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An efficient method of preparation of pheophytin *a*—Divalent metal pheophytinates

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Abstract. An efficient and rapid method of preparation of pheophytin *a* from leaves using silica gel as column material is described. The different divalent metallo, Co, Ni, Cu and Zn derivatives of pheophytin have been prepared and characterised. The optical absorption and emission, and ^{1}H NMR spectral data of pheophytin *a* and its metal derivatives are presented.

Keywords. Pheophytin *a*; metal pheophytinates; ^{1}H NMR spectra.

1. Introduction

Chlorophyll *a* (Chl *a*) and its metal-free derivative pheophytin *a* (Phe *a*) have been recognized as the electron donor-acceptor system in the primary photosynthetic process. In the development of artificial photosynthetic devices employing different metal derivatives of the cell-free pigments to investigate their electron donor-acceptor abilities and their relative stabilities, it became necessary to isolate spectrally pure Phe *a*. A variety of procedures are available in literature for the extraction, isolation and purification of the plant pigments from chloroplast (Omata and Murata 1980; Svec 1978; Hynninen 1977; Hynninen and Assandri 1973; Strain and Sherma 1972; Strain and Svec 1966, 1969). A necessity for several methods arose since a large number of pigments are intimately associated with chlorophyll (Chl) in the chloroplast. Moreover, it has been observed that during the extraction and separation procedures using different solvents, column materials and employing a variety of experimental conditions lead to alteration products of the *in vivo* pigment molecules which are either isomerized, demetallated or oxidized. A few such identified products are demetallated Chl (Phe) (besides the Phe which is intrinsically present) chlorophyllide, pyrochlorophyll, isomer of Chl (Chl') (figure 1), oxidized components of auxillary pigments carotenoids and xanthophylls.

It is customary to employ mild adsorbents such as sugar, cellulose, sepharose and starch to reduce the number of alteration products during chromatographic procedures. Usage of strong adsorbents like silica gel, alumina, kieselguhr are known to cause alteration of the *in vivo* pigments (Sherma 1971; Strain and Svec 1969; Bacon and Holden 1967; Strain *et al* 1967). The difficulties encountered in the prescribed procedures are that they are often laborious and time consuming calling for repeated chromatographic runs resulting in the larger extent of formation of altered products.

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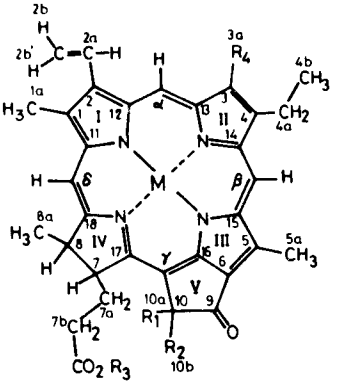

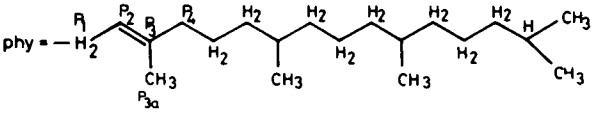

1. R_1= H , R_2= $COOCH_3$, R_3= Phy , R_4 = CH_3 (a) M = Mg : Chlorophyll a
   (b) M = H_2 : Pheophytin a
2. R_1 = $COOCH_3$, R_2 = H , R_3= Phy, R_4=CH_3 (a) M =Mg : Chlorophyll a'
   (b) M = H_2 : Pheophytin a'
3. R_1 = H , R_2= $COOCH_3$, R_3= H , R_4 = CH_3 (a) M = Mg : Chlorophyllide a
   (b) M = H_2 Pheophorbide a
4. R_1 = H , R_2= H , R_3 = Phy , R_4 = CH_3 (a) M = Mg . Pyrochlorophyll a
   (b) M = H_2 : Pyropheophytin a
5. R_1 = H , R_2 = $COOCH_3$, R_3= Phy, R_4=CHO (a) M = Mg : Chlorophyll b
   (b) M = H_2 : Pheophytin b

Figure 1. Structure of chlorophyll *a* and its major altered products.

Thin layer chromatography on cellulose plate is recommended; however it is a microtechnique. Separation on a column of sepharose is a good method but is costly for the preparation of pigments in large quantities. Despite a great deal of experimentation in the direction of extraction, separation and purification of Chl, no one method combines simplicity, wide applicability and high sensitivity. Further, many efforts that have been made are towards the isolation of pure chlorophylls. Our interest lies in the preparation of metalloderivatives which requires the isolation of Phe *a* in fairly large quantities (> 250 mg).

