SCIENTIA gerundensis, 19: 23-34 (1993)

SEPARATION OF BACTERIOCHLOROPHYLL *c* AND CHLOROPHYLL *a* FROM MIXED EXTRACTS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Borrego, C.M. and L.J. Garcia-Gil

University of Girona, Institute of Aquatic Ecology, Pl. Hospital, 6. 17071 Girona.

RESUM

Es descriu un mètode de Cromatografia Líquida d'Alta Resolució (HPLC) per a la separació de Bacteriocloroíil.la *c* (de bacteris fototròfics del sofre) de la Clorofil.la *a*. L'aplicació d'aquest mètode ofereix l'avantatge d'evitar la saponificació prèvia de les mostres alhora que millora la resolució en les anàlisis d'extractes que continguin una mescla d'ambdós pigments.

El gradient utilitzat en la separació té una durada de 40 minuts i és més dràstic que d'altres emprats anteriorment en aquest tipus d'anàlisi. La Bacteriocloroíil.la *c* s'elueix en el minut 21 en forma de dos pics, corresponents a dues formes homòlogues d'aquest pigment. Per la seva banda, la Clorofil.la *a* és eluïda en el minut 35 oferint una clara separació respecte la Bclor *c*. Els resultats obtinguts són comparats amb d'altres trobats en la bibliografia especialitzada i analitzats d'acord amb les característiques mol.leculars de cada pigment.

RESUMEN

En el presente trabajo se describe un método de Cromatografía Líquida de Alta Resolución (HPLC) para la separación de la Bacteriocloroíila *c* (de bacterias fototróficas del azufre) de la Clorofila *a*. La aplicación de este método ofrece la ventaja de evitar la saponificación previa de las muestras al mismo tiempo que mejora la resolución de los análisis de extractos que contengan una mezcla de ambos pigmentos.

La duración del gradiente utilitzado es de 40 minutos y es más drástico que los utilizados anteriormente en este tipo de análisis. La Bacteriocloroíila *c* se eluye en el minuto 21 en forma de dos picos, correspondientes a dos formas homólogas de este pigmento. Por su parte, la Clorofila *a* se eluye en el minuto 35, ofreciendo una clara separación respecto la Bclor *c*. Los resultados obtenidos son comparados con los encontrados en la bibliografía especializada y analizados de acuerdo con las características moleculares de cada pigmento.

ABSTRACT

A reversed-phase HPLC method for the separation of Bacteriochlorophyll *c* of phototrophic sulphur bacteria from Chlorophyll *a* in mixed extracts is described. The application of this method avoid the time-consuming saponification treatment of the samples and provides a reliable results on the analysis of extracts containing both prokaryotic and eukaryotic pigments.

Pigments were separated using a drastic solvent gradient during 40 minutes. Bchl *c* was eluted at 21st minute in a form of two peaks, related to the two homologous Bchl *c* molecules. Chl *a* was eluted at 35th minute, offering a clear separation in respect bacterial chlorophyll. The results obtained are compared with those reported in literature and analyzed according to the molecular characteristics of each pigment.

Keywords: Bacteriochlorophyll *c*, Chlorophyll *a*, HPLC, Pigment separation, Phototrophic Sulphur Bacteria.

INTRODUCTION

The determination of pigment concentrations in aquatic ecosystems has been generally used by microbial ecologists as an estimation of biomass, primary production and photosynthetic activity of planktonic phototrophic microbial populations. However, the low resolution of standard techniques on the precise determination of algal and bacterial chlorophylls is an important limitation on the study of aquatic environments where eukaryotic and prokaryotic photosynthetic planktonic population are spatially coinciding (Jacobsen et al., 1990). The co-occurrence of both groups of microorganisms is very frequent in anoxic waters, where phototrophic sulphur bacteria grow and algal cells are either growing or sinking from oxic waters. This distribution results in samples containing a mixture of eukaryotic and prokaryotic chlorophylls. The similar chemical estructures of these photosynthetic pigments (Figure 1) results in the overlapping of absortion spectra that can both prevent a correct determination using spectrophotometric or fluorimetric techniques and cause large errors in posterior calculations (Talling and Driver, 1961; Rai, 1973:Korthals and Steenbergen, 1985; Jacobsen et al., 1990).

