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A Method of Storage of Leaf Samples for Chlorophyll Analysis

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Souhrn

Byla vyzkoušena metoda, kterou lze uchovávat vzorky listů po 1 až 3 měsíce, aniž poklesne množství chlorofylu ($a + b$) o více než 5 % respektive o 10 %: Kotoučky o průměru 8 mm, vyříznuté z listů, se po 10 až 15 dají do zkumavek velikosti 10 až 12 × 85 až 110 mm. Přidá se špetka $MgCO_3$ a 2 ml bezvodého předestilovaného acetonu. Zkumavka se asi do poloviny ponoří do vody teplé cca +80° C. Jakmile se aceton začne vařit, zkumavka se rychle uzavře zátkou prosycenou teplým parafinem a ochladí se ponořením do studené vody. Zkumavky se skladují ve tmě při teplotách okolo 0° C.

Probíhající změny v obsahu barviv nesníží výsledky kolorimetrického stanovení, pokud nejde o kyselá pletiva (begonie, červené odrůdy krmné kapusty). Metoda je rychlá, provozní a lepší než ostatní zkoušené jednoduché postupy. Osvědčila se pro tykev, slunečnici, krmnou kapustu (některé odrůdy), cukrovku, jetel a tabák. Lze jí užít s výhodou pro analysu chlorofylu při zemědělských pokusech většího rozsahu.

Summary

In the course of experiments with leaf samples of pumpkin, sunflower, leafy fodder kale, sugar beet, tobacco, clover and begonia several methods of storing material were compared with the object of determining the amount of chlorophyll ($a + b$) after 1 to 3 months. Chlorophyll analyses checked by paper chromatography provided evidence that the most reliable method of serial analyses is the following: Leaf discs are heated in very pure anhydrous acetone to its boiling point (with some $MgCO_3$ present), rapidly closed with a paraffin stopper and then kept in the dark at temperatures just above 0° C. 50 leaf samples can thus be prepared for storage within 30 minutes. The

decrease in total chlorophyll ($a + b$) content after 28 days of storage does not exceed 5%, after 84 days of storage 10% of the initial value. Chemical conversions of chlorophyll take place here which do not appreciably affect the spectrum of the acetone extract. The method cannot be applied to plants with acid-reacting cell juice (begonia, red varieties of fodder kale and the like). Other practicable methods of storage (a different storage in acetone, storage of fresh samples at +2 to +3° C, drying at +50° C, storage at laboratory temperature) bring about more serious destruction of the pigments.

Introduction

When extensive experiments are carried out, especially during the vegetation season, it is often convenient to postpone chlorophyll analyses to a later time. For this reason numerous experiments were carried out with leaf sample storage with the aim of ensuring minimal changes in labile leaf pigments.

WILLSTÄTTER and STOLL (1913) recommend the drying of the "more resistant" leaves (*Urtica*, *Galeopsis)* at a slightly increased temperature (above a stove), the "less resistant" ones (*Sambucus*, conifers) in a vacuum exsiccator over sulphuric acid. HARRIMAN (1930) gives a more exact temperature for soya and *Nasturtium* drying +45° (+30°) to +60° C; exsiccation over sulphuric acid is suitable for four days at 70 mm. Hg. Temperatures between 0° and +30° C are not recommendable as a number of enzymes has a destructive effect in that range. This was confirmed by SCOTT and KRAMER (1949) who did not find significant differences between 0° and +20° C for asparagus storage; at all the temperatures tested chlorophyll was severely destroyed in relation to time from 0 to 7 days. However, two-day storage of grasses at room temperature did not affect the chlorophyll content (HARBERTS et al. 1955). According to DELEANO and DICK (1938) leaves of *Salix fragilis* L. are more conveniently dried in the shade than in the light where 43% of the initial chlorophyll content is decomposed within 120 hours at +34 to +38° C. For drying times of 6 hours, 3·4% is decomposed at +40° C, 76·6% at +65° C and practically the entire amount above +80° C. Thus the authors disagree about the optimal temperature for drying: in addition to +45 to 50 °C (COSGROVE and GUTH 1954; MARY et al. 1954) also +70 to +80° C is given (GENEST 1955, for spinach).

Of sub-zero temperatures only those at which the sample freezes thoroughly, i. e. —10° to —40° C are reported to be of importance (GUTHRIE 1929; HARRIMAN 1930). GUTHRIE succeeded in keeping sunflover and potato leaves for 7 to 28 months. Losses were not proportional to time and amounted to 0—12%. This method is often used (MCNULTY and NEWMAN 1956 stored fruit-tree leaves for 3 weeks at —17·8 to —12·2° C; WAGENKNECHT et al. 1952 stored green peas at —18 to —23° C, etc.).

Blanching (submersion in boiling water for 1 min. to inactivate enzymes prior to storage) is occasionally recommended (WAGENKNECHT et al. 1952a, 1952b, for frozen peas) but also rejected (DUTTON et al. 1943, for dehydrated spinach). It depends on the type of storage and on the water content of samples. According to STRAIN (1954) blanching like drying at 20° C brings about the formation of chlorophyll isomers (a', b').

