Leerstoel voor Algemene en Vergelijkende Bosbouw
Prof. dr. M. Van Miegroet

A QUANTITATIVE DETERMINATION OF THE PLASTID PIGMENTS IN HIGHER PLANTS

by

J. Thas

1. Introduction

O.D.C. 160.22

Because determination of the influence of light on leaves (light reaction) is very important for the growth of plants, pigments analyses could contribute to these problems. Between the different plastid pigments chlorophyll is the most important and perhaps the only pigment responsible for photosynthesis in green plants.

It is also possible to characterize ecological plant types and the difference between sun and shade leaves by determination of the pigment contents. Shade leaves of every plant contain more chlorophyll and less carotenoids as sun leaves when the pigment content is expressed per gram weight. The reverse is true when the pigment content is expressed per leaf surface. Shade leaves generally are very thin.

It therefore is desirable to express the pigment content per leaf surface and per weight (dry or fresh) and it is obvious too that besides the total plastid pigment content also quantitative determinations of the different pigments are necessary.

Every higher plant contains two kind of pigment : plastid or chloroplast pigments and pigments of the cytoplasm. The chloroplast pigments are closely connected with the photosynthetic process : chlorophylls or green pigments as energy acceptors and carotenoids or yellow pigments probably as sensibilators of the photosynthesis. Chlorophyll a, chlorophyll b and protochlorophyll (this pigment especially in younger plant organs) belong to the chlorophyll group. The carotenoids can be divided in a group of pigments builded up of carbon and hydrogen, (hydrocarbons), the carotenes, and a group of pigments consisting of hydrogen, carbon and oxygen (hydroxycarbons), the xanthophylls or carotenols. The carotene group contains α-carotene, β-carotene, γ-carotene and lycopene. The isomers α- and γ-carotene are not always present,

β-carotene represents usually 90 % of the total carotene content and lycopene is almost identical with β-carotene concerning structure and spectrum curve. The xanthophyll group is represented by many pigments of which a great part is not yet determined.

The purpose is to execute quantitative determinations of different plastid pigments : chlorophyll a, chlorophyll b, carotenes and xanthophylls. Thus can be determined :

Chlorophyll a content	$= C_a$
Chlorophyll b content	$= C_b$
Total chlorophyll content	$= C_{a+b}$
Carotene (β) content	$= C_c$
Xanthophyll content	$= C_x$
Carotenoid content	$= C_{c+x}$
Total plastid pigment content	$= C_{a+b+c+x}$

Mostly the amounts of pigment are expressed in mg per gram fresh or dry weight or in percentage of the fresh or dry weight or in mg per cm^2 or dm^2 leaf surface.

Further molecular ratios are also important :

Chlorophyll a/chlorophyll b	$= Q\frac{a}{b}$
Xanthophyll/carotene	$= Q\frac{x}{c}$
Proportion green-yellow pigments	$= Q\frac{a+b}{c+x}$

Following averages are accepted for these molecular ratios :

$$Q\frac{a}{b} = 3$$

$$Q\frac{x}{c} = 2$$

$$Q\frac{a+b}{c+x} = 3{,}5$$

These proportions are not genetically constant and they can undergo changes depending on the light quality and the light quantity.

No less important than the quantitative plastid pigment determinations are :

1) The light characteristics of the stand (light quality, light intensity and energy distribution);
2) The absorption and reflection of light on leaf surfaces;
3) The energy storage by photosynthesis.

A synthesis of this research can lead to determine the chromatic and energetical adaptation of the pigment system in function of the light factor.

2. Method

The present method was studied using fresh needles of Picea Abies KARST. The content of chlorophyll a and chlorphyll b and the carotenoid content is determined by a spectrophotometric method after extraction with acetone. The carotenes (β-carotene) are separated from a petroleumether extract by column chromatography. On the basis of a calibration curve relating optical densities to concentrations of β-carotene the amount of β-carotene can be determined. The difference between the carotenoid content and the β-carotene content gives the amount of xanthophylls.

3. Determination of the chlorophyll a (C_a), The Chlorophyll b (C_b) and the Carotenoid content (C_{c+x})

3.1. *Preparation of the extract*

The fresh needles (about 1 gram) were crushed, immediately after cutting, in cold acetone p.a. and anhydrous aluminium oxide was used as a grinding agent. After decanting the filtrate was centrifugated and filtered trough paper. The needles were extracted several times with the cold solvent till the residue was white and this filtrate was consequently brought up to a volume of 1 l. To obtain a total extraction of the plastid pigments it is desirable to add 1 % methanol to the solvent.