The application of High Performance Liquid Cromatography (HPLC) on the identification and separation of photosynthetic pigments from natural phototrophic microbial populations has been developed in the past few years for avoiding these drawbacks, (Mantoura and Lewellyn, 1983; Korthals and Steenbergen, 1985; Bidigare et al., 1985; Caraco and Pucoon, 1986;Yacobi et al., 1990; Hurley and Watras, 1991). The high precission and accuracy of this technique provides rapid and reliable results on the separation and identification of algal and bacterial pigments. However, these chromatographic methods are not evolved sufficiently up to date for their application in a routine way.

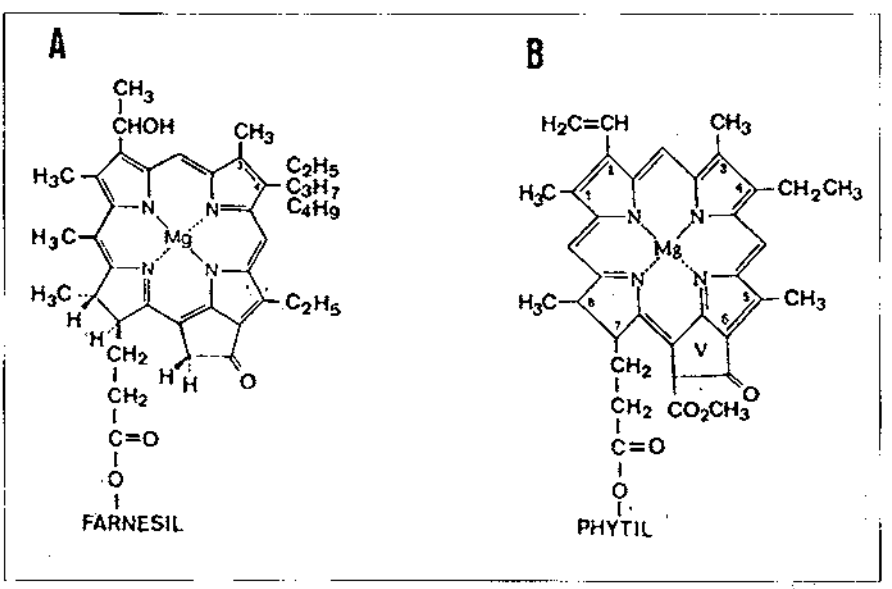

Figure 1. Chemical structure of Bacteriochlorophyll *c* and Chlorophyll *a*.

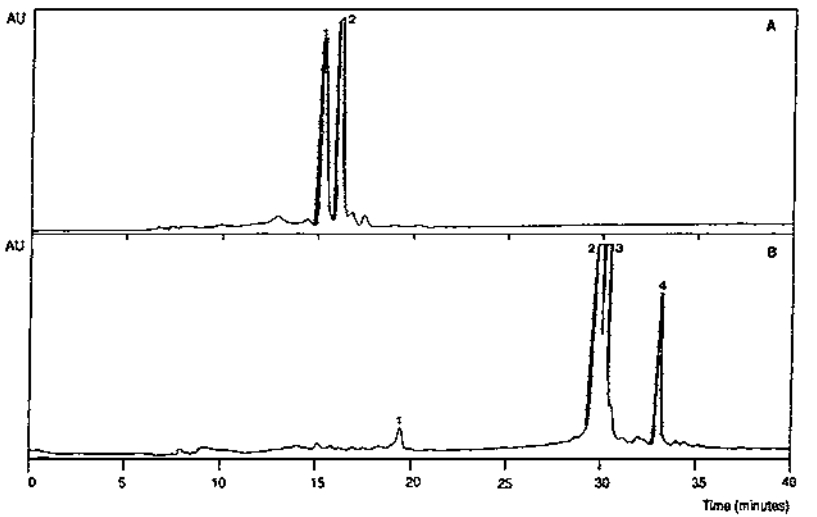

Figure 2. Chromatographic profiles of saponified (A) and untreated Bchlor *c* (B) obtained with the gradient reported by Yacobi et al. (1990). Absorbance chromatograms were performed at 365 nm.

The aim of this work is to provide a more simply and reliable way to separate a prokaryotic pigment (Bchl *c*) and eukaryotic one (Chl *a*) from mixed extracts. The method described achieves a good separation of both pigments, eluted at minute 21st and 35th respectively using a drastic solvent gradient. Furthermore, the application of this method avoids the time-consuming saponification treatment of the samples before to HPLC injection and, at the same time, enhances the resolution of pigment determination.

