Journal of Chromatography, 369 (1986) 125–131
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 999

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF DEUTERATED PHOTOSYNTHETIC PIGMENTS FROM THEIR PROTIO ANALOGUES

R. BAWEJA*
School of Pharmacy, University of Wyoming, Laramie, WY 82071 (U.S.A.)
(First received May 13th, 1986; revised manuscript received August 6th, 1986)

SUMMARY

A high-performance liquid chromatographic method with a visible method of detection is described for the facile baseline separation of deuterated chlorophylls from their protio analogues. A quaternary solvent system comprising of water–organic phase and an octadecyl-silica (C_{18}) column were used. In every instance the deuterio compound eluted ahead of its protio analogue indicating that Van der Waals forces are operational during the separation process. Specificity, sensitivity, and reproducibility make these methods particularly suitable in plant chemistry for semi-preparative purification processes and methodologies.

INTRODUCTION

Many experiments in photosynthesis require pure chlorophylls. The role of chromatography in the studies of chlorophylls is apparent from many investigations[1]. Studies of the leaf pigments lead not only to Tswett's perfection of column chromatographic procedures but also to the discovery of additional chlorophylls. Most active adsorbents like alumina, magnesia, and charcoal alter the chlorophylls so that they cannot be eluted and recovered. Polysaccharides such as starch, and cellulose have been used extensively since they do not alter the chlorophylls and separation of the chlorophylls from one another is achieved. A recent investigation showed Sepharose CL-6B to be the best adsorbent for the separation of chlorophylls amongst four adsorbents studied[2]. Still the most frequently used separation method for extracts of chloroplast pigments is adsorption chromatography on sugar–starch mixtures as the stationary phase[3]. In the last few years high-performance liquid chromatography (HPLC) has served as the most popular method for the separation of chloroplast pigments[4–15].

A clear understanding of the biological effects of deuterium can be obtained by studying the effect of isotopic substitution at the molecular level[16]. Interest in

* Work carried out at: Chemistry Division, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, U.S.A.

0021-9673/86/$03.50

conducting studies related to the biological effects of deuterium started early. In 1959 it was demonstrated that it was possible to grow organisms under fully deuterated conditions[17]. In other words prior to this period it was believed that the replacement of hydrogen by deuterium was incompatible with life. Not so widely recognized is that many fully deuterated compounds of biological importance, whose syntheses are quite beyond the resources of modern synthetic organic chemistry, can be readily obtained by biosynthesis. Many species of microorganisms have been cultivated in fully deuterated form, and some species of higher plants and mammals have been partially deuterated. The first successful cultivation of a deuterated living system was accomplished in 1960 when two species of green algae, *Chlorella vulgaris* and *Scenedesmus obliquus* were grown in media containing 99.7% heavy water[18]. The chloroplast pigments contained deuterium instead of hydrogen. Many other species of algae have been grown since that time. As facilities now exist, many deuterated compounds of great biological interest are now available for chemical and biological investigation[19–21]. The effect of deuterium substitution on the chromatographic behaviour of numerous compounds (carbohydrates, steroids) as well as deuterium isotope effects is accounted for in a review[22]. Fully deuterated pigment were not separated from ordinary hydrogen containing pigments on columns of powdered sugar.

Interest in the fractionation of compounds containing tracer ^{2}H, ^{3}H, and ^{14}C soon led to the discovery that complete resolution of mixtures of ^{1}H and ^{2}H compounds could be achieved by gas–liquid chromatography. The first total resolution of a fully deuterated compound from its ^{1}H analogue was demonstrated for ^{1}H and ^{2}H labeled β-carotene by open column liquid chromatography on activated magnesia[23]. Subsequently, a number of ^{1}H and ^{2}H labeled aromatic hydrocarbons and fatty acids were separated by reversed-phase (RP) HPLC[24]. This study extends to the development of the separation of ^{1}H and ^{2}H chlorophylls, and related compounds. There is clear indication that RP-HPLC may become a general procedure for the separation of mixtures of photosynthetic compounds of differing hydrogen isotopic composition.

