

Methods for the Determination of the Chlorophylls and their Derivatives

Chi-Ming Yang^(1,2), Kuo-Wei Chang⁽¹⁾, Ming-Horng Yin⁽¹⁾ and Hsu-Men Huang⁽¹⁾

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ABSTRACT: We present a flow chart for the determination of eleven intermediates of chlorophyll biosynthesis and degradation pathways. This flow chart is organized from six literatures. We just add a step to link two unrelated procedures to determine the content of pheophytin a and b. It is convenient for the people who want to determine the content of chlorophylls and their derivatives. This flow chart makes it possible to determine the content of eleven pigments of more than 100 samples in one work day.

KEY WORDS: chlorophyll, carotenoid, biosynthesis, degradation, intermediates.

INTRODUCTION

There are many pigments in the higher plants, such as chlorophyll (Chl), carotenoid (Car), phytochrome, flavonoid, anthocyanin, tannin, and so on. Chlorophyll contains two different chemical structures, a and b, while the former contains CH₃ at the position 7 of ring B; the latter contains CHO at the same position. The major absorbance of both Chl a and b is in the blue and red visible light. All carotenoids absorb blue light. Phytochrome transforms its structure between red and far red light. Flavonoids, including anthocyanins and tannins, absorb green and ultra-violet light (Goodwin, 1988). These pigments offer an all-oriented network of interception, reflection and protection for plant body, allowing plant to use sunlight and cut off its photodamage. The biosynthesis of plant pigments are very complex processes from their precursor to final products. Plant pigments are degraded into small molecules recycling into the nature after completing their mission. More than fifteen biochemical reactions are involved in the biosynthesis of chlorophyll originated from precursor glutamate and eight steps in the degradation pathway from chlorophyll to pheophorbide (Fig. 1, Matile *et al.*, 1996, Reinbothe and Reinbothe, 1996).

Since so many intermediates are involved in the biosynthesis and degradation pathways of chlorophyll, it is difficult to determine the content of all intermediates in one experimental step. However, it could be found in the literature that some individual procedure used the same organic solvent to extract different intermediates (Lichtenthaler, 1987). In this short article, we collect methods and organize them in a flow chart linking seven experimental procedures to determine eleven intermediates of chlorophyll, including seven and four compounds in the biosynthesis and degradation pathways, respectively. Meanwhile, this flow chart can separate polar and less polar pigments.

1. Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China.

