Sci. Rep. Fac. Agr. OKAYAMA Univ. (43), 75—84 (1974)

Simultaneous Determination of Total Chlorophyll and Total Carotenoid in Detergent Solubilized Chloroplasts*

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Received December 1, 1973

Introduction

In the study of the possible molecular roles of carotenoids in the chloroplasts of green leaves, precise determination of the pigments in various subcellular preparations is the primary basis of all lines of experiments. The presently established method for quantitative evaluation of pigment composition of various living systems generally involves extraction of lipids with a series of organic solvents, removal of water-soluble components, followed by careful concentration and a chromatographic separation of each lipid or group of lipids, which is then determined usually by spectrophotometric means[1,2,3]. The stepwise procedure employed is highly laborious, time consuming and often cannot be excempted from loss of particular components.

The present paper describes a simple and reliable method of direct and simultaneous determination of chlorophlls and carotenoids in chloroplasts by optical measurements of detergent-solubilized preparations without previous pigment extraction.

The method takes advantage of the solubilizing ability of cationic detergent, by which turbid chloroplations can be transformed to clear solutions, thus making spectrophotometric analysis possible.

The results of comparative examinations of solubilizing activities of various types of detergents, optical characterization of detergent-solubilized plant pigments, as well as of solubilized chloroplasts, and the theoretical and experimental analyses of absorption to yield the quantitative evaluation of carotenoids in the presence of chlorophylls are described.

The direct determination method developed here is believed to be potentially useful in the study of these biologically active pigments.

Materials and Methods

1. *Carotenoids and chlorophylls*

Total lipid was prepared from spinach leaves in accordance with our method previously reported[4]. Chlorophylls and carotenoids were isolated respectively from the total lipid preparation by silica gel (Wakogel C-200, Wako Chemicals) column chromatography using the solvent sequence shown in Table 1.

Visible absorption spectra of fractions 1, 4 and 5 are given in Fig. 1. Thin layer chromatography (TLC) showed that no pigments other than carotenoid or chlorophyll were contained in all fractions.

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* Biochemical Studies on Carotenoids Part Ⅲ.

Table 1. Preparation of chlorophylls and carotenoids from spinach leaves by silica gel column chromatography

Solvent (v/v)	Elution volume (ml)	Fraction	
		No.	Pigments found
Benzene	50	1	Carotenes
Benzene : Ether (9 : 1)	100	2	No pigment
〃 : 〃 (8 : 2)	150	3	Chlorophyll a
〃 : Ethyl acetate (5 : 5)	200	4	Chlorophylls a and b
〃 : EtAc* : MtOH* (6 : 3 : 1)	200	5	Xanthophylls

Column ; 2 cm × 15 cm

* EtAc represents Ethyl acetate and MtOH, methanol.

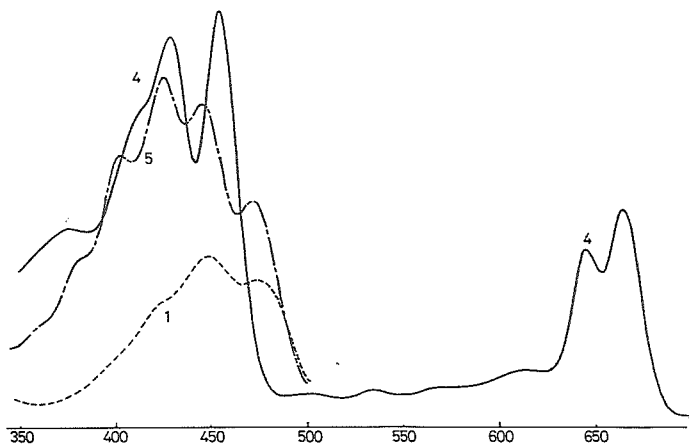

Fig. 1. Absorption spectra of fractions in ether obtained by silica gel column chromatography
1. Carotenes; 4. Chlorophyll a & b; 5. Xanthophylls

2. *Thin layer chromatography of chlorophylls and carotenoids*

TLC of chlorophyll and carotenoid was carried out on Kiesel gel G (Merck) of 0.25 mm thickness with benzene : ethyl acetate : methanol = 6 : 3 : 1.

