The yield of the HPLC step is 73 % for Bchl a (incomplete dissolution of Bchl a in solvent ?) and close to 100 % for Bchl b.

The overall yield can be estimated using the molar extinction coefficients given in (31) for BChl a and in (24) for Bchl b. Extraction of 1 g (wet weight) of cells yields about 1.9 mg Bchla and 1.7 mg BChl b.

By using several Sep Pak cartridges and the preparative column, these quantities can be easily increased without a corresponding increase in preparation time : between 40 and 70 mg can be prepared per day.

The elution diagrams for analytical runs on a μ Bondapak C_{18} column (Fig. 3 a/b) indicate that the preparations contain only very little or no impurities in terms of other pigments absorbing at this wavelength (inferior to 0.6 % in the case of Bchl a and a 0.7 % in the case of Bchl b).

The percentage of Bchl a' (isomer of Bchl a on carbon 10, separated from Bchl a on reversed phase column) has been estimated to be lower than 0.04 %.

The absorbance spectra of the Bchl a and Bchl b in ether are shown in Fig. 4 They are similar to those reported in the litterature (24), concerning the wavelengths of maxima and the ratio of the absorbance peaks. Especially, the very low absorption at 680 nm indicates the absence of oxydation products

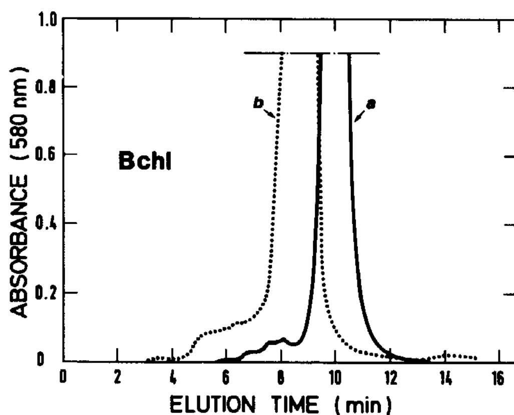


Figure 2 : Preparative Rp-HPLC of Bchl a (——) from Rs rubrum (a) and Bchl b (....) from Rp viridis (b). Conditions : column Partisil 10 ODS2 (Whatman) 9 x 500 mm. Flow rate 6 ml/min. Eluent : ethanol-water 88 : 12 (v/v) for Bchl a and 92 : 8 for Bchl b. Detector set at 580 nm.

Downloaded At: 15:19 24 January 2011

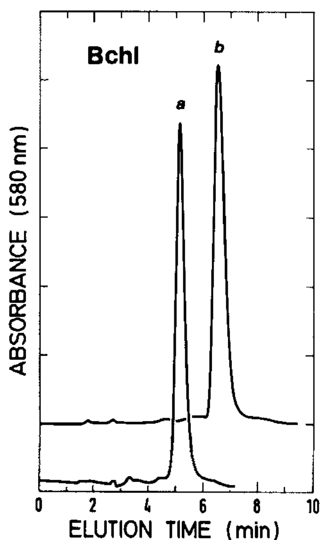


Figure 3 : Analytical Rp-HPLC of BChl a from Rs. rubrum (a) and BChl b from Rp. viridis (b). Conditions : column 3.9 x 300 mm, u Bondapak C_{18}, (Waters Assoc.). Flow rate 1 ml/min. Eluent : ethanol-water 88 : 12 (v/v). Detector set at 580 nm.

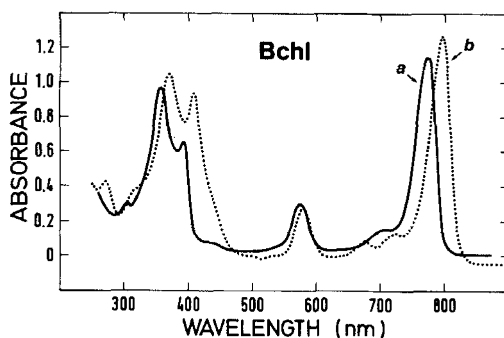


Figure 4 : Absorption spectra of rp-HPLC purified BChl a (——) and BChl b (....) in ether.