2. Corresponding author.

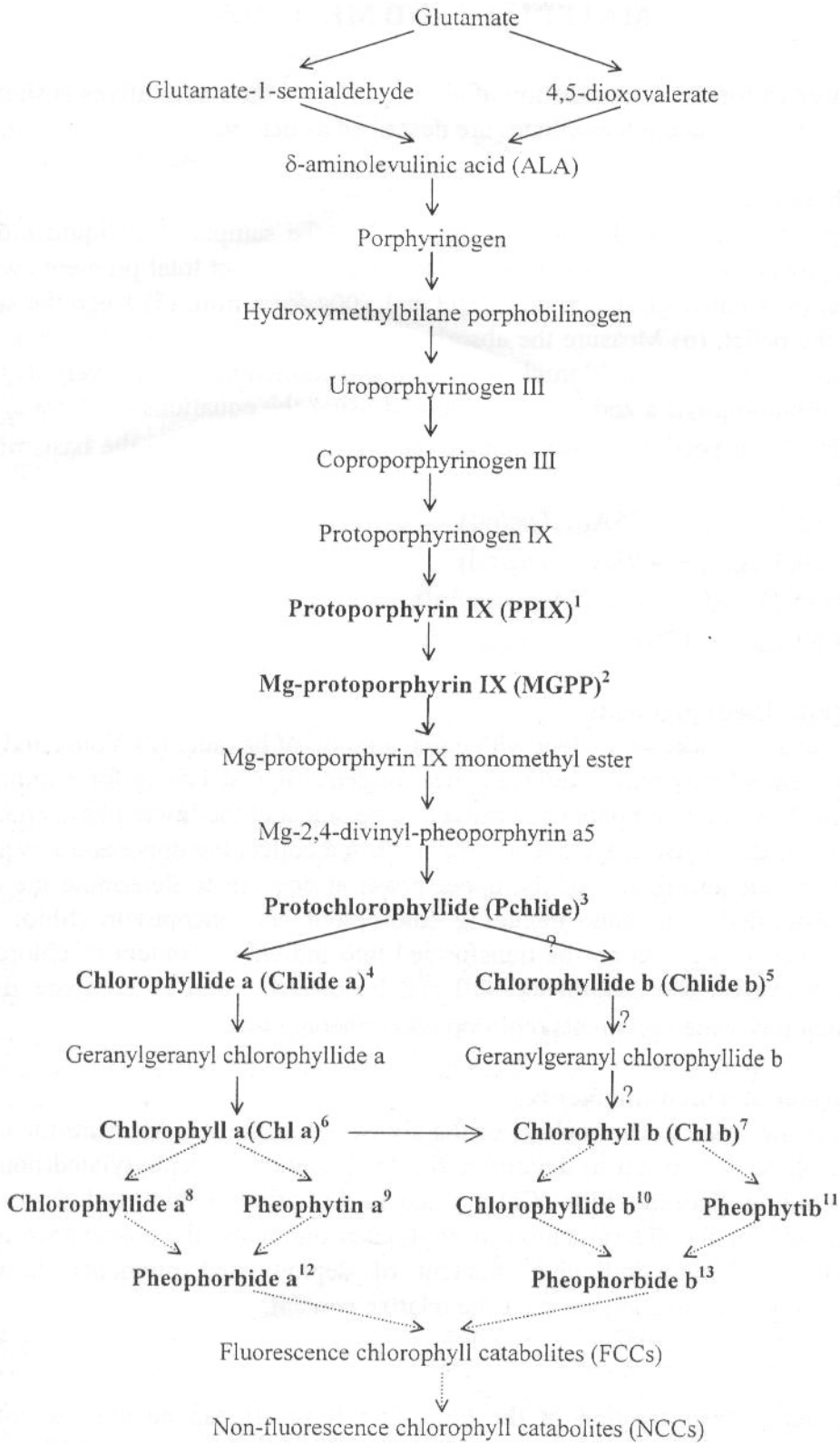


Fig. 1. The pathways of chlorophyll biosynthesis (→) and degradation (···→). The number and bold face indicate the intermediates that can be determined in the following flow chart. The question mark indicates uncertain pathway.

MATERIALS AND METHODS

The flow chart for the determination of chlorophyll and their derivatives is shown in Fig. 2. The detail steps for each intermediates are described as below.

Chl a, Chl b and carotenoid

(1) Weigh the plant sample about 0.25g; (2) Dry the sample with liquid nitrogen and grind it into powder with pestle and mortar; (3) Grind and extract total pigments with 5ml of 80% acetone; (4) Centrifuge the crude extract at 1,500g for 5 min; (5) Keep the supernatant and discard the pellet; (6) Measure the absorbance at 663.6, 646.6 and 440.5 nm, which are the major absorption peaks of chlorophyll a and b and carotenoid, respectively; (7) Calculate the content of chlorophyll a and b and carotenoid using the equations of Porra *et al* (1989) and Holm (1954), respectively; (8) Calculate the pigment contents on the basis of $\mu\text{g Chl/g}$ fresh weight.

$$\text{Chl a} = 12.25A_{663.6} - 2.55A_{646.6} (\mu\text{g/ml})$$

$$\text{Chl b} = 20.31A_{646.6} - 4.91A_{663.6} (\mu\text{g/ml})$$

$$\text{Chl a+b} = 17.76A_{646.6} + 7.34A_{663.6} (\mu\text{g/ml})$$

$$\text{Car} = 4.69A_{440.5} - 0.267\text{Chl a} + \text{b} (\mu\text{g/ml})$$

Phytylated(esterified) pigments

(1) Mix the above acetone extract with equal amount of hexane; (2) Vortex and then keep the mixed solvent silently until interface appear or centrifuge at 1,500g for 3 min; the upper phase contains less polar compounds dissolved in hexane and the lower phase contains more polar compounds dissolved in acetone; (3) Separate and collect the upper and lower fractions; (4) Measure the the absorbance of the upper phase at 661 nm to determine the content of phytylated (esterified) pigments, including chlorophyll and pheophytin (Shioi and Sasa, 1986). The value of A_{661} can not be transformed into individual content of chlorophyll and pheophytin. However, the absorbance still can be directly used to compare the relative content of total phytylated pigments (chlorophyll + pheophytin).