3. *Estimation of absorption spectrum*

Visible absoption spectra in various solvents were obtained with a Shimazu MPS spectrophotometer and 1 cm light path cells.

Results and Discussion

1. *Solubilization of free carotenoids*

(1) *Screening of solubilizing agents*

An aliquot of ether solution of β-carotene with the concentration of about 2×10^{-4} percent was placed in a test tube and the solvent was evapolated by aeration. Into this test tube were added 5 ml each of various detergents in water at 0.01 M concentration, and shaked gently.

The results of solubilization tests are given in Table 2, which shows that carotenoid was solubilized with amphoteric and cationic detergents, while it was only emulsified

with nonionic or anionic detergents even though their concentrations were increased. The tests with lutein instead of β-carotene gave essentially identical results.

(2) *Detergent concentrations required to solubilize carotenoids*

The critical concentrations of the detergents which were required to solubilize carotenoid as follows. Two-tenth milliliters each of ether solution of carotenoid (0.0108 mg as β-carotene) was taken into a test tube. After the removal of the solvent, 5 ml of detergent aqueous solutions at different concentrations were added and gently shaked.

Table 2. Solubilization of carotenoids with various detergents

Detergent		Result*
Name	Rational formulae	
Anon LG	$R{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}NH{\cdot}CH_2{\cdot}CH_2{\cdot}NH{\cdot}CH_2{\cdot}COONa$	Solubilized solution
Anon BF	$R{\cdot}N^+{\cdot}(CH_3)_2$ with $CH_2{\cdot}COO^-$ on N	〃
Cetyltrimethyl ammonium chloride (PB 40)	$[C_{16}{\cdot}H_{33}{\cdot}N^+{\cdot}(CH_3)_3]Cl^-$	〃
Cetylbenzyldimethyl ammonium chloride (CBDAC)	$[C_{16}{\cdot}H_{33}{\cdot}N^+{\cdot}(CH_3)_2]Cl^-$ with CH_2–(benzene ring) on N	〃
Triton X-100	$C_9{\cdot}H_{19}$–(benzene ring)–$(O{\cdot}CH_2{\cdot}CH_2)_{10}\,OH$	Emulsion
Tween-80	Additive compound of 8 mole ethylene oxide to sorbitol	Unstable emulsion
Nonion NS-221	$C_9{\cdot}H_{19}$–(benzene ring)–$(O{\cdot}CH_2{\cdot}CH_2)_{21}\,OH$	〃
Sodium dodecyl sulfate	$C_{12}{\cdot}H_{25}{\cdot}O{\cdot}SO_3^-{\cdot}Na^+$	〃

* "Solubilized solution" means clear solution, "emulsion" are not clear but stable upon standing for more than 2 days and "unstable emulsion" results seperate layers upon standing for about one night.

Optical densities at 450 nm of the supernatant solutions were estimated after standing for 10 min, and modified optical densities were obtained by using that of no detergent solution as a control. The results are shown in Fig. 2. Below 3 mM of the detergent (dotted line in the figure), the solubilization was imcompletely carried out, thus in these cases the thin upper layer which was composed of slightly contaminated solvent and a part of carotenoid was observed. Considering these results it was suspected that the critical concentration of this detergent (PB-40) required to the solubilization of carotenoids in chloroplasts (0.0108 mg/5 ml) might be more than 3 mM. Higher carotenoid concentrations would naturally require detergent concentrations.