Downloaded At: 15:19 24 January 2011

in the case of Bchl b and the absorbance ratio $A_{357\ nm}/A_{770\ nm}$ of 0.85 for Bchl a and $A_{369\ nm}/A_{794\ nm}$ of 0.83 for Bchl b indicates the purity of the material prepared by this method (24).

When a mixture of BChl a from Rs. rubrum and BChl b from Rp. viridis is injected onto a rp-HPLC column, BChl a is eluted before BChl b (fig. 5) (while Chl a is eluted after Chl b under the same conditions). The esterifying alcohol at the propionic acid side chain is geranylgeraniol in the first case (fig. 1 a) (32) and phytol in the second one (fig. 1 b). It is known from a mixture of pheophytins esterifed with different alcohols, that retention time on rp-columns decreases with the number of double bonds in the alcohol (33,34). The comparison of the two BChl's shows that the influence of the side chain alcohol is predominant over that of the ethylidene group.

Quantitative fatty acid analysis of the filtered methanol extracts of pelleted cells on GLC revealed that 3.8 mg fatty acid methyl esters in 1 mg BChl a (= 380 %) and 8.5 mg in 1 mg BChl b (= 850 %) could be detected (see table 1). By applying the sample onto a Sep Pak C_{18} cartridge, contaminating hydrophilic (e.g. some proteins) and hydrophobic (e.g. carotenoids) material was removed. However, no drastic reduction in the fatty acid content could be analyzed (compare table 1).

For the removal of contaminating phospholipids, preparative HPLC of the BChl on a reversed phase octadecyl silica µ-Bondapak column proved to be a very efficient purification step. Only small amounts of fatty acids could be detected in the final products, i.e. a total amount of 60 µg/mg BChl a (6 %) and 200 µg/mg BChl b (20 %).

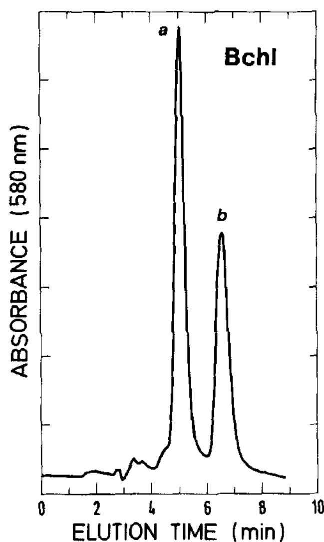


Figure 5 : Elution profile of a mixture of bacteriochlorophyll a and b. Conditions : column 3,9 x 300 mm (μ-Bondapak C_{18}, Waters Assoc.), eluent : ethanol-water (88 : 12, v/v), flow rate : 1 ml/min, detector set at 580 nm.

Downloaded At: 15:19 24 January 2011

TABLE 1

Content of fatty acids after transesterification with 2 N HCl in methanol, estimated on a SE-54 WCOT 25 m glass capillary column for different purification steps of BChl a and BChl b.

	BChl a	BChl b
estimation after purification	% (w/w)*	
1. bacterial methanol extract	380	850
2. Sep PAK C_{18}	280	260
3. rp-HPLC	6	20
Sepharose**	54	n.d.

* Sum of Δ-16 : 1, 16 : 0, Δ-18 : 1, and 18 : 0 in per cent of the respective BChl

** Purified according to T. Omata and N. Murata (26) ; n.d. not done

In addition to the analyzed fatty acid methyl esters several peaks could be identified by their retention time on GLC and characteristic mass spectrometric fragmentation pattern on GC/MS (m/z = 278, 123 (base peak), 109, 95 and 81) to derive from isomeric degraded phytolmoiety of BChl b (data not shown).

