Acta Biologica Szeged 17 (1—4), pp. 67—77 (1971)

SEPARATION OF CHLOROPHYLLS AND CAROTINOIDS BY THIN-LAYER CHROMATOGRAPHY

I. Maróti and Éva Gabnai

Department of Botany, Attila József University, Szeged

(Recived July 20, 1971)

Introduction

Owing to its well-known advantages, — thin-layer chromatography (TLC) has become a prevailing method for separating the chemical components originating from the inanimate nature and the living organisms. This up-todate branch of separation technique has so far not shown entirely its favourable properties in the field of vegetable pigments. As a result of the chemical nature of pigments, several problems are to be solved in the course of preparative separation. It seems necessary to elaborate some methods with which the extraction of pigments and the separation of the single components do not take more than about fourty minutes altogether.

For the quantitative determination revealing the ratio of the single components, it is desirable that in the course of a single wash the chlorophylls, carotenes, and xantophylls should be clearly separata from one another on one and the same sheet, and suffer but a minimum decomposition and transformation. In case of serial measurings, indispensable for the investigations, of metabolism and plant improvement, it is rather essential to obtain reliable measuring values even from a small quantity of material (0,2—2 g gross weight).

Trying to achieve a maximum degree of precision, a fast process like this, can not be expected but from a method that goes without washing, filtering, drying, etc. These requirements preclude, as a matter of course, the possibility of column- and paper-chromatographies.

We have compared the methods dealing with the thin-layer chromatographic investigation of pigments, as described in literature, and have found that no uniform method of comparable advantages has been elaborated as yet for the separation of other matters. The composition of layers is mostly complicated, the extraction and separation demand special conditions making the execution circumstantial and slow.

Taking into consideration the labile chemical nature of the colouring agents, we have elaborated a process permitting to separate and identify quite simply more components simultaneously — carotenes, xantophylls, and chlorophylls. The clear-cut separation makes possible quantitative identification in addition to the qualitative identification of the single pigments.

Materials and Methods

1. The material investigated

We have endeavoured to test our separation technique on the leaves of species of as many different taxons as possible. When applying other methods described in literature — HAGER (1962, 1966), EGGER (1962) — we have noticed that the succesful separation of pigments depends upon the plant object investigated, as well. Leaf pigments have been separated at the following plants: *Solanum laciniatum* AIT., *Solanum dulcamara* L., *Verbascum phlomoides* L., *Spinacia oleracea* L., *Triticum aestivum* L., *Oryza sativa* L.

Our micromethod is first of all suitable for the investigation of ecological effects and metabolism. (For column-chromatographic separations one generally starts from 40—100 g raw material [Müller, 1964].)

1.1 Sampling

For making extracts we have used 0,05—1 g fresh leaf. Four parallel measurements were made from every sample. Because of the little material, sampling was made most carefully. Leaves of the same degree of development and of the same position were compared, since pigment content depends largely on the position and age of leaves. The main rib of leaf, and the leaf-stalk was not measured in the sample. For the pigment extraction we have taken disks of 0,5—1 cm ∅ from one half of the leaf, while the other half of the leaf was used for determining the dry matter.

1.2 Pre treatment of the fresh leaf materials

For denaturating the protein colouring agent complexes, the leaf material is dipped by several investigators into hot water before extraction. Leaves were kept by KOSKI and SMITH (1948) in a water of 90 °C for five minutes, by KALER and SHLYK (1962) for two minutes. HAGER—MEYER—BERTENRATH (1966) deem it necessary to inactivate the enzymes only in case of young leaves, suggesting to dip them into hot water for one minute. High temperature, however, induces the decomposition of chlorophylls into pheophytins and causes the transformation of carotinoids so we made no pre treatment. The material collected was processed in a short time or stored in a dark place (refrigerator) at +4 °C for 1—2 hours.