EXPERIMENTAL

Materials

Samples of protio and fully and partially deuterio chlorophyll *a*, chlorophyll *b*, bacteriochlorophyll *a* (from cultures of *Rhodospirillum rubrum* and *Rhodospirillum spheroides*) and pyrochlorophyll *a* were obtained according to procedures reported earlier[1].

Various solvents were used in the chlorophyll studies; all solvents were distilled in glass (HPLC grade) and were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetonitrile, tetrahydrofuran, methanol, and acetone were employed in experiments with chlorophylls. Water was incorporated in some separation systems and was prepared by a commercial deionization system. All samples and solvents were used without further purification.

In all chlorophyll studies, 1 mg of the sample was accurately weighed and dissolved in 2 ml of HPLC grade tetrahydrofuran. This was equivalent to a concentration of approximately 0.5 mg/ml. All samples were vortexed and were syringe filtered using a Waters organic sample clarification kit (No. 26870). A volume of 90

μl of a solution of one isotopic form was mixed with 100 μl of the solution of the other isotopic form. Mixing unequal volumes of isotopic forms further facilitated the identification of peaks in the chromatogram. A volume of 2 μl of the sample mixture representing approximately 500 ng of each isotopic form was injected into the chromatograph. Chlorophyll *a* studies were monitored at 663 nm, chlorophyll *b* at 642 nm, pyrochlorophyll *a* at 663 nm, and bacteriochlorophyll *a* at 773 nm. Chart speed was kept at 1 cm/min and the flow-rate was set at 1 ml/min.

Chromatography conditions

All of the RP-HPLC separations were carried out on a Beckman Model 332 liquid chromatograph system equipped with two Model 110A metering pumps under the control of a No. 421 microprocessor. A Hitachi UV–VIS variable-wavelength spectrophotometer (Model 155-40) was used as the detector. Samples were introduced by a Model 210 syringe loading injector. Ultrasphere C_{18} ODS (Beckman), 5 μm, 250 mm × 4.6 mm columns were used. A 0.5-μm pre-filter was always inserted before the column and careful attention was directed to the removal of particulate matter from the samples and solvents.

Mobile phases consisted of mixtures of HPLC-grade acetonitrile, methanol, tetrahydrofuran, and water. Experiments were conducted to optimize the eluent composition.

RESULTS

The deuterio and the protio analogues of chlorophyll (chl) *a* were baseline separated in 22 min (Fig. 1). Two very small peaks for the stereoisomers were seen at 18.2 min ($k' = 5.7$) for deuterated chl *a'* and at 20.6 min ($k' = 6.6$) for the protio analogue. The isotopic forms of chlorophyll *b* were resolved in 16 min (Table I) with the deuterio compound eluting in 12.1 min ($k' = 3.2$) followed by the protio analogue in 13.3 min ($k' = 3.7$). Bacteriochlorophyll (Bchl) *a* and pyrochlorophyll *a* studies were also conducted with the mixture but with slightly different composition for A:B. In each instance baseline separation was accomplished; retention times were noted and the capacity factors calculated.

Partially deuterated chlorophylls were obtained from green algae grown in media containing 50% ^2H_2O, 80% ^2H_2O, and 90% ^2H_2O. Stated another way, deuterated chlorophyll compounds were isolated from organisms grown in media con-

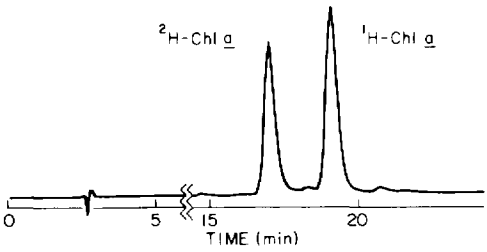

Fig. 1. Reversed-phase separation of deuterated chlorophyll *a* and protio chlorophyll *a* on a 25 cm × 4.6 mm I.D. C_{18} Ultrasphere ODS column. The mobile phase consited of water–methanol–acetonitrile–tetrahydrofuran (5:28:38:23, v/v). Flow-rate was 1 ml/min and detection set at 663 nm.