All the processes must occur in a room screened from direct light to prevent a possible decomposition of chlorophylls and carotenoids. The fresh leafy material should only be stored if that is unavoidable and the filtrate may be kept in the dark and the cold for a few hours, but not overnight because carotene is unstable in the presence of chlorophyll.

3.2. *Quantitative determination of* C_a, C_b *and* C_{c+x}.

The extinctions (E_{661}, E_{644} and $E_{440,5}$) of the clear pigment solution at λ_{661}, λ_{644} and $\lambda_{440,5}$ were measured in a spectrophotometer Beckman DB (1 cm cells and narrow slit program).

From these measurements data and following equations the chlorophyll a (C_a), chlorophyll b (C_b) and carotenoid content (C_{c+x}) can be calculated.

(1) $C_a = (9{,}78 \times E_{662}) - (0{,}99 \times E_{644})$

(2) $C_b = (21{,}40 \times E_{644}) - (4{,}65 \times E_{662})$

(3) $C_{c+x} = (4{,}69 \times E_{440{,}5}) - (C_{a+b} \times 0{,}267)$.

The concentrations C_a, C_b and C_{c+x} are expressed in mg/l.

4. Determination of the β-carotene content (C_c)

4.1. *Relation between extinction* E_{448} *and the* β*-carotene content* C_c.

As standard solution synthetic β-carotene was used (10 mg) diluted in 500 ml petroleumether 25-70° p.a.. From this volume different concentrations of β-carotene were made up :

1 mg β-carotene/250 ml petroleumether 25-70° p.a.
0,8 mg " "
0,5 mg " "
0,4 mg " "
0,2 mg " "
0,15 mg " "
0,12 mg " "
0,08 mg " "
0,04 mg " "
0,02 mg " "

The extinctions of the dilutions were measured at $\lambda_{max} = 448$ mμ (fig. 1) and from these data a linear regression (4) and tabulation (tab. I) can be calculated (fig. 2).

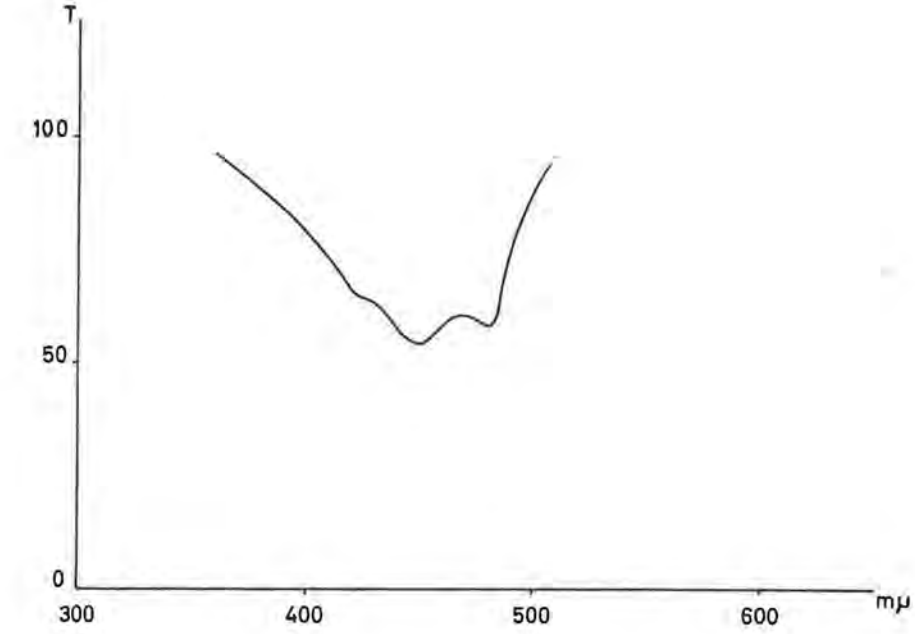
(4) $\boxed{C_c = 1{,}0648\ E_{448}}$

$r = 0{,}996$

C_c = β-carotene content in mg for a volume of 250 ml.

E_{448} = extinction at $\lambda_{max} = 448$ mμ.