2. Extraction of pigments

2.1 Choice of solving material

With a view to extractability of the single pigments, the choice of the extracting solving materials and the way of extraction is very important. Number of research workers use alcohol — first of all in case of algae. STEEMAN—NIELSEN (1961) prefers methanol to acetone for extracting Chlorella pigments. COHEN—BAZIRE (1957) used an acetone-methanol mixture (7 : 2) for extracting the bacteriochlorophyll. STRAIN (1958) achieved the minimum decomposition of pigments by an absolute methanol — petroleum ether combination (2 : 1). For extracting chlorophyll from leaf tissue, WICKLIFF and ARONOFF (1962) prefer an 80 p. c. cooled ethanol. We found that in case of higher plants wet alcohols are not suitable for extraction since chlorophyllase has a strong effect within them, causing the decomposition and transformation of chlorophyll. According to STRAIN (1958), in 6—24 hours, methyl-chlorophyllids a and b come into being in the methanolic extraction of the fresh leaf. We have also

observed that, the pigments, first of all chlorophylls, suffer considerable enzymatic, autooxidative, and isomerizational changes in alcoholic extract. Petrol, benzene, petroleum ether cannot be used for extraction, as owing to them the fission of the protein pigment complexes is slight, and, as a consequence, the full dissolution of pigments, first of all of chlorophylls and xantophylls, does not succeed. The extracts containing water are light-sensitive in a higher degree, the colouring agents they contain are fading soon or, in the presence of oxygen, allomerized chlorophylls are produced.

In the research of plant pigments, 80 per cent acetone as a primary extracting medium is widely spread and, in the opinion of most investigators, it is actually the most efficient dissolvent. Chloroform Free from hydrochloric-acid was used — by HAGER—MEYER—BERTENRATH (1966), MÜLLER (1964), and others — this is, however, not a current method.

When the purpose of the pigment investigation is only a quantitative determination of carotinoids, many researchers apply other methods and dissolvents. The extraction of carotinoid stains from ensiled and dried vegetable matters was carried out by Mrs. RÉTALJI and Mrs. JAKABFI (1969) with ethanol or a combination of ethanol-ether-acetone at a ratio 1 : 1 : 1. For extracting the carotene content of fresh green plants and hays, WALGER and MRS. THURÁNSZKY (1962, 1965) used a combination of petrol-petroleum ether at a 2 : 1 ratio.

Even in connection with the acetonic extraction, that proved to be the most practicable for extracting the total pigment, some difficulties arise:

a) An acetonic extract cannot be decanted safely because the pulverized cell particles remain in suspension. Therefore some of the researchers — HAGER—MEYER—BERTENRATH (1966), IHÁSZ (1960) — filtrate it through a glass filter or filter paper and others — MÜLLER (1964) — centrifuge it, but this, of course, requires more time for preparative work, and involves also some loss in material.

b) By acetone the materials of proteic and lipoid character are also dissolved, — particularly from young cells — and at a following chromatography they cause a „sticking" at the starting point and a blurred, smudged separation of the pigments. On the other hand, the pigments in acetonic phase can generally not be separated by adsorption chromatography since adsorption is highly impeded by acetone. Therefore, the most investigators: WORKER (1957), IHÁSZ (1966), BOOTH (1967), SHERMA (1967), and others take over the pigments from the acetonic solution into a petroleum ether phase, or endeavour to achieve a full acetone-freedom by watery-methanolic washing.

2.2 The total pigment extraction applied

Contrary to the methods described so far in literature, the process suggested by us tries to utilize the favourable properties of acetone and petroleum ether together and jointly as well as successively.

The samples were put in a china mortar of 5 cm diameter, adding to them some quartz sand and a few miligrammes of $MgCO_3$. According to our experience, $MgCO_3$ is more suitable for neutralizing plant acids than $CaCO_3$, or the hydroquinine suggested by BOOTH (1967), is. With $MgCO_3$ the pH value 7,1—7,2 can easily be obtained.