Here we report an efficient method for the separation of Phe *a* directly from the plant material using silica gel as the column material. This method avoids the preparation of Chl *a* and its subsequent acidification to yield Phe *a* which is time consuming and leads to possible photobleaching. The metallo derivatives prepared from the purified Phe *a* are characterized by visible absorption, emission and ^{1}H NMR spectral studies.

2. Experimental

2.1 *Materials*

Leaves of *Amaranthus caudatus*, a plant that is richly grown around all seasons was employed for the extraction of plant pigments. Silica gel-G was procured from Achme Synthetic Chemicals (Bombay). GR grade methylene chloride (BDH, India), commercial acetone and benzene were purified according to standard procedures (Vogel 1968). The acetates of Co(II), Cu(II) and Zn(II) were employed to prepare their respective pheophytinates. 2,4-pentananedionato nickel(II) was prepared according to known procedures (Fackler 1966) and it was used to prepare Ni(II) pheophytinate. All other reagents were of GR grade.

2.2 *Methods*

Fresh green leaves devoid of mid ribs (500 g), were cut into small pieces and treated with 3 l of boiling water for 2 min to remove the water soluble pigments and oily material. This treatment also helps to coagulate the proteins present in the leaves. The leaves were then dried using a towel. The dried leaves (300 g) were then suspended in 4 l of acetone and stirred with a mechanical stirrer at full speed for 5 min to hasten the extraction of the pigments into the solvent. The suspension was allowed to settle for 5 min. The dark green supernatant liquid was filtered through a plug of cotton to remove the suspended leafy materials. A second extraction was carried out with 1 l of acetone to completely remove the greenness of the leaves. The solvent was removed from the extract batch-wise (employing not more than 500 ml of extract) by distillation under reduced pressure at 40–50°C. After the removal of acetone, the pigments were transferred to 20 ml of diethyl ether. A total of 200 ml of ether extract was collected in this fashion. Sodium chloride solution (100 ml of 20% aqueous) was employed to enhance the transfer of pigments to ether layer. The ether extract was shaken well with 20 ml of 1 N hydrochloric acid in a separating funnel for 1 min and the blue acid layer was removed immediately. The brown ether layer was washed repeatedly with 200 ml water each time to remove the traces of acid. Emulsion formation at this stage was overcome with the addition of 200 ml of 20% aqueous sodium chloride solution. The ether extract containing crude Phe was dried over anhydrous sodium sulphate. Ether was evaporated under reduced pressure. Traces of water remaining in the brown coloured material sticking to the glass were removed by repeated codistillation with dry carbontetrachloride under reduced pressure. The crude Phe (2 g) thus obtained was used for chromatographic separation.

The Phe (2 g) was dissolved in minimum amount of benzene (10–15 ml) and loaded on to a column of silica gel packed in benzene (height of the column = 42 cm, diameter = 4·5 cm, height of silica gel packed = 30 cm). Initially the flow rate of benzene was maintained at 1 ml/min till the brown extract was completely adsorbed on the silica bed and the eluent on the top of the column bed was colourless. Increasing the flow rate to 4 ml/min results in the fast separation of yellow carotenoids while the brown coloured band of Phe moves slowly. Polarity of the eluent was gradually increased by adding different amounts of acetone/alcohols (the proportion of the solvents is shown in figure 2) and the pigment was eluted in increasing order of their adsorbability. The visible absorption spectrum was recorded for each 2–4 ml of the eluent fraction. The Phe *a* fraction was rechromatographed twice to obtain pure Phe *a*. The Phe *a* can