The sample correlation coefficient r tested for P = 0,01 and P = 0,05 was significant.



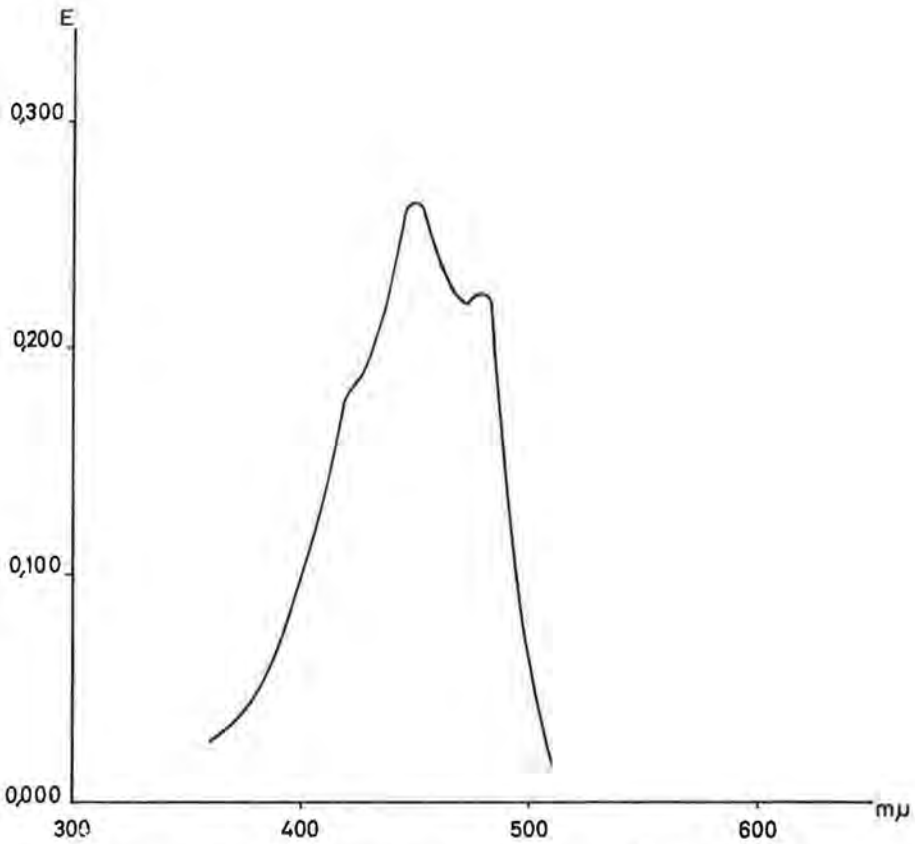


Fig1 Transmission and absorption spectrum of synthetic β-carotene in petroleumether 25-70° p.a.