Because of inherent difficulties with a photometric protein estimation the following method was applied : increasing amounts of the respective BChl were applied onto a SDS-PAGE and stained for protein with Coomassie Brilliant Blue G. After the first methanol extraction some proteins, most likely from the light harvesting complex could be hardly visualized. No proteins, however, could be detected after Sep Pak C_{18} purification in spite of overloading the gel. The detection limit according to standard proteins was estimated to be lower as 0.5 % of the respective BChl.

In conclusion, a rapid extraction and purification of BChl a and b at the 50 mg scale can be achieved by combination of Sep Pak cartridge prepurification and rp-HPLC. The quantity and quality of the preparations are sufficient for optical and electrochemical experiments and they can be prepared almost immediately before use, avoiding storage degradation.

Downloaded At: 15:19 24 January 2011

ACKNOWLEDGMENTS

The authors would like to thank Dr. H.-W. Wollenweber (MPI Freiburg) for helpful discussions and performing the GLC, D. Borowiak (MPI Freiburg) for performing the gas chromatography mass spectrometry analysis, Dr. J. Breton (CEN, Saclay) and Prof. Dr. W. Kreutz (Freiburg) for encouragement and continous support of our work and Mlle P. Belle for typing this manuscript. One of us (W.G.M.) is indebted to the EMBO (European Molecular Biology Organization) for a short term fellowship.

REFERENCES

1. Strain, H.H., Cope, B.T., and Svec, W.A., Analytical procedures for the isolation, identification, estimation and investigation of the chlorophylls. Methods in Enzymology, 23, 452, 1971.

2. Schwartz, S.J., and von Elbe, J.H., High performance liquid chromatography of plant pigments. A review. J. Liq. chrom., 5, 43, 1982.

3. Watanabe, T., Nakazato, M., Mazaki, H., Hongu, A., Konno, M., Saitoh, S., and Honda, K., Chlorophyll a epimer and pheophytin a in green leaves. Biochim. Biophys. Acta, 807, 110, 1985.

4. Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M., and Saitoh, S., Preparation of chlorophylls and pheophytins by isocratic liquid chromatography. Anal. Chem., 56, 251, 1984.

5. Iriyama, K., Yoshiura, M., and Shiraki, M., Micro-method for the qualitative and quantitative analysis of photosynthetic pigments using high-performance liquid chromatography. J. Chrom., 154, 302, 1978.

6. Rahmani, M., and Csallany, A.S., Mise au point d'une méthode de chromatographie liquide à haute performance pour la détermination des pigments chlorophylliens dans les huiles végétales. Rev. Fran. Corps Gras, 32, 257, 1985.

7. Yoshiura, M., Iriyama, K., and Shiraki, M., High performance liquid chromatography of chlorophylls and some of their derivatives. Chem. letters, 281, 1978.

8. Shoaf, W.T., Rapid method for the separation of chlorophylls a and b by high pressure liquid chromatography. J. Chrom., 152, 247, 1978.

9. Braumann, T., and Grimme, L.H., Single step separation and identification of photosynthetic pigments by high performance liquid chromatography. J. Chrom., 170, 264, 1979.

10. Liebezeit, G., Chlorophyll a in marine phytoplankton : separation by HPLC and specific fluorimetric detection. J. High. resol. Chrom., 3, 531, 1980.

11. Davies, D., and Holdsworth, E.S., The use of high pressure liquid chromatography for the identification and preparation of pigments concerned in photosynthesis. J. Liq. Chrom., 3, 123, 1980.

12. Scholz, B., and Ballschmitter, K., Preparation and reversed phase high performance liquid chromatography of chlorophylls. J. Chrom., 208, 148, 1981.

13. Falkowski, P.G., and Sucher, J., Rapid, quantitative separation of chlorophylls and their degradation products by high performance liquid chromatography. J. Chrom., 213, 349, 1981.

Downloaded At: 15:19 24 January 2011

14. Braumann, T., and Grimme, L.H., Reversed phase high performance liquid chromatography of chlorophylls and carotenoids. Biochim. Biophys. Acta, 637, 8, 1981.