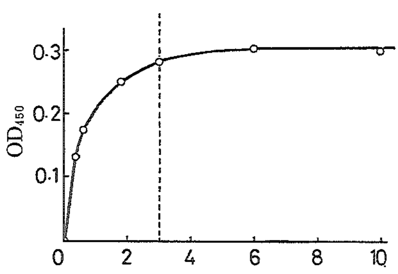

Fig. 2. Solubilization of carotenoids by a cationic detergent, PB-40

An essentially identical result to that with PB-40 was obtained with cetylbenzyldimethyl ammonium chloride (CBDAC). The solubilization test with Triton X-100 or SDS for

carotenoids was impossible because of the reason shown in Table 2.

(3) *Absorption spectrum and its maximum wavelengths of solubilized carotenoids*

Since the absorption spectrum is generally effected by solute environments, the possible changes of the spectra of carotenoids by the addition of cationic detergents were examined. The absorption maxima of solubilized carotenoids were found to have shifted to longer wavelengths from those in ether or benzene up to 6 to 10 nm, and their fine structures had been weakened (Table 3).

Table 3. The shift of λ_{max} of β-carotene and spinach leaf carotenoids solubilized with PB-40

Carotenoid	Solvent	λ_{max} (nm)
β-carotene	Ether	432, 453.5, 481
〃	Aqueous PB-40	440, 462, 487.5
Spinach leaf carotenoids	Ether	424, 445, 472
〃	Aqueous PB-40	433, 454, 481

2. *Solubilization of chlorophylls*

(1) *Solubilizing agents*

The identical experiments to those with carotenoid were carried out and the essentially identical results were obtained. Cationic and amphoteric detergents were found to be useful for the solubilization of chlorophylls.

(2) *Detergent concentrations required for the solubilization of chlorophylls*

Two-tenth milliliters of total chlorophyll ether solution (37.8 mg as chlorophyll) was taken into a test tube, and after the removal of the solvent, 5 ml of a cationic detergent (PB-40) of various concentration was immediately added, followed by gentle shaking. Solubilized solutions thus obtained were subjected to spectrophotometric analysis at 665 nm. The results are shown in Fig. 3.

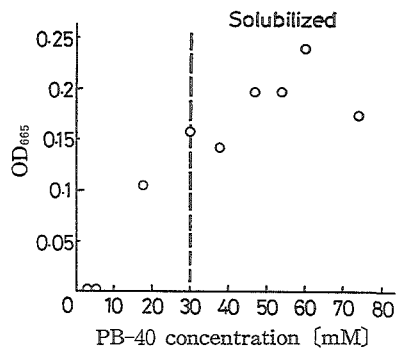

Fig. 3. Solubilization of chlorophylls by a cationic detergent, PB-40

Under the experimental condition employed, chlorophylls were solubilized at the detergent concentrations higher than 30 mM which were at least ten times higher than those required for the solubilization of carotenoids.

(3) *Visible absorption spectrum and λ_{max} of solubilized chlorophylls*

The visible absorption spectra had also been shifted to longer wavelengths by the range of 5 to 8 nm from those in ether as well as carotenoid, and the fine structures were also weakened (Table 4).

Table 4. λ_{max} of solubilized chlorophyll in ether and in a cationic detergent

Ether	PB-40	Ether	PB-40
383 nm	385 nm	455.5 nm	467 nm
415	422	650	658*
432	440	666	673

* shoulder

3. *Determination of carotenoids by the elimination method in the presence of chlorophylls*

Of the sample solution containing two compounds, the quantitative determination of one compound has been able to carry out spectrophotometrically[5,6,7,8] by the elimination of the other.

Since the major pigments of green leaf may be chlorophylls and carotenoids, the chloroplast contains also both as major pigments[9,10].

Thus the determination of total carotenoid in chloroplast was taken place by the following procedures. Two wavelengths with the identical optical densities were primarily determined on the spectrum of the solubilized total chlorophyll solution. Next, on the spectra of the mixtures of total chlorophyll with constant concentration and total carotenoid with various concentration, those optical densities at the two wavelengths above determined were estimated, and it was resulted that the differences of these optical densities were proportional to the amounts of total carotenoid at first order.