Dephytylated(nonesterified) pigments

(1) Collect the lower acetone phase of the above procedure; (2) Measure the absorbance of the lower phase at 666 nm to determine the total content of dephytylated(nonesterified) pigments including chlorophyllide (Chlide) and pheophorbide (Shioi and Sasa, 1986). As mentioned above for the determination of phytylated pigments, the absorbance of A_{666} can not be transformed into individual content of dephytylated pigments, however, the absorbance can be used to compare their the relative content.

Porphyrins

(1) The same lower fraction of the procedure II or III can be used to measure the absorbance at 575, 590 and 628 nm; (2) The equation of Kahn *et al* (1976) is hired to determine the content of protoporphyrin IX (PPIX), magnesium-protoporphyrin IX (MGPP) and protochlorophyllide (Pchlide).

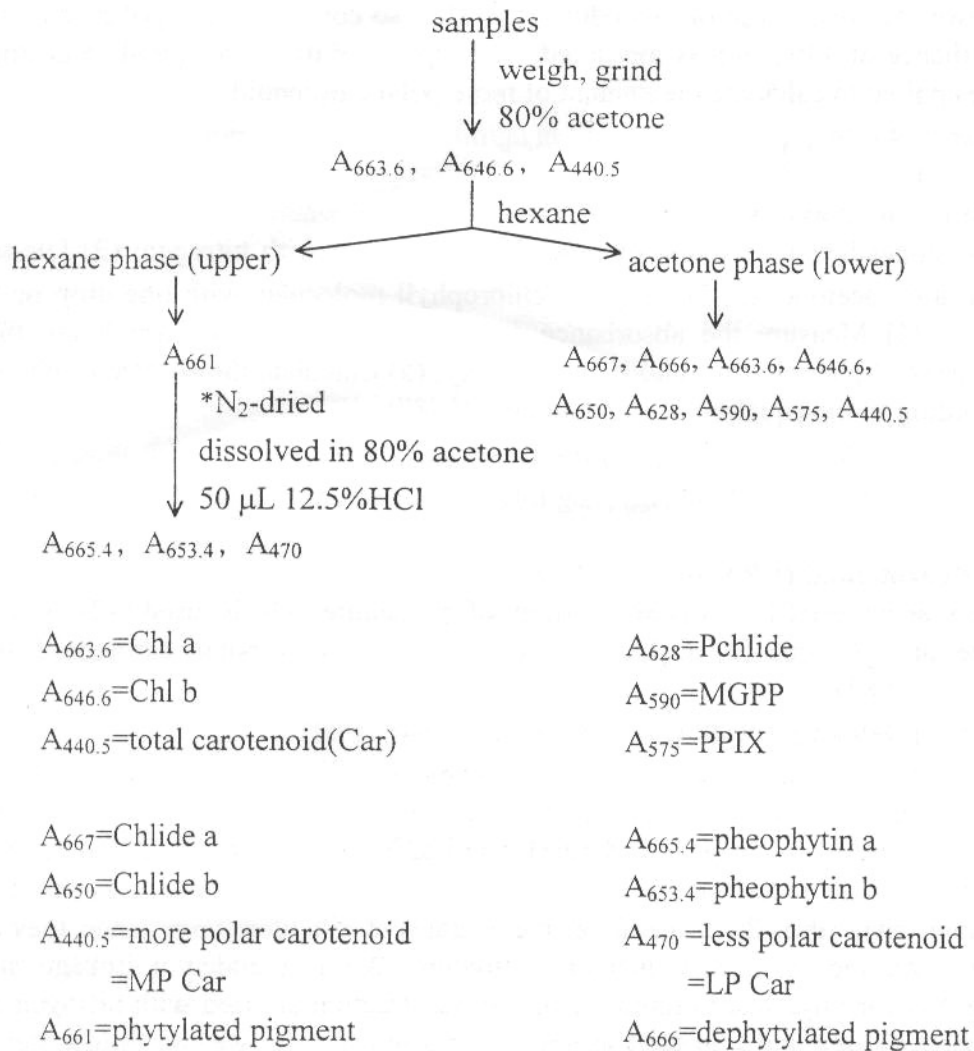


Fig. 2. The flow chart for the determination of plant pigments and their derivatives. Star indicates the linkage step that the authors added.