We extract the 0,5 g fresh leaf material with 2 ml 100 p. c. cooled acetone and about 10 ml petroleum ether (of 80 °C boiling-point) in the following way:

a) We add to the 0,5 g material weighed in previously cooled friction mortars about 1 ml cooled acetone and smear it thoroughly for about one minute.

b) Then, we add about 2 ml petroleum ether and homogenize it entirely in one minute or two.

c) The homogenized material is given a rub („washing") with about one ml petroleum ether, after being carefully ground, and the dark green pigment solution (cca. 1 ml) is decanted into a graduated measuring eprouvette.

d) Then the precipitate in the mortar is rinsed through several times (four-five times) in a way that first we rub it over with a few drops of acetone, dissolving the residual pigment from the side of the rubbing mortar, and decant it after rinsing through with one ml petroleum ether. We set the pigment extract in this way to 5 ml.

According to our comparative investigations, in this way there may remain much less pigment in the homogenized leaf material than the loss of pigment decomposed or transformed during filtration, centrifuging, washing, and drying.

The pigment extract obtained in that way contains nearly in full quantity and almost unchanged the carotenes, xantophylls, and chlorophylls. It can be directly used for chromatographic separation. The extract may be preserved — practically without any decomposition — in a dark place and at $\pm 4°C$ for days.

For deciding, how the quantity of chlorophyll and β-carotene is influenced by light during pigment extraction, we have carried out parallel extractions in dark (with green lamp) and in light, and have investigated the extracting peculiarities of acetone and petroleum ether both cooled and of room-temperature.

3. Preparation of thin-layer sheets

Thin-layers of different composition are used for separating pigments. We have compared the separation techniques described in literature: HAGER-BERTENRATH (1962), HAGER-MEYER-BERTENRATH (1966), EGGER (1962), MÜLLER (1964), RANDERATH (1962), SHERMA-ZWEIG (1967), and we have found two as the best ones. HAGER-BERTENRATH (1962), HAGER-MEYER-BERTENRATH (1966) use a method based on the distributive thin-layer chromatography, for separating chlorophylls and carotinoids. The composition of their layer is:

12 g Kieselgur G (Merck 8129)
3 g Kieselgel „umder 0,08 mm" (Merck 7729)
3 g $CaCo_3$ (Merck 2066)
0,02 g $Ca(OH)_2$ (Merck 2047)
50 ml aqueous ascorbic-acid solution, 8×10^{-3} m.

The layer is made with a mixing apparatus, to be 0,125 mm thick. The wash liquid is: 100 ml petrol (Kp 100—140), 12 ml isopropanol and 0,25 ml distilled water.

The other sheet-making technique found to be satisfactory is EGGER's method (1962): mixing 10 g Kieselgur G (Merck) and 1 g gypsum with 15 ml dioxane into a soft pulp and diluting it with 10 ml water. He makes the layer to be 200—300 μ thick, drying the leaves for several hours at 80—100 °C and then impregnating them with the 7 p. c. petroleum ether solution of subacidic plant oil. He interrupts the impregnation is interrupted when the front approa-

ches the edge of the sheet to 3—4 cm. Then he lets it dry on 70 °C for about one hour, and applies the pigment mixture to the non-impregnated part. The development takes place in the dark, with the combination of methanol-acetone-water at a ratio of 20 : 4 : 3.

The isomerous carotinoids (e. g., α- and β-carotenes or lutein and zeaxanthin) also get separated on the impregnated sheets. A disadvantage of impregnation is the necessity of removing the impregnating material before spectrophotometric determination or else the spectrum would be influenced thereby.

For making the layer, we have used the mixture of Cellulose powder MN 300 and Silica gel G to Stahl. We have mixed 20 g Silica gel and 10 g Cellulose powder with 100 ml distilled water (with an electric mixer) to a homogeneous pulp in one minute. 8 ml of the pulp was put with a pipette on a 18×5 cm degreased and dried glass plate. The smearing was performed by means of a glass rod. The thickness of the layer was 200—300 μ. The 20×20 cm plates were prepared with a smearing apparatus. The plates were left to desiccate on a horizonal and dust-free place for eight hours, and then activated at 120 °C. After being cooled they can be immediately used. It is the best to store the ready plates in exsiccators.