The detailed procedures were as the following; three-tenth milliliters of total chlorophyll solution in ether (18.9 mg % concentration) were taken into six test tubes, followed by addition of carotenoid ether solution (5.4 mg % concentration) of various volumes from 0.1 to 0.35 ml. After removal of the solvent (Drying up should be avoided), the solubilizations were carried out by addition of 2 ml of 40 % PB-40 and then the total volumes were filled up to 7 ml with distilled water.

In these cases each solution should be completely clear. If the addition sequences of pigments and the detergent were reversed, the solubilization would not be completed. The absorption spectra of these solubilized solutions were estimated with the control of water. The two wavelengths with the identical optical densities were determined to 443 and 401 nm in this study, and so by reading of the optical densities at the both wavelengths on the spectra, the quantitative relationship between the differences of both optical densities ($OD_{443}-OD_{401}$) and carotenoid concentrations was established. The relationships are shown in Fig. 4. From these results the following equation can be set up. Therefore, the quantitative determination of total carotenoid in the presence of total chlorophyll was able to be carried out by this method.

$$C=\frac{OD_{443-401}}{0.7};\ C=\text{carotenoid concentration (mg \%)}.$$

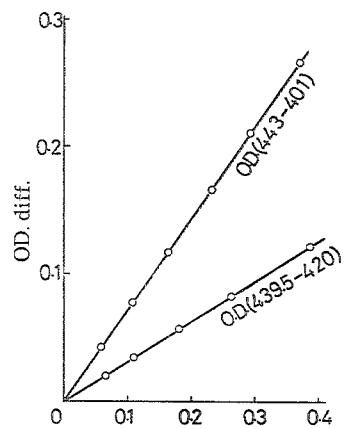

Fig. 4. Standad curve for the determination of total carotenoid solubilized by PB-40

Even if the spectrum might be shifted on either side by the reasons of the estimation technique or otherwise, the accurate results have been obtained by the following method. As the normal solubilized chlorophyll has the λ_{max} at 667 nm and the shift from its normal standard λ_{max} is considered to reflect the wavelengths above mentioned the difference of the estimated λ_{max} from 667 nm may be added to or subtracted from each of the two wavelengths, 443 nm and 401 nm, respectively.

4. *Effect of the chlorophyll concentration on thequantitative analysis of the total carotenoid*

The influences of various chlorophyll concentrations to the total carotenoid determination were investigated. The constant quantitative values of the total carotenoid should be obtained at the constant carotenoids concentration even if the sample solutions had various chlorophyll concentrations. To each 0.2 ml of total carotenoid ether solution (7.58 mg %), various volumes (0.05 to 0.5 ml) of total chlorophyll ether solution (35.44 mg %) were added, and the solvent was almost removed.

Two milliliters of 40 % PB-40 aqueous solution were added to the pigment mixtures for solubilization, and the solubilized solutions were messed up to 5 ml with distilled water, followed by estimation of visible absorption spectrum. The carotenoid quantities (concentrations) determined from the difference of the two optical densities are given in Table 5. It was seen that the total carotenoid determination was not effected by the chlorophyll concentrations.

Table 5. Effects of the total chlorophyll concentration on the determination of the total carotenoid

Chlorophyll / Carotenoid ratio	Total carotenoid determined (mg %)
1.2	0.24
2.4	0.24
4.8	0.24
7.2	0.24
9.6	0.22
12.0	0.22

5. *Effects of detergent concentrations on the determination of the totalcarotenoid*

In order to investigate the effects of detergent concentrations on determination of the total carotenoid, both the patterns of spectra of the solubilized total chlorophylls and the differences of the two optical densities (443 and 401 nm) were checked.

Two-tenth milliliters of the total chlorophyll ether solution were taken respectively and the solvent was removed. The varied volumes from 0.5 to 2.0 ml of 40 % PB-40 were added for solubilization, followed by filling up to 6 ml with distilled water. In the visible spectra of these solubilized chlorophylls λ_{max} were completely identical with each other and the differences of the optical densities at 443 and 401 nm were zero respectively. Thus it was found that the detergent concentrations would not effect on the spectra of the solubilized total chlorophyll and therefore on the determinations of the total carotenoid.