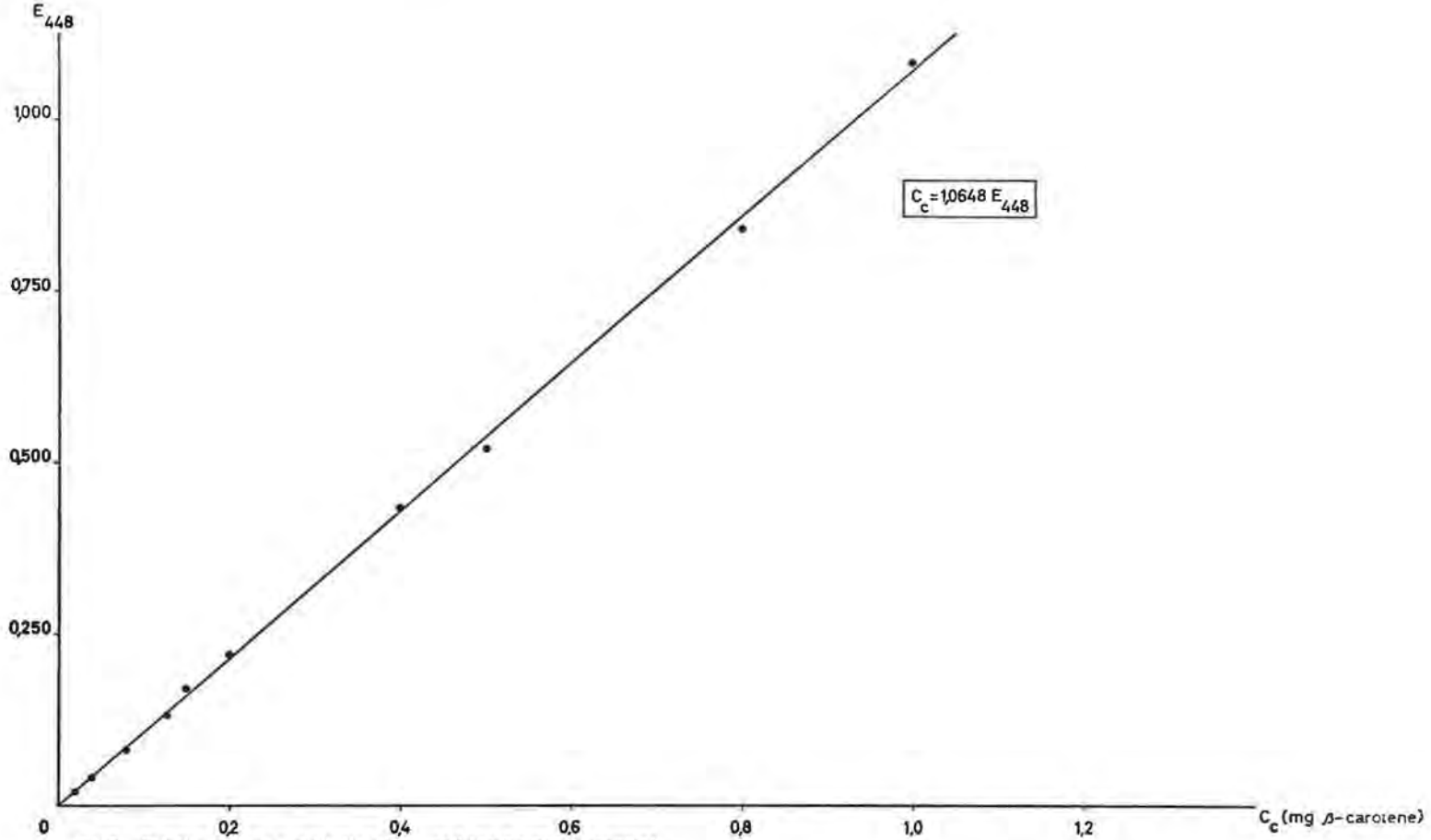


Fig 2 Relation between extinction E_{448} and the β-carotene content.

TABEL I

Tabulation of C_c in mg for a solvent volume of 250 ml

E_{448}	C_c	E_{448}	C_c	E_{448}	C_c	E_{448}	C_c
0,005	0,0053	0,155	0,1650	0,305	0,3248	0,455	0,4845
0,010	0,0106	0,160	0,1704	0,310	0,3301	0,460	0,4898
0,015	0,0160	0,165	0,1757	0,315	0,3354	0,465	0,4951
0,020	0,0213	0,170	0,1810	0,320	0,3407	0,470	0,5005
0,025	0,0266	0,175	0,1863	0,325	0,3461	0,475	0,5058
0,030	0,0319	0,180	0,1917	0,330	0,3514	0,480	0,5111
0,035	0,0373	0,185	0,1970	0,335	0,3567	0,485	0,5164
0,040	0,0426	0,190	0,2023	0,340	0,3620	0,490	0,5218
0,045	0,0479	0,195	0,2076	0,345	0,3674	0,495	0,5271
0,050	0,0532	0,200	0,2130	0,350	0,3727	0,500	0,5324
0,055	0,0586	0,205	0,2183	0,355	0,3780	0,505	0,5377
0,060	0,0639	0,210	0,2236	0,360	0,3833	0,510	0,5430
0,065	0,0692	0,215	0,2289	0,365	0,3887	0,515	0,5484
0,070	0,0745	0,220	0,2343	0,370	0,3940	0,520	0,5537
0,075	0,0799	0,225	0,2396	0,375	0,3993	0,525	0,5590
0,080	0,0852	0,230	0,2449	0,380	0,4046	0,530	0,5643
0,085	0,0905	0,235	0,2502	0,385	0,4099	0,535	0,5697
0,090	0,0958	0,240	0,2556	0,390	0,4153	0,540	0,5750
0,095	0,1012	0,245	0,2609	0,395	0,4206	0,545	0,5803
0,100	0,1065	0,250	0,2662	0,400	0,4259	0,550	0,5856
0,105	0,1118	0,255	0,2715	0,405	0,4312	0,555	0,5910
0,110	0,1171	0,260	0,2768	0,410	0,4366	0,560	0,5963
0,115	0,1225	0,265	0,2822	0,415	0,4419	0,565	0,6016
0,120	0,1278	0,270	0,2875	0,420	0,4472	0,570	0,6069
0,125	0,1331	0,275	0,2928	0,425	0,4525	0,575	0,6123
0,130	0,1384	0,280	0,2981	0,430	0,4579	0,580	0,6176
0,135	0,1437	0,285	0,3035	0,435	0,4632	0,585	0,6229
0,140	0,1491	0,290	0,3088	0,440	0,4685	0,590	0,6282
0,145	0,1544	0,295	0,3141	0,445	0,4738	0,595	0,6336
0,150	0,1597	0,300	0,3194	0,450	0,4792	0,600	0,6389