MATERIALS AND METHODS

Samples

Bchl *c* was extracted from a pure culture of the Green Sulphur Bactera species, *Chlorobium limicola* DSM 249 grown in batch culture at the laboratory using Pfennig medium and saturation light intensities.

Because of the easiness of extraction and the high pigment concentration attained, Chl *a* extracts were obtained from *Spinascia olearacea* leaves.

Pigment extraction

For bacterial chlorophyll, 100 ml of *Chlorobium* culture was centrifuged at 10,000 rpm during 10 minutes. The pellet was resuspended in 25 ml of 100% acetone and disrupted with a B-Braun Labsonic 2000 during 15 seconds. After this treatment, the extract was preserved from light and stored at -40°C during 24 hours. After-

Table 1. Solvent gradient program used.

Time Periods (minutes)	% solvent B
0 to 5	60 up to 85
5 to 15	85 hold
15 to 25	85 up to 100
25 to 40	100 hold

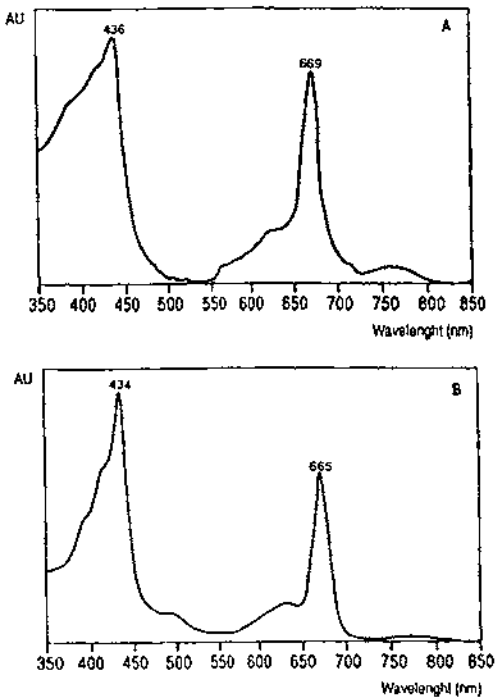

Figure 3. Absorbance scanning of peaks effluents from chromatographic profiles of saponified Bchl *c* (A) and untreated one (B). Absorbance spectras were performed at wavelenght range of 350-850 nm.

Table 2. Retention times, identification and absorbance maxima of respective peak effluents of chromatographic analysis of saponified Bchl *c* (A) and untreated one (B) with the original gradient program reported by Yacobi et al. (1990). Parenthesis values are the previously found by Yacobi and co-workers. Spectral scanning was performed between 350 and 850 nm.

Run	Peak nº	Rt (minutes)	Tentative Identification	Absorption maxima (nm)
A	1	15.4 (14.7)	Bchlor c_1	436 (436) - 668 (668)
	2	16.2 (15.4)	Bchlor c_2	436 (436) - 669 (668)
B	1	-	Not identified	-
	2	29.8	Bchlor c_1	435-668
	3	30.2	Bchlor c_2	435-669
	4	-	Not identified	-

Table 3. Retention times, identification and absorbance maxima of respective peak effluents of chromatographic analysis of Bchl *c* (see Figuere 4A).

Peak nº	Tentative Identification	Rt (minutes)	Absorption maxima (nm)
1	Not identified	13.2	-
2	Bchlor c_1	21.1	436-667
3	Bchlor c_2	21.9	436-667
4	Not identified	28.8	-

wards, the extract was centrifuged (10' at 15,000 rpm) and the green supernatant was filtered through a glass fiber filter and eventually injected in the HPLC without further treatment.

For saponified samples of Bchl *c*, bacterial extracts were evaporated by means of nitrogen bubbling. The pellet was resuspended in 2 ml of Methanol-KOH (5%) and kept in the dark at room temperature during two hours. After this period, 3 ml of petroleum spirit was added to the sample. Hypophase containing Bchl *c* was