6. *The quantitative method of the solubilized total chlorophyll*

Though the quantitative determinations of the total chlorophyll in ether or 80 % acetone etc. have been established[1,2], such methods could not be applied to these solubilized chlorophyll aqueous solutions. Thus a quantitative method for the solubilized total chlorophyll should be designed to establish.

The total chlorophyll quantities in the ether solution were primarily determined according to the ordinary method[11]. On the basis of these data, the quantitative standard curve of the solubilized total chlorophyll was able to be drawn as follows. Aliquots of the total chlorophyll ether solution with varied volumes were taken into 8 test tubes. After the removal of solvent, 5 ml of 0.04 M PB-40 aqueous solution

were added for solubilization. The optical densities of the solubilized solutions at 667 nm and 620 nm were estimated. The first order relation between the differences of the two optical densities ($OD_{667}-OD_{620}$) and the total chlorophyll concentrations was obtained as shown in Fig. 5. From the results the following equation can be set up.

$$C = \frac{OD_{667-620}}{0.24}\ ;\ C = \text{total chlorophyll concentration (mg \%)}.$$

These relations might be able to be utilized for the determinations of the solubilized total chlorophyll.

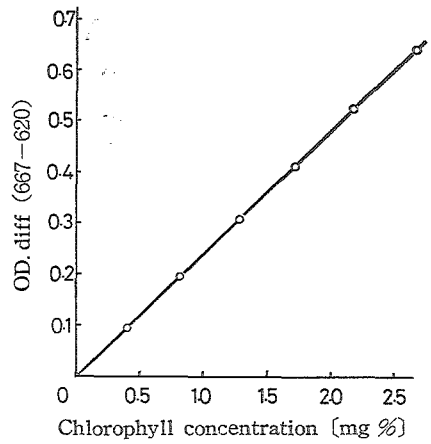

Fig. 5. Standard curve for the determination of total chlorophyll solubilized by PB-40

7. Solubilization of chloroplasts and the determination examples of the total carotenoids and the total chlorophylls of various chloroplasts

(1) *Solubilization of chloroplast*

For the solubilization of chloroplast Triton X-100[12], SDS[13] and Nonidet P-40[14] have been frequently used, but cationic and amphoteric detergents have been hardly utilized for its purpose. In this case the critical concentrations of the cationic detergents for the solubilization of chloroplasts were determined.

Five-tenth milliliters of a suspension of spinach leaf chloroplast (1.4 mg N/ml), which was prepared by ordinary method, were taken into test tubes. Tenth milliliters of 0.25 M PB-40 and 5.9 ml of 0.01 M Tris buffer (containing 0.5 M sucrose, pH 8.0) was added toeach test tube, followed by the gentle stirring. After standing for 5 min in dark place, 0.5 ml of the filtrates were diluted with 5 ml of dist. water, followed by estimations of the optical densities at 660 nm.

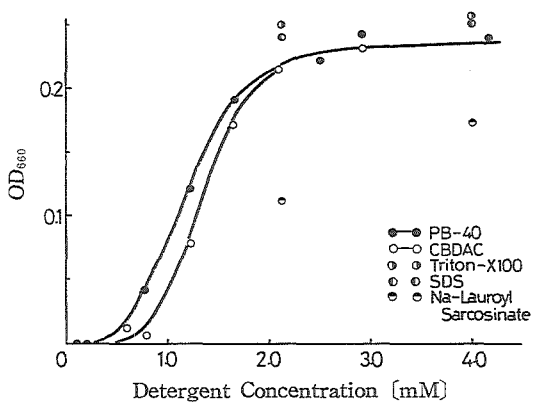

Fig. 6. Solubilization curve of chloroplast in PB-40 aq.