TABLE I

RETENTION TIME AND CAPACITY FACTOR CHARACTERISTICS FOR VARIOUS DEUTERATED CHLOROPHYLLS AND THEIR PROTIO ANALOGUES

Compound	*Mobile phase (A:B)**	*Retention time (min)*		*Capacity factor (k')*	
		Deuterio	*Protio*	*Deuterio*	*Protio*
Chlorophyll *a*	5:95	17.0	19.0	5.3	6.0
Chlorophyll *b*	5:95	12.1	13.3	3.2	3.7
Bacteriochlorophyll *a* (geranylgeranyl chain)	10:90	16.6	18.6	4.7	5.4
Bacteriochlorophyll *a* (phytyl chain)	7:93	17.4	19.2	5.1	5.7
Pyrochlorophyll *a*	0:100	8.2	8.9	1.8	2.1

* A = Water; B = [methanol–acetonitrile–tetrahydrofuran (30:40.5:24.5, v/v)].

taining different amounts of ^2H_2O. (As there is discrimination against ^2H in biosynthetic processes, the resultant chlorophylls contain somewhat less ^2H than the $^2H/^1H$ ratio that would be obtained in the absence of isotopic fractionation effects.) All of the partially deuterated chlorophylls were run individually against ^1H-chl *a* and in each case baseline separation was achieved. The system developed for chl *a* was employed in separating ^1H-chl *a* from 90% ^2H-chl *a* (Fig. 2). Baseline separation was accomplished in 24 min with retention times as follows: 90% ^2H-chl *a*, 19 min ($k' = 5.6$) and chl *a*, 21 min ($k' = 7.4$). Similarly, 80% ^2H-chl *a* eluted in 19.6 min and 50% ^2H-chl *a* had a t_R of 20.4 min when paired separately with chl *a* ($t_R = 21.4$ min).

A mixture of ^1H-chl *a*, 50% ^2H-chl *a* and ^2H-chl *a* was resolved along with their respective stereoisomers (Fig. 3). For a separate mixture consisting of 100% ^2H-chl *a*, 90% ^2H-chl *a*, 80% ^2H chl *a*, 50% ^2H-chl *a* and ^1H-chl *a* the solvent system provided a separation for three peaks, *viz.*, one for ^1H-chl *a*, the other for 50% ^2H-chl *a*, and the third was an asymmetric peak composed of 100% ^2H-chl *a*, 90% ^2H-chl *a* and 80% ^2H-chl *a*. Baseline separation was not accomplished. The asymmetric peak eluted first with a retention time of 20.8 min ($k' = 6.3$), followed by

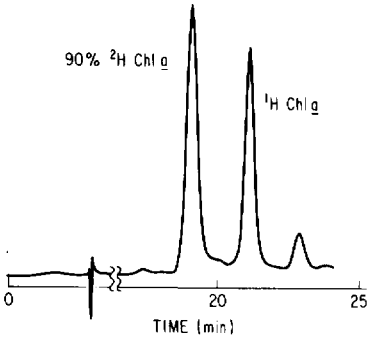

Fig. 2. Separation of 90% deuterated chlorophyll *a* from protio chlorophyll *a*. Experimental conditions were as in Fig. 1.