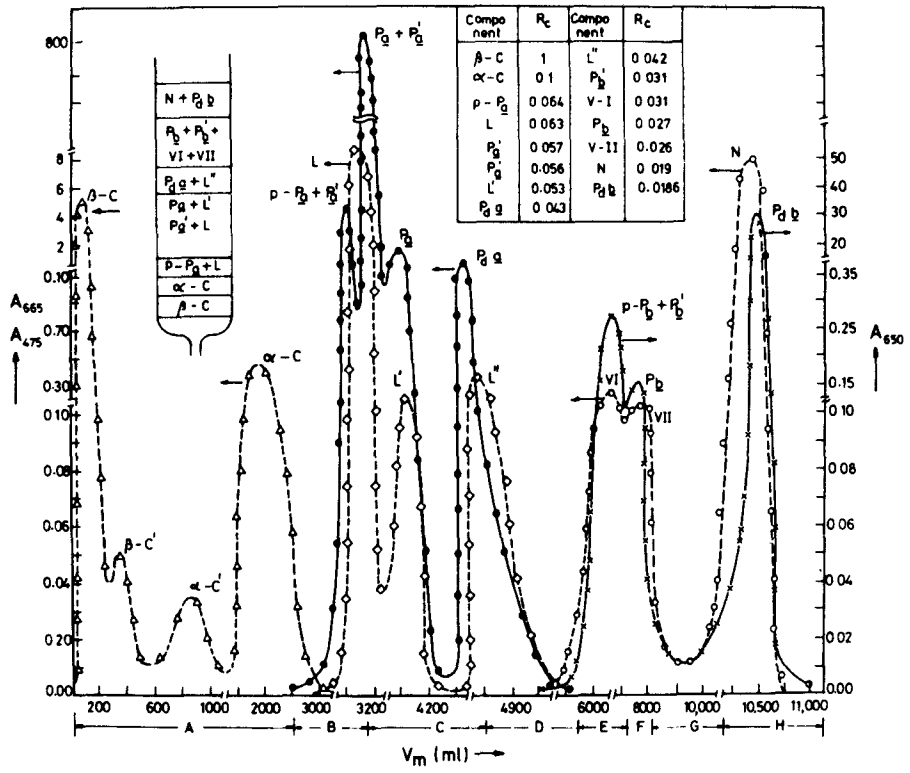

Figure 2. Separation of acidified leaf extract on a silica gel column. V_m = eluent volume. The different solvents used are marked as A = benzene; B = 1% acetone in benzene; C = 2% acetone in benzene; D = 2% *n*-propanol in benzene; E = 4% *n*-propanol in benzene; F = 10% *n*-propanol in benzene; G = 25% ethanol in benzene and H = methanol. R_c value is defined in the text. The upper left hand part of the figure shows the separation of various pigments and the R_c values are given in the upper right hand part. β-C = β-carotene; α-C = α-carotene; p-p*a* = pyropheophytin *a*; P*a*′ = pheophytin *a*′; P*a* = Pheophytin *a*; L = Lutein; Pd*a* = pheophorbide *a*; p-P*b* = pyropheophytin *b*; P*b*′ = pheophytin *b*′; P*b* = pheophytin *b*; VI = violoxanthin fraction I; VII = violoxanthin fraction II; Pd*b* = pheophorbide *b*; N = neoxanthin. β-C′ and α-C′ are altered products.

conveniently be stored after the removal of the solvent under N_2 in a sealed tube. The material should be protected from light and kept in a refrigerator.

2.3 *Preparation of divalent metallo (Co, Ni, Zn and Cu) pheophytinates*

A solution of 50 mg of Phe *a* in 100 ml of chloroform was refluxed with excess of metal acetate/acetylacetonate (200 mg) dissolved in acetic acid for about 30 min under N_2. The bluish-green solution was cooled and washed repeatedly with water and then dried over anhydrous sodium sulphate. The solution was concentrated to 5 ml and loaded on to a silica gel column packed in benzene and eluted with benzene containing 0–6% of acetone. The brown material was left behind in the column and the metallopheophytin coloured blue was collected. This solution was again chromatographed to obtain pure metallopheophytinates. The metal derivatives were stored in sealed tubes. The entire

operation of extraction and separation of the pigments was carried out under dim light and in an atmosphere of N_2.

The optical absorption spectra of Phe *a* and its divalent metal derivatives were recorded using CH_2Cl_2 as solvent on a Beckman model 25 spectrometer. Fluorescence spectra of the solutions were recorded on a Perkin-Elmer MPF-44A spectrofluorimeter. The concentration of solutions employed for optical absorption and emission measurements was 10^{-5} M and 10^{-6} M respectively. The ^{1}H NMR spectra of Phe *a* and its divalent metal derivatives in $CDCl_3$ (0·05 M) were recorded on a Brucker WH 270 MHz FT NMR spectrometer using TMS as an internal standard.