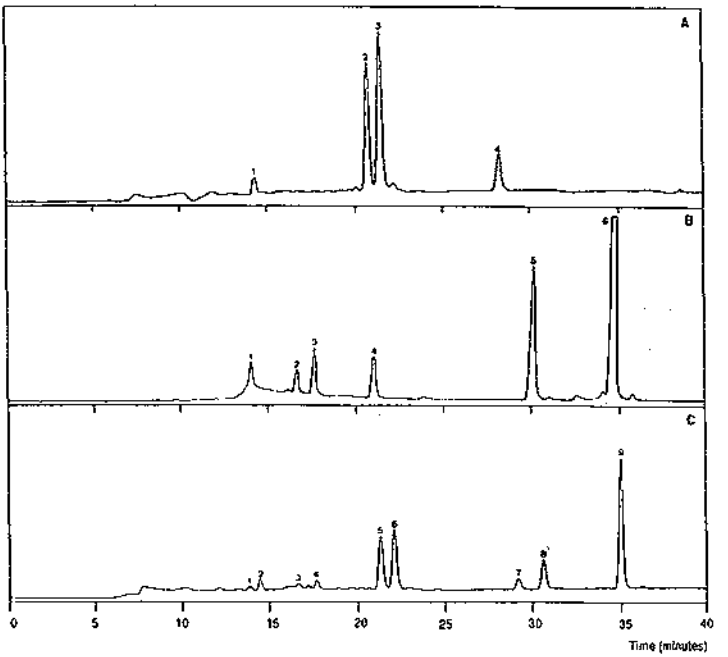

Figure 4. Chromatographic profiles of Bchl *c* (A), Chl *a* (B) and mixed extract (C) obtained with the modified method. Absorbance chromatograms were performed at 365 nm.

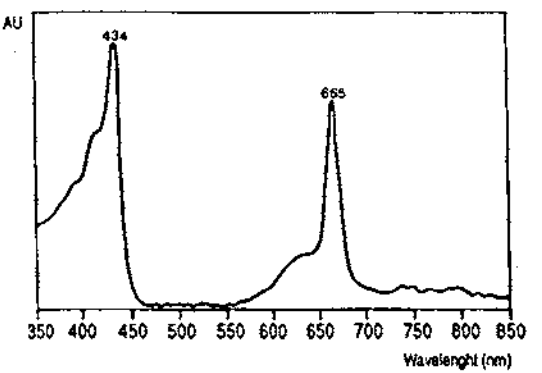

Figura 5. Absorbance spectra of peaks effluents from chromatographic profile of Bchl *c* (see Figure 4A). Unidentified peaks are not represented. Scannings were performed at wavelenght ranger of 350-850 nm.