$$\text{PPIX} = 196.25 A_{575} - 46.6 A_{590} - 58.68 A_{628} \text{ (nmole)}$$

$$\text{MGPP} = 61.81 A_{590} - 23.77 A_{575} - 3.55 A_{628} \text{ (nmole)}$$

$$\text{Pchlide} = 42.59 A_{628} - 34.22 A_{575} - 7.25 A_{590} \text{ (nmole)}$$

Chlorophyllide (Chlide) a and b

(1) The lower acetone fraction of procedure II or III is used to measure the absorbance at 667 and 650 nm; (2) The Beer-Lambert law ($A = \epsilon cl$) and method of McFeeters (1971) is used to calculate the content of chlorophyllide a and b.

$$\text{Chlide a} = A_{667}/74.9 \text{ (mM)}$$

$$\text{Chlide b} = A_{650}/47.2 \text{ (mM)}$$

More polar carotenoid (MP Car)

The lower acetone phase of procedure II or III also contains more polar carotenoid and their absorbance at 440.5 nm is measured. The equations of Holm (1954) and Porra *et al* (1989) are applied to calculate the content of more polar carotenoid.

$$\text{MP Car} = 4.69A_{440.5} - 0.267\text{Chl a} + \text{b}(\mu\text{g/ml})$$

Pheophytin (Phe) a and b

(1) The upper hexane fraction of procedure II is dried with nitrogen; (2) Dissolve the pellet with 80% acetone; (3) Destroy the chlorophyll molecules with one drop or 50 μl of 12.5% HCl; (4) Measure the absorbance at 665.4 and 653.4 nm, which are the major absorption peak of pheophytin a and b, respectively; (5) Calculate the content of pheophytin a and b according to the equation of Lichtenthaler (1987).

$$\text{Phe a} = 22.42A_{665.4} - 6.81 A_{653.4} (\mu\text{g/ml})$$

$$\text{Phe b} = 40.17A_{653.4} - 18.58 A_{665.4} (\mu\text{g/ml})$$

Less polar carotenoid (LP Car)

(1) The acetone-HCl-dissolved solution of procedure VII is used; (2) Measure the absorbance at 470 nm; (3) Calculate the content of less polar carotenoid by using Lichtenthaler (1987).

$$\text{LP Car} = (1000A_{470} - 4.28A_{665.4} - 4.78A_{653.4})/164(\mu\text{g/ml})$$

CONCLUSION

The flow chart (Fig. 2) is based on the linkage of six literatures, since they all used acetone and hexane to extract different pigments. We just added a linkage step after measuring A_{661} for phytolated pigments, i.e. hexane fraction is dried with nitrogen and then dissolve pheophytin a and b in 80% acetone. In our experience, we can follow steps of this flow chart to determine the content of eleven pigments for more than 100 samples in 8 h. We followed this flow chart in the study of Chl degradation in the Yuenyang Lake Nature Preserve and obtained useful data presented in 1997 International Long-Term Ecological Research and Biodiversity Studies Conference (Yang *et al.*, 1997a and 1997b).

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一套測定葉綠素及其衍生物含量的方法

楊棋明^(1,2)、章國威⁽¹⁾、鄞銘宏⁽¹⁾、黃淑滿⁽¹⁾

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摘 要

我們提出一個測定葉綠素生合成及崩解途徑的十一種中間代謝物之實驗流程。此實驗流程是根據六篇測定不同色素的文獻而聯繫起來，之所以可以聯繫的原因是它們都以丙酮或正己烷為溶劑萃取不同的色素。我們唯一改進之處是測 A_{661} 後以氮氣吹乾，並以丙酮溶解 pheophytin a 和 b。此流程非常方便，經驗顯示，在一個工作天內可以完成至少一百個樣品。

關鍵詞：葉綠素、類胡蘿蔔素、生合成、崩解、中間代謝物。

1. 中央研究院植物研究所。
2. 通訊聯絡員。