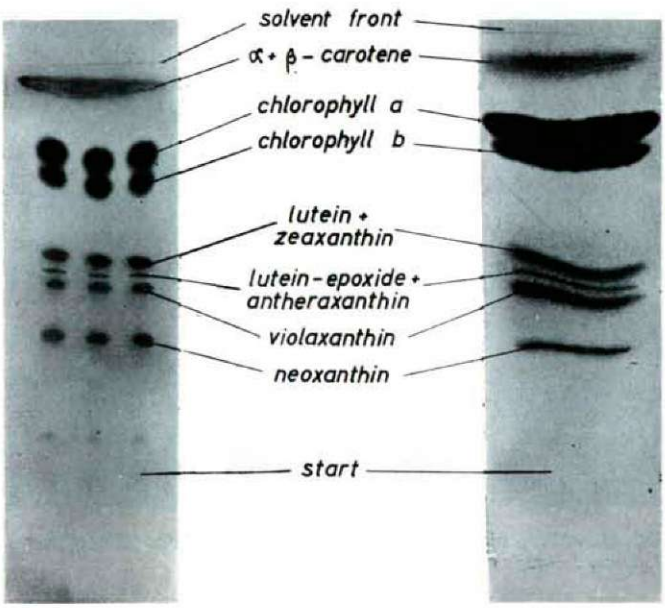

Fig. 1. Photograph of thin layer chromatogram of plastid pigments from *Verbascum phlomoides* L.

The cellulose is of neutral reaction, it does not isomerize the xantophylls like the aluminium-oxide, magnesium-oxide or silicaceous adsorbents. It has the further advantage of better mechanical adhesiveness and stronger resistance to fracture, but it cracks when peeled off. Its disadvantage consists in poor adsorption, therefore the carotinoids do not separate well.

The pure Silica gel is of slightly acidic reaction — Szász (1964) — to it isomerizes the pigments or transforms chlorophylls into pheophytin. It is one of its major advantages that it separates the single components.

The mixture system Silica gel-cellulose powder at a ratio of 2 : 1, used by us, combines the advantages listed above without the disadvantages.

4. Application of the pigment extract

The petroleum ether pigment extract was applied to the thin-layer with a micropipette small quantities of the material should better be applied on stains of 6—8 mm diameter, major quantitio, however, on a line. The starting point is 3 cm from the edge of the plate. Owing to the quick evaporation of the petroleum ether, the material can be applied to two plates without interruption. For developing the chromatogram the stains must not be desiccated.

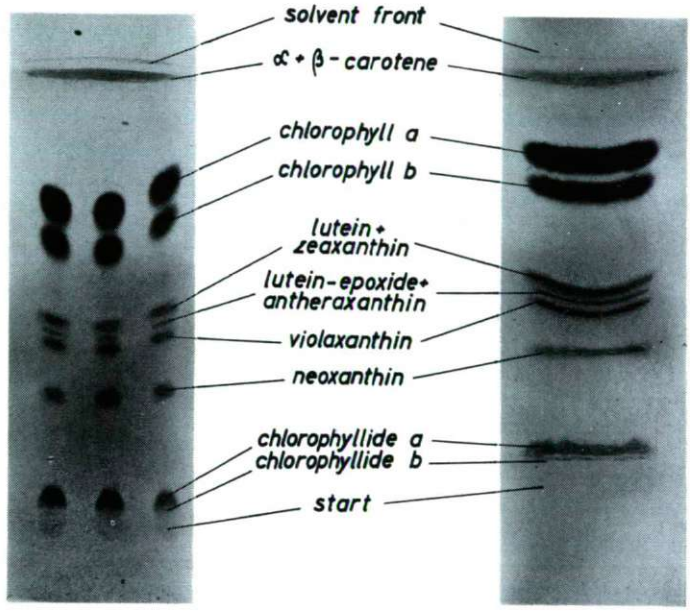

Fig. 2. Distribution of various pigments on the plate cromatoplate. The pigments are from the leaf of *Solanum laciniatum* Ait., kept in darkness.