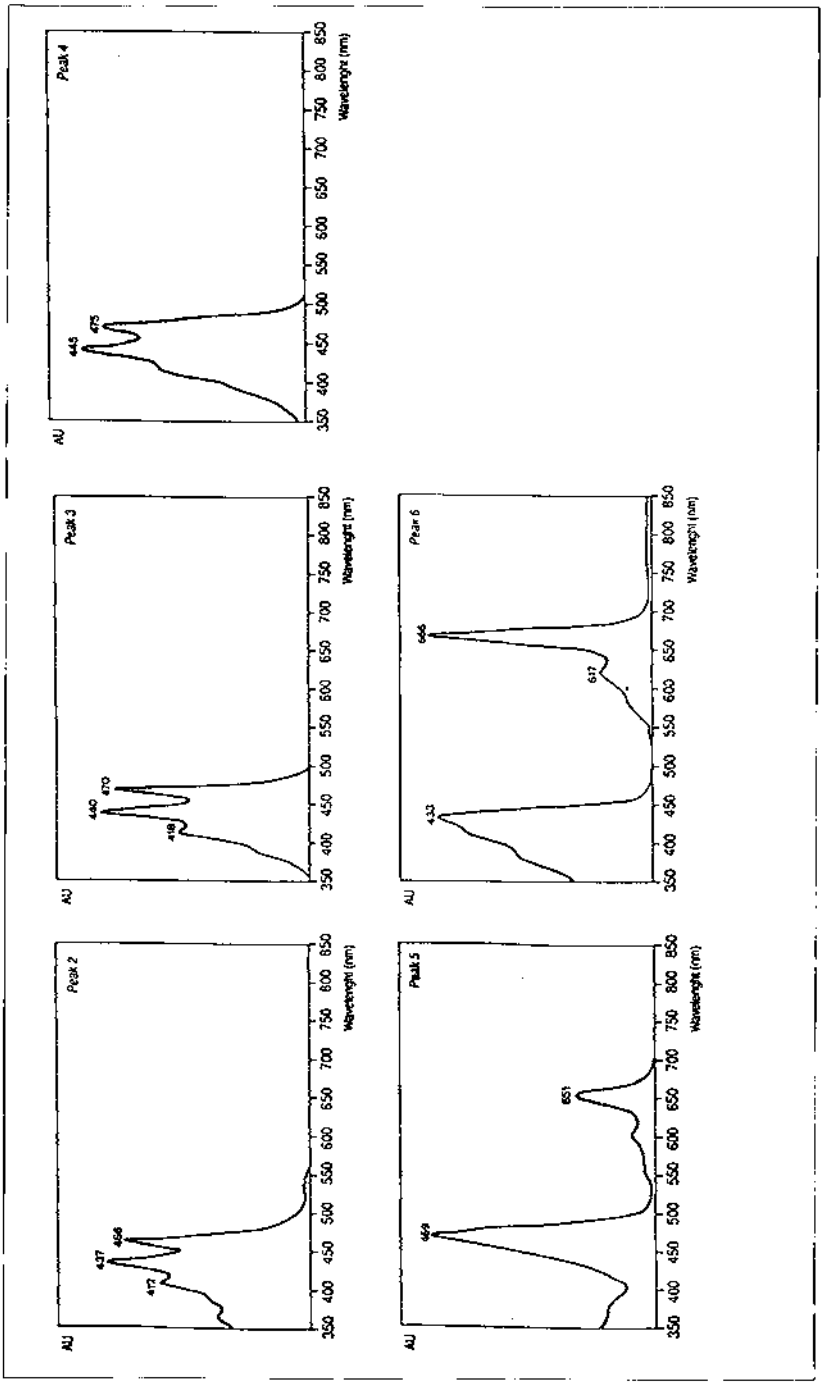

Figura 6 Absorbance spectra of peaks effluents from chromatographic profile of Chl *a* (see Figure 4B). Unidentified peaks are not represented. SAbsorbance Spectras were performed at wavelenght range of 350-850 nm.

Table 4. Retention times, identification and absorbance maxima of respective peak effluents of chromatographic analysis of Chl *a* (see Figure 4B).

Peak nº	Tentative Identification	Rt (minutes)	Absorption maxima (nm)
1	Not identified		-
2	Neoxanthine	16.2	412-437-466
3	Violaxanthine	17.2	418-443-470
4	Luteine	20.6	(420)-445-475
5	Chlorophyll *b*	29.8	469-651
6	Chlorophyll *a*	34.5	433-466

separated from epiphase containing bacterial carotenoids through a decantation funnel. Saponified Bchl *c* extract was then let to dry and resuspended in 100% acetone prior to the HPLC analysis.

Chlorophyll *a* was obtained from a 100% acetone extract of *Spinascia oleracea* leaves. Before to HPLC injection the extract was processed in the same way as described above.

HPLC Analyses

The separations were carried out with a reversed-phase HPLC system formed by LKB 2249 gradient solvent pump, a Rheodyne 7125 syringue loading injector and a C-18 S5 ODS2 (5 μm silica particle size) 25x4.6 mm column protected with a S5 ODS2 5x4.6 mm column guard. Pigments were detected with a LKB 2510 SD detector setting at 365 nm. Chromatograms were recorded with a LKB 2221 integrator.

The pigments were separated using a modification of the method reported by Yacobi et al. (1990). The modification of the program was designed to obtain a better resolution with natural pigment samples. The solvent program consists in a linear increase of solvent B from 60 to 85% in 5 minutes, a hold at 85% for 10 minutes, a linear increase from 85 to 100% solvent B from 15 to 25 minutes, followed by a hold of 100%B for 15 minutes (Table 1). Solvents used for the separation were the same reported by Yacobi and co-workers, namely, Solvent A (70% methanol and 30% ammonium acetate (1M)) and Solvent B (70% methanol and 30% ethyl acetate). Solvents were bubbled with Helium before its use. The best results were obtained with a flow rate of 0.5 $ml.m^{-1}$. The final pigment identification was based on both, its absorbance spectra and its relative retention time. Absorption spectras were performed using a Milton-Roy 3000 spectrophoto-meter, setting a wavelength range between 350 and 850 nm.