The linear regression for an arbitrary volume V is :

$$(5) \quad \boxed{C_c = 0{,}004259\ E_{448}V}$$

C_c = β-carotene in mg.

V = volume of the solvent in ml.

4.2. *Preparation of the extract*

Before extraction of the carotenes the needles were boiled during one minute in aq. dest. After drying and cutting into bits the material was crushed in cold petroleumether 25-70° p.a. By this way a first pigment extract was obtained with 50 ml petroleumether. Then a new extraction was made with anhydrous aluminium oxide and 3 × 10 ml acetone p.a. This extract, evaporated at low temperature by blowing a current of dry air over the surface of the fluid, was added to the first extract and generally

a third extraction of the material with petroleumether is necessary. All preparations must occur in dim light and the filtrate can be stored for several days in a deep freeze installation.

4.3. *Separation of β-carotene from other pigments*

β-carotene can be removed chromatographically from unwanted pigments on a column made of soluble starch. The starch together with petroleumether is added to the column by means of a vacuum pump. The chromatographic tube is about 3,5 cm inside diameter by 50 cm high and is washed with petroleumether before the extract is added. The soluble starch has the property to separate hydrocarbons from oxy- or hydroxycarbons so that all pigments except β-carotene should be absorbed near the top of the column. The column must remain saturated with the solvent and must be washed to elute all the β-carotene. The β-carotene can be left in the petroleumether solution for photometric determination. When a large column is used it is possible to re-use the same column for other absorptions after washing it with petroleumether.

4.4. *Quantitative Determination of* C_c

The extinction of the β-carotene solution at $\lambda_{max} = 448$ mμ is measured in a spectrophotometer Beckman DB (1 cm cells and narrow slit program). By using linear regression (4) or tabulation (tab I) the β-carotene content in mg can be immediately determined for a solvent volume of 250 ml and the linear regression (5) gives the β-carotene content in mg for any other volume.

The xanthophyll content can be calculated by substracting the β-carotene content from the carotenoid content.

$$C_{c+x} - C_c = C_x$$

5. Procedure for a practical determination of the chlorophyll a content (C_a), chlorophyll b content (C_b), β-carotene content (C_c) and xanthophyll content (C_x)

C_{a+b}, C_{a+b}, C_{c+x}

1. Extraction of the pigments with acetone.
2. Volume of the pigment solution : 1 l.
3. Measurement of the extinction E_{662}, E_{644} and $E_{440,5}$.

4. Determination of $\boxed{C_a}$, $\boxed{C_b}$, $\boxed{C_{a+b}}$ and $\boxed{C_{c+x}}$ by using the equations (1), (2) and (3).

$\boxed{C_c \text{ and } C_x}$

1. Extraction of the pigments with petroleumether 25-70° and acetone.
2. Separation of β-carotene from other pigments on a column. Absorbent : soluble starch.
3. Volume of the β-carotene solution : 250 ml or any other volume.
4. Measurement of the extinction E_{448}.
5. Determination of $\boxed{C_c}$ by using regression (4) or tab 1 for a volume of 250 ml and regression (5) for any other volume.
6. $\boxed{C_x} = C_{c+x} - C_c$.