5. Development of the chromatogram

The development takes place in a refrigerator, in the dark, with the following combination: benzene-petroleum ether-absolute alcohol-water, at a ratio of 5 : 5 : 1 : 0,5. We have applied a one-dimensional ascendant method. The distance of development is 10—14 cm. The wash-time is about 30 minutes, in case of 5×18 cm plates, 45 minutes for 20×20 cm plate size.

6. Elution of colouring agents

For spectrophotometric investigations the colouring agent zones are to be peeled off when still wet, as the epoxidic carotinoids suffer a quick change. A blade will be the most suitable for peeling purposes. The single colouring agents were eluted in ethanol and then centrifuged.

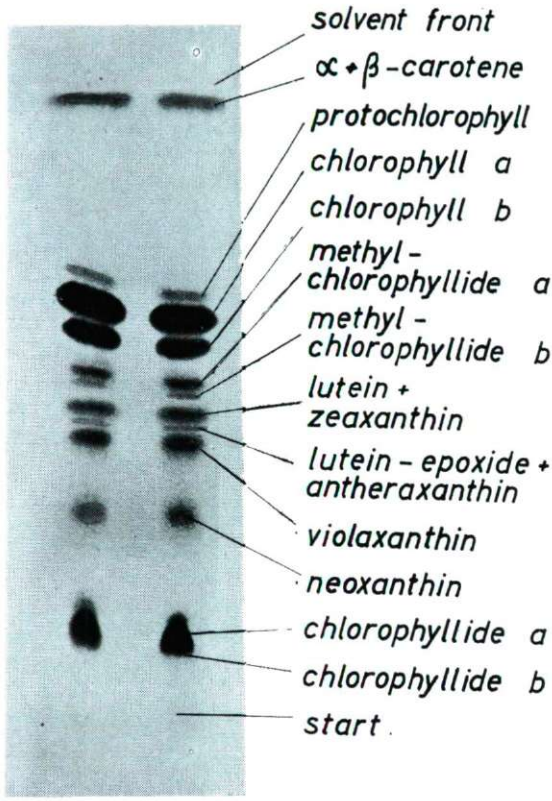

Fig. 3. Thin layer chromatographic (TLC) extraction of plastid pigments extracted by Methanol–aceton (1 : 10). The pigments are from the young leaf of *Oryza sativa* L., kept in darkness.

Results and discussion

1. Succession of the separation of pigments

On the plate the three groups of colouring agents — carotenes, xantophylls, and chlorophylls — are distinctly separated. They can easily be recognized by their colour, their reciprocal site and their Rf-value (Fig. 1).

In case of a cellulose — Silica gel system, the chlorophylls are localized on the plate between carotenes and xantophylls. In an impregnated layer, ho-

wever — MÜLLER (1964) — they take place among the xantophylls. The sequence of pigments according to the Rf-values, is inverse on the non-impregnated plates, as compared with the plates impregnated by EGGER (1962) and RANDERATH (1966). Depending on the number of substituents the speed of migration is changed in opposite way by the two systems of separation, in case of carotinoids with oxygen (hydroxyl, resp. epoxyl) content (Fig. 2).

Tab. 1. Rf-values of pigments in the thin-layer

A = Kieselgur layer impregnated by RANDERATH (1962) and EGGER (1962) with subacid vegetable fat. Wash liquid: methanol-acetone-water, at a ratio of 20:4:3.

B = Silica gel-Cellulose powder, ratio 2:1, a non-impregnated layer used by us. Wash liquid: petroleum ether-benzene-absolute alcohol-water, at a ratio of 5:5:1:0,5.