RESULTS

Formerly, a separation of the pigments using the solvent program reported by Yacobi et al., was assayed. In our case, the results obtained by Yacobi and co-workers with bacterial chlorophyll were repeated only after the saponification of pigment extracts. Figure 2 shows the chromatographic charts of both saponified and not saponified Bchl *c*. In both analyses, bacterial chlorophyll was eluted forming a "cluster" of two peaks. It should be pointed out the lower resolution of HPLC analysis of untreated Bchl *c* (Figure 2B). On the other hand, the retention times obtained with this sample were higher than those found with saponified Bchl *c* (Table 2). Absorption spectra of respective peaks effluents of both Bchl *c* samples collected after the elution from HPLC detector are shown in Figure 3.

The modification of the method was designed both to avoid the saponification process and achieve a good separation of photosynthetic pigments. HPLC analysis of untreated Bchl *c* extracts using the modified method also showed the peculiar two peaks cluster pattern (peaks numbers 2 and 3) but their elution time was earlier (Figure 4A). Bchl *c* was eluted at minute 21st approximately. The respective retentions times and the identification of each peak are reported in Table 3. Absorbance spectra of two homologous peaks effluents were identical and corresponded exactly to the expected Bchl *c* (Figure 5). However, peaks numbers 1 and 4 were unidentified due to the fact that the concentration in the peak effluent was below the resolution power of the spectrophotometer.

Figure 4B shows the chromatographic chart of *Spinascia olearacea* extract. The main pigments of this species were identified except ß-carotene. The major xantofiles: Neoxanthine, Violaxanthine, Luteine and two chlorophylls, Chlorophyll *b* and Chlorophyll *a* (Peak numbers 2, 3, 4, 5 and 6 respectively) were identified in the basis of the spectral characteristics of the respective peak fractions (Figure 6). Retention

Table 5. Retention times, identification and absorbance maxima of respective peak effluents of chromatographic analysis of Bchl *c* - Chl *a* extract (see Figure 4C).

Peak nº	Tentative Identification	Rt (minutes)	Absortion maxima (nm)
1	Not identified	13.3	-
2	Not identified	14.5	-
3	Neoxanthin	16.6	412-437-466
4	Violaxanthine	17.3	418-443-470
5	Bchlor c_1	21.5	436-667
6	Bchlor c_2	22.3	436-667
7	Not identified	29.5	-
8	Chlorophyll *b*	30.9	469-451
9	Chlorophyll *a*	35.3	433-666

times and tentative identification of each peak are reported in Table 4. It is noticeable that Chl *b* and *a* were eluted considerable late in the chromatographic run (minutes 29 and 35, respectively).

Figure 4C shows the chromatogram obtained from a Chl *a*-Bchl *c* mixed extract. The obtained elution pattern of each pigment was the same previously found in samples containing pigment alone. Except luteine, all pigments of both species were identified from the mixture from both, their retention times and their respective absorbance spectra (Table 5).

DISCUSSION

The overlapping of absorption spectra between Bchl *c* and Chl *a*, that have the same absortion maxima (434-665), difficults their discrimination in mixed extracts as for example natural samples containing both pigments (Figure 7). The principal differences between Bchl *c* and Chl *a* lie in the diverse substituents of the porfirinic central group and the long lateral hydrophobic terpenoid chain (farnesyl group in Bchl *c* and phytil group in Chl *a*) esterified with the propionic acid of IV ring of the macrocycle (Figure 1). Apparently, these structural changes do not determine significant differences in light absortion properties, but in contrast they provide a good separation in chromatographic analyses.

Bacterial chlorophylls *c*, *d* and *e* from phototrophic Green and Brown sulfur bacteria, exist as complexs of homologous forms with different substituents in the macrocycle (see Figure 1) (Gloe et al., 1975). The two peaks elution pattern showed in chromatographic profiles was due to the similar spectral characteristics of the two homologous forms of Bchl *c* (Bchl *c1* and *c2*). The differences observed between the elution times of saponified Bchl *c* cluster (Figure 2A) in respect to the untreated one (Figure 2B) was probably due to the loss of farnesyl chain produced during the saponification process. This cleavage convert Bchl *c* in Bacteriochlorophyllide *c*. This structural change decreases the interaction of pigment molecules with the octadecyl groups of the column and therefore, its retention time is lower. On the other hand, untreated Bchl *c* keeps its structure, increasing the interaction

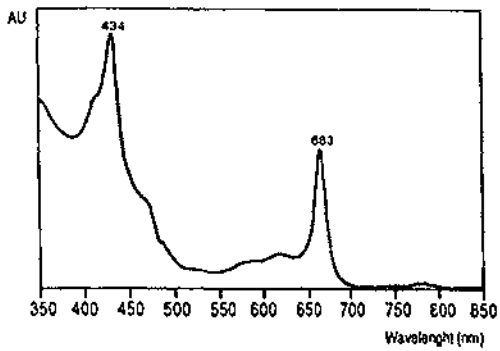

Figure 7. Absorbance spectra of a mixed extract containing Bchlor c and Chlor a. Scanning were performed at wavelenght range of 350-850 nm.

strenght with the column groups and consequently, the respective elution time was higher (30 minute approx.) (Figure 2B).