3. Results

The different pigments in the eluent were identified by making use of their characteristic absorption spectra and their R_F values. The maxima at 475 nm were used to represent the carotenes, xanthophylls and lutein (Nelson and Livingston 1967). The isomeric forms of these pigments, however, have not been collected individually in the eluent. The presence of Phe *a* and Phe *b* in the eluent was monitored at the absorption maxima 665 and 650 nm respectively. Owing to the closeness of these absorptions, the spectra of the eluents containing these components often appear as a band with an accompanying shoulder. It was found necessary to dilute the eluent to an appropriate volume for the absorbance measurements. The absorbance values corrected for the

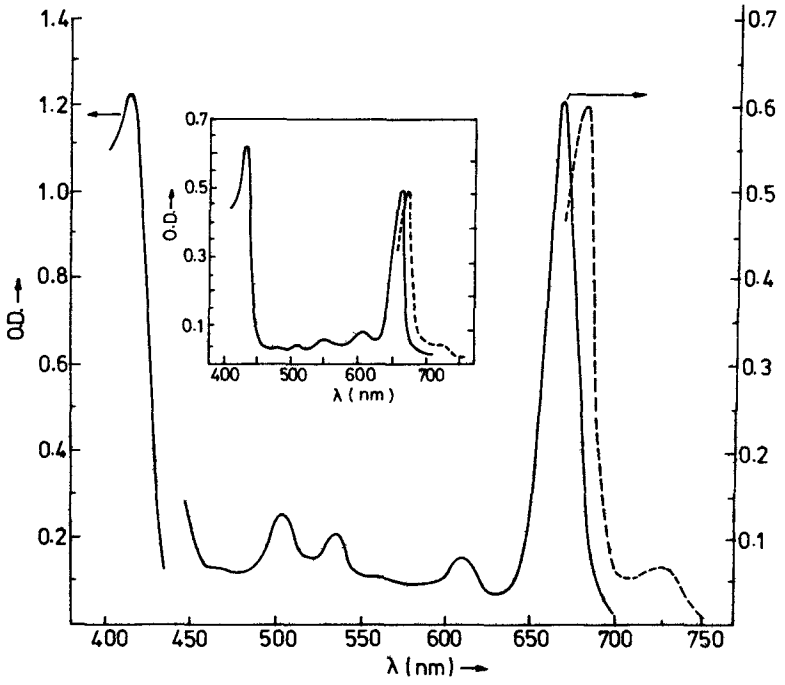

Figure 3. Absorption (——) and fluorescence (– – –) spectrum of Phe *a* in CH_2Cl_2 at 25°C. Excitation at 665 nm Inset; absorption (——) and fluorescence (– – –) spectrum of ZnPhe *a* in CH_2Cl_2 at 25°C. Excitation at 654 nm. Fluorescence intensities are in arbitrary units.

Table 1. Visible absorption spectral data[a] of pheophytin *a* and its divalent metal derivatives in methylene chloride at 25°C.

Compound	λ_{max} nm (red)	λ_{max} nm (blue)	$\frac{I_{max}\text{ (blue)}}{I_{max}\text{ (red)}}$	I_{max} (red)
Phe *a*	665	412	2·05	56,666
	(667)	(409)	(2·09)	(61,000)
Zn Phe *a*	654	421·5	1·32	45,424
	(658)	(426)	1·30	
Cu Phe *a*	650·5	421·5	1·29	40,196
	(654)	(425)	(1·18)	
Ni Phe *a*	649	421·5	1·34	33,856
	(652)	(423)	0·95	
Co Phe *a*	650·5	422	1·36	37,903

[a] The values in parenthesis are those reported in literature: Pennington *et al* (1964); Boucher and Katz (1967).

dilution are shown in figure 2. The relative order of elution of the pigments with increasing polarity of the eluent, is the same as that observed in the sugar column (Pennington *et al* 1964). The R_c values of different pigments were calculated as the ratio of the eluent volumes with respect to the first eluted component β-carotene. The results of the absorption spectral measurements at different volumes of the eluent and the R_c values of the various components are depicted in figure 2. The volume of the eluent just before the elution of the first component β-carotene corresponds to zero in figure 2. The appearance of shoulders in the elution curve (figure 2) signifies the trailing of major zones with increasing polarity of the solvent. Also, minor zones like pyropheophorbides *a*/*b* and pyropheophytin *b* are not seen clearly due to overloading of the column as well as due to the sudden increase in polarity of the eluent before their elution. Owing to the closeness of the R_c values and similar absorption spectral features, the isomers *a* and *a'* of Phe were collected in the same eluent volume.