Spinach chloroplast suspension 0.5 ml (0.7 mg-N); Total volume 6.5 ml; Five-tenth of the filtrates were diluted with 5 ml of dist. water, followed by colorimetric estimation

From these results shown in Fig. 6, the critical concentration of PB-40 for the solubilization of the chloroplast was found to be 3.0 mM. The results with Triton X-100, SDS and CBDAC were also summarized in Fig. 6, and thus it was found that these detergents had the almost identical abilities to those of PB-40 in the solubilizations of chloroplast.

(2) *Visible absorption spectra of solubilized chloroplasts and the transformations of these spectra with time course*

The visible absorption spectra of the spinach chloroplast solubilized by PB-40, CBDAC, Triton X-100 and SDS were given in Fig. 7.

Using the detergents of the identical functional groups, λ_{max} and the

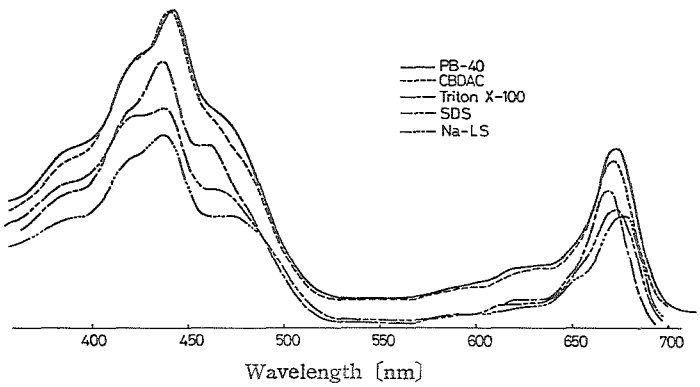

Fig. 7. Absorption spectra of spinach total chloroplast solubilized by various detergents

fine structures of these spectra were found to be similar, but in the cases with the different functional groups, both patterns of spectra and λ_{max} were found to considerably differ. Therefore, when the carotenoid determination is carried out by the method mentioned in this report, it will be essential to define the species of detergents.

Furthermore, the spectra patterns of the solubilized chloroplast were found to be transformed with time course, that is, the ratios of λ_{max} of 442 nm against that of 417 nm became gradually lower on standing after the solubilization (Fig. 8). From this fact it was deduced that chlorophylls or carotenoids would be faded owing to the solubilization.

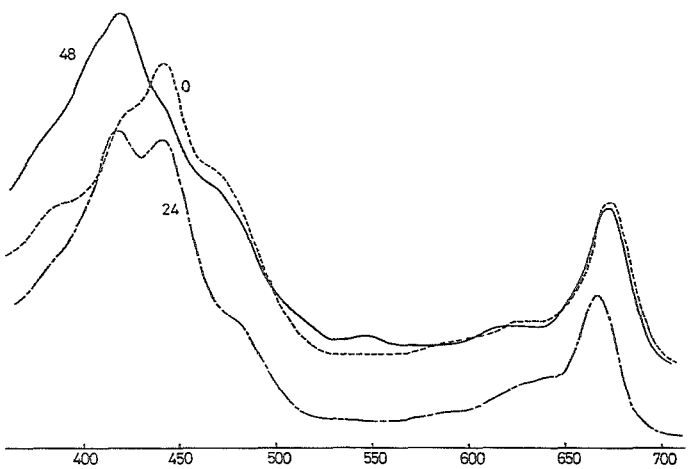

Fig. 8. Absorption spectra of spinach total chloroplast solubilized by PB-40 and the transformation with time course
0 : indicates immediately after solubilization; 24, 24 hr. after solubilization; 48, 48 hr. after solubilization

Since the color of the solubilized chloroplast was found to change from green to orange yellow with naked eye, this phenomenon would be considered to arise from the decrease of a chlorophyll. But on these spectra the maximum absorptions at about 670 nm were hardly changed, thus the fading of only a chlorophyll would not

occured. The details of these fading phenomena were not clarified, but as the spectra of the solubilized chloroplast were transformed with time course, the colorimetric estimations for the total carotenoid determination would be needed to carry out as soon as the chloroplast was solubilized with a detergent.