15. Shioi, Y., and Sasa, T., Separation of protochlorophylls esterified with different alcohols from inner seed coats of three cucurbitaceae. Plant Cell Physiol., 23, 1315, 1982.

16. Gleixner, G., Karg, V., and Kis, P., Rapid preparation of pure chlorophyll a. Experientia, 38, 303, 1982.

17. Steiner, R., Wieschhoff, H., and Scheer, H., High performance liquid chromatography of bacteriochlorophyll b and its derivatives as an aid for structure analysis. J. Chrom., 242, 127, 1982.

18. Liebezeit, G., and Bartel, J., Reversed phase HPLC separation of fossil chlorins. J. High. Resol. Chrom., 6, 573, 1983.

19. Hamamoto, C.M., and Castelfranco, P.A., Separation of monovinyl and divinyl protochlorophyllides from etiolated and phototransformed cucumber cotyledons. Plant Physiol., 73, 79, 1983.

20. Schaber, P.M., Hunt, J.E., Fries, R., and Katz, J.J., High performance liquid chromatographic study of the chlorophylll allomerization reaction. J. Chrom., 316, 25, 1984.

21. Wright, S.W., and Shearer, J.D., Rapid extraction and high performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton. J. Chrom., 294, 281, 1984.

22. Shioi, Y., Doi, M., and Sasa, T., Separation of non esterified chlorophylls by ion suppression high performance liquid chromatography. J. Chrom. 298, 141, 1984.

23. Braumann, T., Vasmel, H., Grimme, L.H., and Amesz, J., Pigment composition of the photosynthetic membrane and reaction center of the green bacterium Prosthecochloris aestuarii Biochim. Biophys. Acta, 848, 83, 1986.

Downloaded At: 15:19 24 January 2011

24. Steiner, R., In vivo und in vitro Untersuchungen an den Bakteriochlorophyll B-haltigen organismen. Ectothiorhodospira Halochloris, Ectothiorhodospira Abdelmalekii und Rhodopseudomonas viridis. Ph. D. Thesis, München 1984.

25. Chow, H.S., Caple, M.B., and Strouse, C.E., Polyethylene powder as a stationary phase for preparative scale reversed-phase high performance liquid chromatography. J. Chrom., 151, 357, 1978.

26. Omata, T., and Murata, N., A rapid and efficient method to prepare chlorophyll a and b from leaves. Photochem. Photobiol., 31, 183, 1980.

27. Eskins, K., and Dutton, H.J., Sample preparation for high performance liquid chromatography of higher plant pigments. Anal. Chem., 51, 1885, 1979.

28. Hutner, S.H., J. Gen. Microb., 4, 286, 1950.

29. Cohen Bazire, G., Sistrom, W.R., and Stanier, R.Y., Kinetic studies of pigment synthesis by non sulfur purple bacteria. J. Cell. compar. physiol., 49, 25, 1957.

30. Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680, 1970.

31. Connolly, J.S., Samuel, E.B., and Janzen, A.F., Effect of solvent on the fluorescence properties of bacteriochlorophyll a. Photochem. Photobiol., 36, 565, 1982.

32. Katz, J.J., Strain, H.H., Harkness, A.L., Studier, M.H., Svec, W.A., Janson, T.R., and Cope, B.T., Esterifying alcohols in the chlorophylls of purple photosynthetic bacteria. A new chlorophyll, bacteriochlorophyll (gg) all trans geranyl geranyl bacteriochlorophyllide a. J. Am. Chem. Soc., 94, 7938, 1972.

33. Schoch, S., The esterification of chlorophyllide a in greening bean leaves. Z. Naturforsch., 33C, 712, 1978.

Downloaded At: 15:19 24 January 2011

34. Schoch, S., Lempert, U., Wieschhoff, H., and Scheer, H., High performance liquid chromatography of tetrapyrrole pigments. Pheophytins esterified with different diterpene alcohols, isomeric biliverdins and synthetic bilins. J. Chrom., 157, 357, 1978.

Downloaded At: 15:19 24 January 2011