The visible absorption spectrum of Phe *a* prepared in this fashion is given in figure 3 along with its emission spectrum. The inset in figure 3 shows the absorption and emission spectrum of Zn(II) Phe *a*. The absorption spectral data of Phe *a* and its divalent metal derivatives are given in table 1. The ratio of intensities of the blue to red band has often been used to check the purity of the products. The present values agree with those reported in literature (Boucher and Katz 1967; Pennington *et al* 1964). Excitation of Phe *a* and Zn Phe *a* at their red absorption maxima leads to emissions at 676 nm followed by a shoulder at 726 nm and 665 nm with a shoulder at 713 nm respectively. The ^{1}H NMR spectrum of Phe *a* in $CDCl_3$ is shown in figure 4. The appearance of well-resolved spectrum indicates the purity of the sample and the assignments of the proton resonances are analogous to the reported values (Katz 1973). Presence of *a'* in the sample is clearly seen from the satellite peaks near the meso proton resonances. It has not been possible to estimate the relative amount of *a'* component in the purified sample. The vinyl proton resonances appear as an ABX multiplet with 2*a* proton resonating at 7·98 δ while 2*b* and 2*b'* proton resonances occur at 6·23 δ and 6·27 δ respectively ($J_{AB} \approx 2H_z$, $J_{AX} \approx 18\ H_z$, $J_{BX} \approx 12\ H_z$). The 5*a*, 3*a* and 1*a* proton resonances are clearly seen at 3·69, 3·23 and 3·34 δ respectively. The 10*a* and 10*b* proton resonances are observed at 6·26 δ and 3·88 δ respectively. Exhibition of these resonances

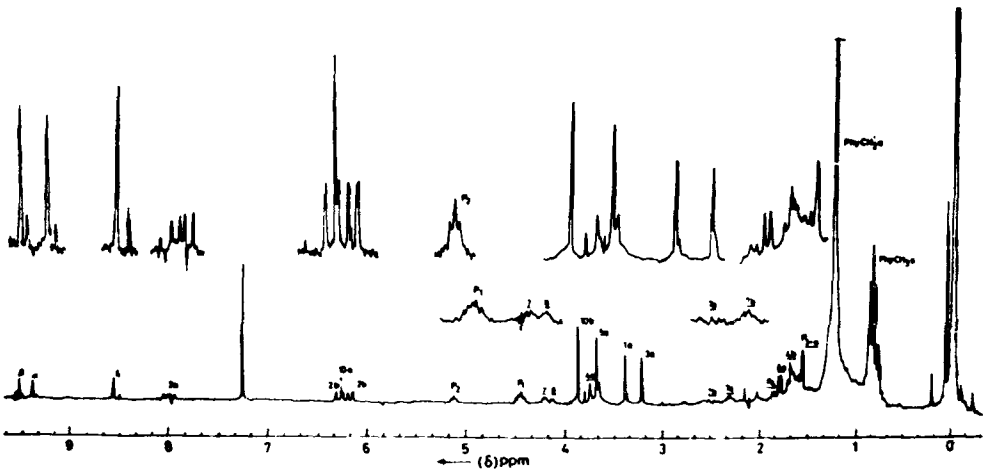

Figure 4. 270 MHz ^{1}H NMR spectrum of pheophytin *a*. The numbering is the same as in figure 1. Numbers underlined indicate tentative assignments. The expanded spectrum is designated above the corresponding proton resonances.

is a clear indication that the Phe *a* sample prepared by the method described is not an oxidized product.

Moreover the appearance phythyl resonances, P_1, P_2, P_3, P_4, P-CH_2 and P-CH_3 occurring at 4·45, 5·14, 1·57, 1·86, 1·25 δ and 0·89 to 0·77 δ respectively provides additional check on the purity of the Phe *a*.

4. Discussion

It is known that carotenoids and xanthophylls undergo isomerization and oxidation when silica gel is used as the adsorbent (Strain *et al* 1967). Thus the elution curve (figure 2) represents the altered products of the pigments besides the major pigments. It is worthy to note that the usage of pure benzene as the eluent separates the major portion of carotenes. The order of sorbability of the different pigments in silica gel matrix is found to be β-carotene < α-carotene < pyropheophytin *a* $\leqslant$ pheophytin *a'* $\leqslant$ lutein $\leqslant$ pheophytin *a* $\leqslant$ lutein' < pheophorbide *a* $\leqslant$ lutein'' < pheophytin *b'* + *b* $\leqslant$ violoxanxthin I & II < neoxanthin < pheophorbide *b*. L' is regarded to be an altered product of lutein. It is not known with certainty whether L'' is the altered product of lutein or the altered product of violoxanthin. The strongly adsorbed pigments neoxanthin, pheophorbide *b* and other oxidized products of Phe are eluted using pure methanol. Owing to the closeness of the band separation of these pigments, the eluent volumes containing a given major pigment are always contaminated with the preceding component. However, repeated chromatography of the eluent fraction essentially yields the desired pigment.