LITERATURE

K. EGLE — Menge und Verhältnis der Pigmente. Handb. der Pflanzenphys. (1960). Band V Teil I p. 444-497.

K. PAECH & P.V. TRACEY — Modern methods of plant analysis (1955). Vol. I p. 208-214; Vol. IV p. 142-197.

J. THAS — A quantitative determination of the total chlorophyll content in higher plants. *Med. Rijksfac. Landbouwwet. Gent* (1966) XXXI nr. 4.

V.H. BOOTH — Carotene. Its determination in biological materials. (1957) Cambridge.

P. HOFFMANN — Zur Physiologie der Photosynthese bei höheren Pflanzen. *Bot. Studien.* (1968) Heft 18.

E.K. GABRIELSEN — Über Lichtabsorption in Photosyntheseorganen. Handb. der Pflanzenphys. (1960) Band V-Teil II p. 8-27.

H. HERZMANN — Pflanzenfarbstoffe (1962). Wittenberg Lutherstadt.

J. CARLES — L'énergie chlorophyllienne. (1953) Paris.

SUMMARY

The pigments of the plastids belong to two groups : the chlorophylls or green pigments and the carotenoids or yellow pigments. The chlorophyll group can be divided in chlorophyll a, chlorophyll b and protochlorophyll and the carotenoid group in carotenes (principally β-carotene) and different xanthophylls. In connection with other research work (light quality and quantity measurements, photosynthesis, ...) it is important to determine the chlorophyll a, chlorophyll b, carotene and xanthophyll content.

According to this method the chlorophyll a, chlorophyll b and carotenoid content is determined by a spectrophotometric method (1) (2) (3) after extraction with acetone. The carotenes, after extraction with petroleumether and acetone and after separating from other pigments by column chromatography, are determined from preleminary quantitative spectrophotometric calibrations with synthetic β-carotene. The difference between the carotenoid content and the β-carotene content gives the amount of xanthophylls.

SAMENVATTING

Kwantitatieve bepaling van de pigmenten van de plastiden bij hogere planten

De pigmenten van de plastiden behoren tot twee groepen : de chlorofyllen of groene pigmenten en de carotenoïden of gele pigmenten. Tot de chlorofyl groep behoren voornamelijk chlorofyl a, chlorofyl b en protochlorofyl en tot de carotenoïde groep de carotenen (hoofdzakelijk β-caroteen) en verscheidene xanthofyllen.

Aansluitend met andere onderzoekingen (lichtkwantiteit en -kwaliteit, fotosynthese, ...) is het belangrijk het chlorofyl a, chlorofyl b, caroteen en xanthofyl gehalte te weten.

Volgens deze methode wordt het chlorofyl a, chlorofyl b en carotenoïde gehalte spectrofotometrisch bepaald (1) (2) (3) na extraktie van het plantenmateriaal met aceton. De carotenen worden, na extraktie met petroleumether en aceton en na scheiding van de overige pigmenten door middel van kolomchromatografie, bepaald (4) (5) (6) aan de hand van voorafgaande kwantitatieve spectrofotometrische ijkingen met synthetisch β-caroteen. Het verschil tussen het gehalte aan carotenoïden en carotenen geeft het xanthofyllen gehalte.

RESUME

Détermination quantitative des pigments des chloroplastes dans les plantes supérieures

Les pigments des chloroplastes appartiennent à deux groupes : les chlorophylles ou les pigments verts et les caroténoïdes ou les pigments jaunes. Dans les plantes supérieures le groupe des chlorophylles comporte la chlorophylle a, la chlorophylle b et la protochlorophylle et le groupe des caroténoïdes les carotènes (surtout le β-carotène) et différentes xantophylles. Par rapport à d'autres recherches (qualité et quantité des radiations lumineuses, photosynthèse, ...) il est important de savoir l'abondance de chlorophylle a, de chlorophylle b, de carotène et de xantophylle.

Cette méthode permet de déterminer la quantité de chlorophylle a, de chlorophylle b et des caroténoïdes à l'aide d'un spectrophotomètre (1) (2) (3) après extraction avec de l'acétone. Les caractères sont déterminés sur la base de données quantitatives avec du β-carotène synthétique (4) (5) (6) après extraction avec de l'éther de pétrole et de l'acétone et séparation des autres pigments par la chromatographie de partage. La différence entre la quantité des caroténoïdes et la quantité du carotène donne la quantité de la xantophylle.