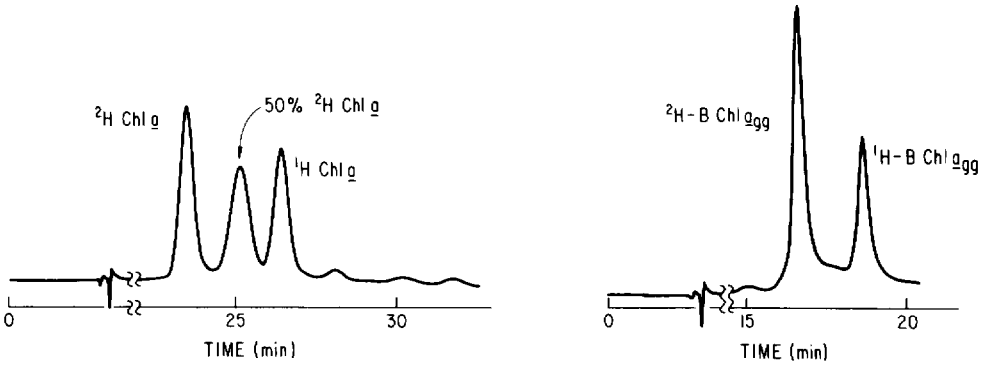

Fig. 3. Baseline separation of deuterated chlorophyll *a*, 50% deuterated chlorophyll *a* and protio chlorophyll *a* using a reversed-phase C_{18} column. Experimental conditions were as in Fig. 1.

Fig. 4. Reversed-phase HPLC separation of deuterio bacteriochlorophyll *a* (geranylgeranyl chain) from its protio analogue. The mobile phase consisted of water–methanol–acetonitrile–tetrahydrofuran (10:27:36:21, v/v). Flow-rate was 1 ml/min and detection set at 773 nm.

50% ^{2}H-chl *a* (t_R = 22.1 min, k' = 6.7), and finally by ^{1}H-chl *a* (t_R = 23.2 minutes, k' = 7.1). A solvent system water–organic (10:90) separated the three peaks at baseline level but the run was extended (approximately 100 min). Again the assymmetric peak failed to resolve further.

Fig. 4 shows the separation of ^{2}H-Bchl $a_{\text{geranylgeranyl (gg)}}$ from ^{1}H-Bchl a_{gg}. A similar separation was possible for the isotopic forms of Bchl a_{phytyl} (Table I). It was also possible to baseline separate protio Bchl a_{gg} (t_R = 7.5 min, k' = 1.6) from protio Bchl a_{phytyl} (t_R = 13.6 min, k' = 3.8) (Fig. 5). Similarly, deuterio Bchl a_{gg} when

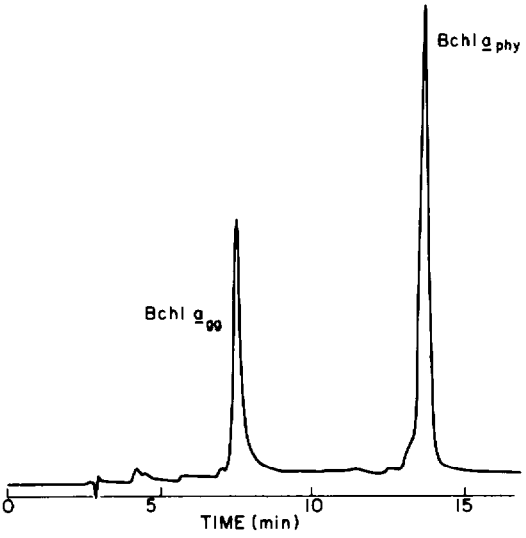

Fig. 5. Separation of protio bacteriochlorophyll *a* (geranylgeranyl chain) from protio bacteriochlorophyll *a* (phytyl chain). The geranylgeranyl chain has three double bonds more (*i.e.*, six hydrogen atoms less) than the phytyl chain.

paired and run with deuterio Bchl a_{phytyl} had a retention time of 7 min ($k' = 1.5$), and the latter eluted in 12.5 min ($k' = 3.4$). The deuterio isotopes remained on the column for a shorter period of time than their respective protio analogues. These studies were designed to observe the effects of the two kinds of tail chains present.