(3) *Examples of the determinaton of the total carotenoid and the chlorophyll in solubilized chloroplasts*

In accordance with the method mentioned in this report, the determinations of the total carotenoid and the total chlorophyll have been taken place with various leaf chloroplasts. Some examples of those results are given in Table 6.

Table 6. Some examples of the determination of the total carotenoid and the total chlorophyll in the solubilized chloroplasts

Date harvested	Source plant	Total-N(mg) in 1 ml of chloroplast suspension	Total carotenoid	Total chlorophlly	Ratio of chlorophyll to carotenoid by weight
June 27 1973	Spinach	—	0.374 mg%	2.45 mg%	6.55
July 10 1973	Clover	0.91	0.073 (0.80×10^{-3})	0.89 (9.87×10^{-3})	12.20
July 13 1973	Corn	0.56	0.078 (1.39×10^{-3})	0.91 (16.25×10^{-3})	11.67
July 14 1973	Mulberry leaf	0.56	0.11 (1.96×10^{-3})	0.96 (17.14×10^{-3})	8.70
July 16 1973	Red perilla	0.80	0.123 (0.44×10^{-3})	1.46 (5.21×10^{-3})	11.90
August 1 1973	Sweet potato leaf	0.40	0.076 (1.90×10^{-3})	0.97 (24.25×10^{-3})	12.70

In parenthesis shows mg%/N-mg.

Acknowledgment. The author wishs to express his gratitude to Nippon Oils & Fats Co. Ltd., for his kind gift of various detergents.

Summary

The simultaneous determination of the total carotenoid and the total chlorophyll in chloroplast was investigatgd. As the chlorophylls and the carotenoids were found to be solubilized by the cationic detergents, the total carotenoid determinations were carried out by the so-called elimination method, in which the both isolated pigments from spinach leaves were used as the standards and only the carotenoid was determined spectrophotometrically. Namely, cetyldimethyl ammonium chloride was used as the solubilizing agent, and in absorption spectra of the solubilized total chlorophyll aqueous solutions the two wavelengths having the identical optical densities (401 and 443 nm) might be arbitrarily determined.

As the first order relationship between the total carotenoid quantities and the differences of these optical densities was able to be established, the carotenoid determinations of the solubilized chloroplasts were also taken place by estimating the optical densities in the two wavalength. The total chlorophyll determinations were carried out by the estimation of the optical densities of the two wavelength (620 and 667 nm).

The determination examples of both pigments in some chloroplasts were carried out with these methods.

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可溶化クロロプラスト中のクロロフィル及び カロチノイドの定量法について*

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要　　約

クロロプラスト中のカロチノイド及びクロロフィルを直接的に同時定量する方法について検討した．クロロフィル及びカロチノイドは陽イオン界面活性剤で可溶化されるので，緑葉から単離した両色素を標準として分光光度計を用いた1物質消去法によりカロチノイドを定量した．

クロロフィルは常法を改変して定量した．すなわち，可溶化剤として Cetylbenzyldimethylammonium Chloride を用い，あらかじめ単離した可溶化クロロフィル水溶液について同一吸光度を示す任意の2波長（この場合 401 nm 及び 443 nm）を求めておく．さらにクロロフィルとカロチノイドを含む可溶化溶液でカロチノイド量と前記2波長の吸光度の差との間に一次比例の関係のあることを確認できれば可溶化クロロプラストについても上記2波長の吸光度を測定することによりカロチノイドを定量することができる．

クロロフィルの定量は赤色領域の吸光度測定によって行なった．この方法を適用して2，3のクロロプラストについての分析例を挙げている．

＊カロチノイドの生化学的研究（第3報）

岡山大学農学部学術報告第43号正誤表

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目　次 （和文）	下から４行	題名の最後へ挿入	（英文）
目　次 （欧文）	上から24行	（XXⅢ）	（XVⅢ）
34	Fig. 9. の縦軸	11 12	12 14
46	下から10行	Lib rary	Library
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