The misgivings of the usage of silica gel as a column material for the separation of chlorophyll *a* from the plant pigments have been cited in literature (Bacon 1966; Bacon and Holden 1967). These essentially point out to the formation of a variety of alteration products of Chl with Phe as one of the components. The identification of the altered products has been based on phase test and the absorption spectra. Despite the

ambiguities involved in the phase tests and similar absorption spectral features, components *viz* 'unknown' or Chl *a*, 'unknown', Chl *a*-1, Chl *a*-2 and Chl *a*-3 have been proposed after a thin layer chromatographic separation of Chl *a* on silica gel. The R_F values and the ratio of the intensities of blue to red bands in the absorption spectra of the different components observed by Bacon seem to suggest that the proposed components can possibly be pyro Chl *a*, Phe *a*, Chl *a*, Chl *a'* and chlorophyllide *a* (and/or pyrochlorophyllide *a*) respectively. The present method of separation starts with the acidified plant extract with the explicit intention of obtaining Phe *a* for the preparation of metalloderivatives. Thus, the caution expressed in literature against the usage of silica gel as an adsorbent is of little significance in the efforts described here. No attempts were made to separate the *a* and *a'* isomers of Phe. The relative amounts of *a'* in a sample of *a* seem to depend on the nature of solvent and temperature. The cooccurrence of *a'* component does not seem to alter the absorption and emission spectral features of Phe *a*. However, it is shown that the CD spectra can be used with advantage to distinguish the presence of *a'*.

The optical absorption and emission spectra of Phe *a* and its Zn(II) derivative (figure 3) assure the purity of Phe *a* obtained in the present study. Moreover, the appearance of resonances of 10a and 10b protons show the absence of oxidized products (*a*-1, *a*-2 and *a*-3). Interestingly the 13-carbon resonance spectra of the Phe *a* and its divalent metal derivatives show a large down field shifts of meso carbons and carbonyl carbon in the Co(II) complex relative to the spin-free Zn(II) complex. Further work is in progress to relate these carbon shifts with their ability to function as π-donors.

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References

Bacon M F 1966 *Biochem. J.* **101** 340
Bacon M F and Holden M 1967 *Phytochem.* **6** 193
Boucher L J and Katz J J 1967 *J. Am. Chem. Soc.* **39** 1703
Fackler Jr J P 1966 in *Progress in inorganic chemistry* (ed.) F A Cotton (New York: Interscience, John Wiley) Vol. 7, p. 361
Hynninen P H 1977 *Acta Chem. Scand.* **B31** 829
Hynninen P H 1981 *Z. Naturforsch.* **36b** 1000
Hynninen P H and Assandri S 1973 *Acta Chem. Scand.* **27** 1478
Katz J J 1973 in *Inorganic biochemistry* (ed.) Eichhorn (Amsterdam: Elsevier) p. 1022
Nelson J W and Livingston A L 1967 *J. Chromatogr.* **28** 465
Omata T and Murata N 1980 *Photochem. Photobiol.* **31** 183
Pennington F C, Strain H H, Svec W A and Katz J J 1964 *J. Am. Chem. Soc.* **86** 1418
Sherma J 1971 *J. Chromatogr.* **61** 202
Strain H H and Sherma J 1972 *J. Chromatogr.* **70** 87
Strain H H and Svec W A 1966 in *The chlorophylls* (ed.) L P Vernon and G R Seely (New York, London: Academic Press) p. 21
Strain H H and Svec W A 1969 *Adv. Chromatogr.* p. 119
Strain H H, Sherma J and Grandolfo M 1967 *Anal. Chem.* **39** 926
Svec W A 1978 in *The porphyrins* (ed.) D Dolphin (New York, London: Academic Press) p. 341
Vogel A 1968 *Practical organic chemistry* (London: ELBS Longmans, Greens and Co.) p. 163