In experiments with medicinal plant leaves (MARY et al. 1954; COSGROVE and GUTH 1954) freeze-drying was found more convenient than drying at +45 to +50° C for 36 hours. The dried samples contained 7 to 25·5% less chlorophyll.

ZSCHEILE et al. (1944) recommend the freezing of maize leaves and keeping them at —20° C for a month. If storage for more than 7 months is desired, then fresh leaves should be kept in acetone. Vacuum drying at +40° C is not suitable.

The aim of this paper was to test a method which would suit field experimenters; which would be easy from the point of view of time and equipment (i. e. without freeze-drying and deep-freeze temperatures). Conservation and storage should not diminish the total chlorophyll ($a + b$) content, as measured by common colorimetry, by more than 10%.

Material and Methods

Plants. Fully developed or ageing leaves of pumpkin of unknown origin, sunflower (firstly, newly selected strain No. 147 Sládkovičovo; secondly, of unknown origin), leafy fodder kale (*Brassica oleracea* var. *acephala*, Markstammkohl and Coulet de Flandre varieties), sugar beet Dobrovická A var.), tobacco (population of *Nicotiana tabacum* L.), clover (selected types from populations of *Trifolium pratense* L.) and begonia (*Begonia rex* PUTZEYS) were used.

Sampling. In order to save space it is not always necessary to store all the material; it suffices to remove a representative sample with a cork-borer (a definite number of discs) which can be compared with the whole leaf in several analyses. If the quantity of chlorophyll in the entire leaf area is calculated from the area of the removed discs a certain error is introduced because the area of leaf nervature is included which contains very tittle chlorophyll.

In the described experiments one sample contained always 12 discs of 8 mm. in diameter (0·5 cm.^{2}), i. e. a total of 6 cm^2. leaf area. Since 64 samples of the same chlorophyll content were always required for the purpose of comparison, they were removed as follows: from each species (with the exception of clover) 12 leaves of approximately the same size were always removed. Each sample contained discs from all parts of the leaf (from base to apex) but each disc originated from a different leaf. Thus in Fig. 1, sample No. 1 was composed from a disc from position 1 of the first leaf, a disc from position 2 of the second leaf etc. until the last disc came from position 12 of the twelfth leaf. Sample No. 2 contained a disc from position 2 of the first leaf, a disc from position 3 of the second leaf etc. until the last disc was taken from position 1 on the twelfth leaf, and so on. This procedure ensured a sufficient homogeneity of all samples (coefficient of variation for sunflower was 2·08, for tobacco 1·59, for sugar beet 5·07 etc.).

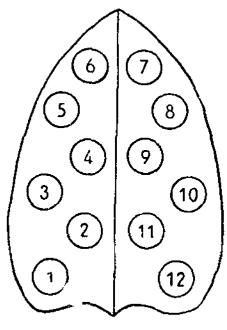

Fig. 1. Diagram of removal of leaf samples (description on p. 289).

Storage methods. A. Discs in test-tubes (10 × 110 mm. or 10—12 × 85 mm.) were covered with 2 ml. anhydrous acetone (pure, redistilled), some $MgCO_3$ added and stoppered with a cork stopper.

B. The same as (A) but prior to stoppering the test-tube was submerged (above half of its length) in water at +80° C. When acetone began to boil (b. p. +56·5 °C) the test-tube was quickly closed with a stopper saturated with warm paraffin, removed from the water bath and cooled by submersion in cold water.

C. Discs in open test-tubes were dried for 36 hours at +50° C in a thermostat.

D. Fresh discs in test-tubes were closed with paraffin-saturated stoppers.

Samples were stored in closed boxes at room temperature or in a cold-chamber at *c.* +2 to +3° C.

Determination of chlorophyll (a + b) content. The discs were ground with a small amount of silica sand, extracted with anhydrous and 85% acetone. The extract was filtered through a G 4 glass sinter and the volume made up to 20 ml. with acetone. The extinction of acetone extracts was measured in 1 cm. cuvettes on a PULFRICH photometer using a S 64 filter. Chlorophyll content was computed from the extinction values with the aid of a calibration curve for a 3 : 1 mixture of chlorophyll *a* and *b*. For calibration crystalline chlorophyll was used obtained from Dr. E. WIEDEMANN, of the Sandoz Co., Basel, Switzerland.

Control of pigment decomposition. Checking was carried out by paper chromatography (cf. ŠESTÁK 1958). Samples on Whatmann paper 1 or 3 were developed unidimensionally in the ascending direction. Size of paper 270 × 135 mm. Extract used was in acetone or ether. Solvent system employed was either tol ene or a mixture of benzin-benzene-chloroform-acetone-isopropanol (50 : 35 : 10 : 0·5 : 0·17) described by HAGER (1955) or a mixture of toluene and methanol (400:1), which is a modified system of CHIBA and NOGUCHI (1954). Time of development was about 60 min. Carotenoid spots were marked with pencil immediately after development (they are oxidized rapidly and disappear), chlorophylls and their derivatives were observed under ultra-violet light.