Nevertheless, the necessary saponification of our samples to obtain a good pigment separation applying the method of Yacobi is unsuited in a routinary way due to their considerable loss of time. The modification of the method was done for avoiding this treatment and, at the same time, improve the separation of pigment extracts. The solvent program proposed is more drastic than original and permits an earlier separation of natural Bchl *c* (Rt= 21') (Figure 4B).On the other hand, eukaryotic chlorophylls were eluted almost at the end of the run (Rt=29' for Chl *b* and Rt=35' for Chl *a*). This fact demonstrate the higher interaction strenght of these pigment with the column groups in respect to bacterial one, basically due to the presence of the phytyl chain (longer than the Bchl farnesyl chain).

The application of the modified method to mixed extracts displays a clear separation of the analyzed pigments (Figure 4C). In the chromatographic profile, the bacterial chlorophyll cluster (21 minutes) and the Chl *b* and *a* peaks (29 and 35 minutes) are easily recognizable. The distance between the two groups of peaks (approximately 13 minutes) demonstrated the high resolution achieved with this method in relation to the spectrophotometrical one (Figure 7).

The high reliability and resolution of the method described on the separation of tested pigments can provide an useful tool for the study of aquatic environments where algal and bacterial photosynthetic microbial populations are found together. Nowadays, further modifications of the method have to be developed in order to achieve the separation of others specific photosynthetic pigments of phototrophic sulfur bacteria (Bchl *a*, *d* and *e*) - and their respective degradation products) from algal chlorophylls. Moreover, the calibration of the method for quantitative analyses and its application to natural samples containing both groups of pigments are the subject of further investigations.

Bibliography

BIDIGARE, R. R., M. C. KENNICUTT and J. M. BROOKS. (1985). Rapid determination of chlorophylls and their degradation products by high performance liquid chromatography. *Limnol. Oceanogr.*, 30: 432-435.

CARACO, N. and A. H. PUCCOON. (1986). The measurement of bacterial chlorophyll and algal chlorophyll a in natural waters. *Limnol. Oceanogr.*, 31: 889-893.

GLOE, A., N. PFENNIG, H. BROCKMAN and W. TROWITSCH. (1975). A new bacteriochlorophyll from brown-colored Chlorobiaceae. *Archives of Microbiology*, 102: 103-109

HURLEY, J. P. and C. J. WATRAS. (1991). Identification of bacteriochlorophylls in lakes via reversed-phase HPLC. *Limnol. Oceanogr.*, 36(2): 307-315.

JACOBSEN, T. R. and H. RAI. (1990). Comparision of spectrophotometric, fluorimetric and high performance liquid chromatography methods for determination of chlorophyll *a* in aquatic samples: effects of solvents and extraction procedures. *Int. Revue ges. Hydrobiol.*, 75(2): 207-217.

KORTHALS, H. J. and C. L. M. STEENBERGEN. (1985). Separation and quantification of pigments fron natural phototrophic microbial populations. *FEMS Microbial Ecology*, 31: 177-185.

MANTOURA, R. F. C. and C. A. LLEWELLYN. (1983). The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high performance liquid chromatography. *Anal. Chem. Acta*, 151, pp. 297-314.

RAI, H. (1973). Methods involving the determination of photosynthetic pigments using spectrophotometry. *Verh. Inter. Verein. Limnol.*, 18: 1864-1875.

TALLING, J. F. and D. DRIVER (1961). Some problems in the estimation of chlorophyll *a* in phytoplankton. 10th Pac. Sci. Congress, Honolulu, pp. 142-146.

YACOBI, Y. Z., W. ECKERT, H. G. TRÜPER and T. BERMAN. (1990). High Performance liquid chromatography detection of phototrophic bacterial pigments in aquatic environments. *Microbial Ecology*, 19: 127-136.