Chlorophylls lose the carbomethoxy group at the C-10 position when heated in pyridine solution to yield green "pyro" derivatives. The spectral properties are similar to those of the parent chlorophyll, With the quaternary solvent system, deuteriopyrochlorophyll *a* emerged in 8.2 min followed by protio pyrochlorophyll *a* in 8.9 min.

DISCUSSION

In all chlorophyll studies the deuterated compound eluted ahead of its protio analogue. When examined in pairs, *i.e.*, deuterio compound versus 100% ^{1}H-chl *a*, baseline separation was observed. There is evidence on the basis of t_R values that the greater the percentage of deuterium in the compound, the faster it will move through the column. In other words the elution pattern is related to the percent ^{2}H character. Fully deuterated chl *a*, partially deuterated chl *a*'s (90% ^{2}H-chl *a*, 80% ^{2}H-chl *a* and 50% ^{2}H-chl *a*) and ordinary chl *a* were mixed and run together. It was observed that it was not possible to separate 100% ^{2}H-chl *a*, 90% ^{2}H-chl *a* and 80% ^{2}H-chl *a* from one another in the mixture. Fifty percent deuterated chl *a* and 100% ^{1}H-chl *a* did separate as individual peaks from the mixture. In another study, 100% ^{2}H-chl *a* and 100% ^{1}H-chl *a* were mixed and chromatographed together. In this instance a clear baseline separation was observed for the compounds. Thus, while it was not possible to achieve baseline separation in a mixture of all five components, a good baseline separation in a mixture of 100% ^{2}H-chl *a*, 50% ^{2}H-chl *a* and 100% ^{1}H-chl *a* can be obtained.

Bacteriochlorophyll *a* obtained from the culture *Rhodospirillum rubrum* possesses a geranylgeranyl$_{(gg)}$ tail chain whereas Bchl *a* from the organism *Rhodospirillum spheroides* has the phytyl tail chain. The geranylgeranyl chain has three double bonds more than the phytyl chain, *i.e.*, six hydrogen atoms less. In a separation study involving Bchl a_{gg} and Bchl a_{phytyl}, the former eluted first. This should suggest then that not only does the tail chain participate in the chromatographic process, but that at the atomic level, the number of hydrogens present also influences Van der Waals attractive forces between the solute and the adsorbent. Therefore, Bchl *a* from *R. spheroides*, which possesses the phytyl chain, adhered strongly compared to Bchl *a* obtained from *R. rubrum* which has the geranylgeranyl chain.

At the outset it appears that such chromatographic behaviour is independent of the size of the molecule. It has been mentioned that the entire molecule need not be considered when dealing with isotope effects[22]. The major contribution for such effects is essentially from isotopically participating bonds and not from vicinal bonds. In other words, the chromatographic behaviour at the fundamental level is the interaction between the C–H bond (for the protio compound) or the C–^{2}H bond (for the deuterated compound), and the stationary phase. The elution of the ^{2}H-isotopic form before its protio analogue suggests then that Van der Waals forces are operational. A C–H bond has a higher oscillation frequency (3300 cm^{-1}) than the C–^{2}H bond (2333.8 cm^{-1}). With the oscillation frequency an electromagnetic field is created

and the electrons in the C–H bond are subject to this field. In turn, this electromagnetic field for a C–H bond creates a large induced field of opposite charge in other molecules around it. The C–H bond induces greater forces of attraction between itself and the stationary phase. The C–^{2}H bond, on the other hand, has a lower oscillation frequency, a less electromagnetic field is created, and the forces of attraction that develop between the C–^{2}H bond and the stationary phase are weaker. Therefore, the C–^{2}H bond with a lower Van der Waals force of attraction elutes ahead of its protio analogue.