Pigments	Rf-values		Number of Substituents	
	A	B	—OH	—O—
Chlorophyllide b	—	0,06	—	—
Chlorophyllide a	—	0,10	—	—
Neoxanthin	0,95	0,24	3	1
Violaxanthin	0,84	0,34	2	2
Lutein-epoxide	0,72	—	2	1
Lutein	0,56	0,38	2	0
Zeaxanthin	0,54	0,40	2	0
Cryptoxanthin	0,07	—	1	0
Chlorophyll b	0,25	0,49	—	—
Chlorophyll a	0,13	0,53	—	—
Protochlorophyll	—	0,57	—	—
Pheophytin b	0,07	0,69	—	—
Pheophytin a	0,01	0,73	—	—
Carotenes	0,00	1,00	0	0

2. Identification of pigments

From the intensive and characteristic colour of pigments we can easily conclude on the single colouring agents. From the intensity of colour and the size of stain we may carry out semi-quantitative evaluations, too. For an exact qualitative determination we have recorded absorption curves from the pure pigments by means of a Unicam SP 800 photometer, in different eluting media. The single colouring agents were identified on the basis of λ-maxima, as well.

Tab. 2. λ-maxima of chlorophylls in mμ, measured in red wavelength range.

Pigment	Eluting media			Metric data
	acetone	ethanol	methanol	
Protochlorophyll	664	666	667	own
Chlorophyll a	663 663	— 665	— 665	Müller's own
Chlorophyll b	645 645	— 650	— 651	Müller's own

The main carotinoids of the leaf get separated on the thin-layer applied without any transformation. The yellow colouring agents are, anyway, to be peeled off immediately after chromatography, before the evaporation of the wash liquid, since they suffer oxidative decomposition even in the dark. Even After standing 5 to 10 minutes, violaxanthin, neoxanthin and lutein-5, 6-epoxide change abready into a blue or bluish-green shade. This is manifested in the shift of maxima of the absorption curves towards the shorter wavelengths, in their general sinking and, later on, in their dissappearance. In case of peeling off in two minutes, these transformations do not occur. In a dissolvent and in the dark, on the other hand, even the epoxidic carotinoids mentioned above can be preserved without any alteration for a rather long time. The value of absorption maxima largely depends on the purity of the eluting medium, particularly on its water content.

We have determined from the separated pigments the absolute and relative quantity of the colouring agents as well as their processes of decomposition depending upon various factors. This, however, will be the topic of succeeding monographs.

Summary

We have elaborated a new extracting and thin layer chromatographic method for separating the leaf pigments.

Our procedure has the following advantages:

1. We use a new extracting technique whereby pigments are but slightly changed. The extract can immediately be used for chromatography, without any other process.

Tab. 3. The absorption λ-maxima of carotinoids in mμ

Metric data: (1)* = MÜLLER (1964),
(1)** = own measurement,
(2—4) = GOODWIN (1960).

Carotinoids	Eluting media			
	1 ethanol	2 chloroform	3 n-hexane	4 benzene
Carotenes	452, 482* 449, 475**	466, 497	425, 451, 482	
Cryptoxanthin		433, 463, 497	425, 451, 483	
Zeaxanthin	451, 480*	429, 462, 494	423, 451, 483	
Lutein	446, 476, 420* 444, 472, 420**	428, 456, 487	420, 447, 477	
Lutein-5, 6-epoxid	423, 445, 473*		442, 471	453, 482
Violaxanthin	441, 443, 471* 440, 468**	424, 451, 482	443, 472	454, 484
Flavoxanthin	422, 446, 400*	430, 459	421, 450	432, 481
Neoxanthin	415, 439, 466* 415, 437, 465**	447, 476	437, 466	447, 477

2. The green and yellow colouring agents show a clearcut separation on a plate, already after a single washing. The single zones can be peeled off neatly, thus permitting the qualitative investigation and quantitative determination of pigments.
3. The separation of colouring agents, together with extraction, does not take more than 45 minutes.

We are grateful to GÁBOR HORVÁTH for his versatile technical assistance.

References

BOOTH, V. H. (1959): The extraction of pigments from plant material. — The Analyst *84*, 464—465.

COHEN-BAZIRE, G., SISTROM, W. R., and STANIER, R. Y. (1957): J. cell. comp. — Physiol. *49*, 25—31.

EGGER, K. (1962): Dünnschichtchromatographie der Chloroplast-Pigmente. — Planta *58*, 664—667.

HAGER, A.—BERTENRATH, T. (1962): Verteilungschromatographische Trennung von Chlorophyllen und Carotinoiden grüner Pflanzen an Dünnschichten. — Planta *58*, 546—568.

HAGER, A.—MEYER—BERTENRATH, T. (1966): Isolierung und quantitative Bestimmung der Carotinoide und Chlorophylle von Blättern, Algen und isolierten Chloroplasten mit Hilfe dünnschichtchromatographischer Methoden. — Planta *69*, 198—217.

IHÁSZ, I. (1960): Gyakrabban előforduló pázsitfüvek és herefélék karotintartalmának meghatározása és értékelése (Determination and evaluation of the carotene content of the frequently occurring grasses and clover species). — Agrokémia és Talajtan *9*, 559—571.

IHÁSZ, I. (1967): Néhány zöld pillangós β-karotin és össztokoferol tartalmának vizsgálata (Investigation of the β-carotene and total tocopherol content of some green *Papilionaceae*). — Agrokémia és Talajtan *16*, 635—643.

KALER, V. L., and SHLYK, A. A. (1962): Biokhimiya *27*, 599—608.

KOSKI, V. M., and SMITH, J. H. C. (1968): Amer. chem. Soc. *70*, 3558—3564.

MÜLLER, F. (1964): Chromatographische Pinteruntersuchungen an röntgeninduzierten Chlorophyllmutanten von *Pisum sativum*. — Planta *63*, 301—315.

RANDERATH, K. (1962): Dünnschicht-Chromatographie. — Verlag Chemie, GMBH, Weinheim.

RÉTALJINÉ és JAKABFINÉ (1969): Adatok kétféle módon tartósított zöld lucerna karotinoid festékeinek összehasonlításával (Data concerning the comparison of the carotinoid colouring agents of green lucerne preserved in two ways). — Agrokémia és Talajtan *18*, 117—131.

SCHERMA, J.—ZWEIG, G. (1967): Separation of chloroplast leaf pigments by thin-layer chromatography on cellulose sheets. — J. Chrom., *31*, 297—306.

STEEMAN—NIELSEN, E. (1961): Physiol. Plant. *14*, 868—876.

STRAIN, H. H. (1958): Chloroplast Pigments and Chromatographic Analysis. — The Pennsylvania State University, University Park, Pennsylvania.

SZÁSZ, Gy. (1964): Papírkromatográfia és vékonyrétegkromatográfia (Paperchromatography and thin-layer chromatography). — Medicina *25*.

WALGER, J. és THURÁNSZKYNÉ (1962): Egyszerű módszer zöldnövények és szénák karotin tartalmának meghatározására (A simple method for determining the carotene content of green plants and hays). — Agrokémia és Talajtan *11*, 443—454.

WALGER, J. és THURÁNSZKYNÉ (1965): Gyorsszárított lucernaliszt karotintartalmának stabilizálása antioxidánsokkal (Stabilization of the carotene content of quickly dried lucerne flour by means of antioxidants). — Agrokémia és Talajtan *14*, 87—94.

WORKER, N. A. (1957): Rapid producere for the chromatographic separation and spectrophotometric estimation of certain pasture lipides: Carotene, xanthophyll and chlorophyll. — J. Sci. Food Agric. *8*, 442—444.

Address of the authors:
Dr. I. MARÓTI
ÉVA GABNAI
Department of Botany,
A. J. University, Szeged,
Hungary