RP-HPLC has been shown to be a versatile tool for separating isotopically analogous compounds. In this study the compounds were identical in all respects except the C–H and the C–^{2}H bond which differ in frequency of vibration. It appears that the larger the aliphatic C–H component in the compound, the easier will separation be effected. With the increased popularity of HPLC and the availability of more efficient columns isolation, purification, and quantification studies can now be performed. Thus, RP-HPLC has the very strong potential of becoming the standard analytical method for the separation of mixtures of photosynthetic compounds of differing hydrogen isotopic composition.

ACKNOWLEDGEMENTS

The author wishes to thank Drs. M. I. Blake (University of Illinois) and J. J. Katz (Argonne National Laboratory) for their assistance. The author is also grateful to Argonne National Laboratory for the fellowship. Work performed under the auspices of the Office of Basic Energic Sciences, U.S. Department of Energy.

REFERENCES

1 H. H. Strain and W. A. Svec, *The Chlorophylls*, Academic Press, New York, 1966, p. 21.
2 K. Iriyama and M. Yoshiura, *J. Liq. Chromatogr.*, 5 (1982) 2211.
3 H. H. Strain and W. A. Svec, *Advances in Chromatography*, Marcel Dekker, New York, 1969, p. 119.
4 N. Evans, D. E. Games, A. H. Jackson and S. A. Matlin, *J. Chromatogr.*, 115 (1975) 325.
5 W. T. Shoaf, *J. Chromatogr.*, 152 (1978) 247.
6 K. Eskins, C. R. Scholfield and H. J. Dutton, *J. Chromatogr.*, 135 (1977) 217.
7 K. Iriyama, M. Yoshiura and M. Shiraki, *J. Chromatogr.*, 154 (1978) 302.
8 T. Brauman and L. H. Grimme, *Biochim. Biophys. Acta*, 637 (1981) 8.
9 K. Eskins and L. Harris, *Photochem. Photobiol.*, 33 (1981) 131.
10 Y. Nakatani, G. Ourisson and J. P. Beck, *Chem. Pharm. Bull.*, 29 (1981) 533.
11 S. Burke and S. Aronoff, *Anal. Biochem.*, 114 (1981) 367.
12 B. Scholz and K. Ballschmitter, *J. Chromatogr.*, 252 (1982) 269.
13 L. Goeyens, E. Post, F. Dehairs, A. Vandenhoudt and W. Bayens, *Int. J. Environ. Anal. Chem.*, 12 (1982) 51.
14 Y. Shioi, R. Fukae and T. Sasa, *Biochim. Biophys. Acta*, 722 (1983) 72.
15 Y. Shioi, M. Doi and T. Sasa, *J. Chromatogr.*, 298 (1984) 141.
16 J. J. Katz, *Am. Sci.*, 48 (1960) 544.
17 H. L. Crespi, S. M. Archer and J. J. Katz, *Nature (London)*, 184 (1959) 729.
18 W. Chorney, N. J. Scully, H. L. Crespi and J. J. Katz, *Biochim. Biophys. Acta*, 37 (1960) 280.
19 R. G. Taecker, H. L. Crespi, H. F. Da Boll and J. J. Katz, *Biotech. Bioeng.*, 13 (1971) 779.
20 E. R. Klein and P. D. Klein, *Biomed. Mass Spectrom.*, 5 (1978) 91.
21 D. L. Hachey, K. Nakamura, M. Kreek and P. D. Klein, in H.-L. Schmidt, H. Förster and K. Heinzinger (Editors), *Stable Isotopes*, Elsevier, Amsterdam, Oxford, New York, 1982, p. 235.
22 P. D. Klein, *Advances in Chromatography*, Marcel Dekker, New York, 1966, p. 3.
23 H. H. Strain, M. R. Thomas, H. L. Crespi and J. J. Katz, *Biochim, Biophys. Acta*, 52 (1961) 517.
24 N. Tanaka and E.R. Thornton, *J. Am. Chem. Soc.*, 98 (1976) 1617.