Results

Changes in chlorophyll (*a* + *b*) content

Sixty-four uniform samples of a total area of 6 cm.² were removed from leaves of sugar beet, sunflower, tobacco and begonia. In 16 samples chlorophyll was determined immediately; in 8 of these directly (control), in 8 after heating with acetone and $MgCO_3$. Sets of 8 samples were preserved by using the methods (A), (B) and (C) and kept in the laboratory, sets of 8 samples were preserved by using the methods (A), (B) and (D) and kept in a cold-room at *c.* +2 to +3° C. From these sets one half (4 samples from each) was removed after 4 weeks, the other half after 12 weeks of storage, and the chlorophyll content was determined. Analytical results are summarized in Table 1. The Table shows the chlorophyll content of the control (statistically

Table 1. The decrease in the content of chlorophyll (*a* + *b*) during different types and periods deviation from the mean of four samples as compared

Plant	K Control in μg. chlorophyll (*a* + *b*) ($\bar{x} \pm 3s_{\bar{x}}$)	K_1 Determined immediately after heating with acetone	A			
			Laboratory		Cold-room	
			28 d.	84 d.	28 d.	84d.
Sugar beet	335·6 ± 3 . 6·03	— 0·5	— 26·8	— 46	— 6	— 29
Sunflower (unknown origin)	535·6 ± 3 . 4·06	0	— 24·2	— 29	— 13	— 16·7
Tobacco	281·2 ± 3 . 1·58	— 3·5	— 23·2	— 29·5	— 2·5	— 12·5
Begonia	182·0 ± 3 . 7·35	— 30·8	— 31·8	— 29·1	— 33·5	— 22

treated) and differences from the control mean value of individual experimental sets. Results varied within the range of experimental error and the differences only rarely exceeded $\pm$ 5%; therefore their statistical treatment is not presented.

It follows from Table 1 that the best results are obtained with the (B) method of storage and samples kept in the cold (with the exception of begonia acid tissue). Therefore the method was tested on other material. Three samples formed an experimental set. Table 2 shows average values as percentages of initial value (pumpkin 347 $\pm$ 3 $\times$ 3·7 μg. chlorophyll (a + b), sunflower 333 $\pm$ 3 $\times$ 5·3 μg., fodder kale var. Markstammkohl 412 $\pm$ 3 $\times$ 6·2 μg.).

Table 2. Values of chlorophyll (a + b) content during storage by method (B) (described on p. 289) in a cold-room. Expressed as % of control, analyzed immediately after removal of samples

Plant	Immediately	After 56 days	After 84 days
Pumpkin	100	96·5	95·5
Sunflower (strain 147 Sládkovičovo)	100	95	95·5
Fodder kale (var. Markstammkohl)	100	92·3	94·5

The same method was applied to storage of clover samples where discs from opposite leaf halves were compared. After 42 days of storage the amount of chlorophyll (a + b) decreased by 5·2% (average of 26 samples), after 77 days by 10% (average of 24 samples).

The method is not suitable for the storage of red varieties of fodder kale (var. Coulet de Flandre) whose red-violet colouring on the reverse side of leaves, due to anthocyans, indicates an acid character of the vacuoles. After 105 days of storage the values oscillated very considerably and the decrease in chlorophyll content lay between 7·7% and 40·2% of the initial value (based on an analysis of 48 samples).

Chromatographic control of pigment conservation

The following conclusions can be derived on the basis of comparison of 41 paper chromatograms:

of storage. A, B, C, D-types of storage (described on p. 289). For K_1, A, B, C, and D only the with the control is given (as % of initial value)

B				C		D	
Laboratory		Cold-room		Laboratory		Cold-room	
28 d.	84 d.	28 d.	84 d.	28 d.	84 d.	28 d.	84 d.
— 10·5	— 31	+ 1·5	— 10·2	— 34·6	— 46·3	— 7·5	— 20
— 13·9	— 25·5	— 3	— 7·3	— 27	— 25·5	— 5	— 11·3
— 15·7	— 28·4	— 2·5	— 7	— 47	— 48	— 9·6	— 9·2
— 35·2	— 31·3	— 31·2	— 22	— 36·3	— 30·7	+ 23·5	+ 16

1. In all methods of storage chlorophyllides are formed, which have practically the same spectrum as the chlorophylls (Holt and Jacobs 1954) and do not affect the determination of chlorophyll in acetone solution. They are formed mostly on storage in acetone, particularly by the method (B).

2. In samples in acetone (A, B) there are more spots of products arising from chlorophyll *a* on standing in the solvent (cf. Angapindu et al. 1958), visible particularly by developing in Hager's (1955) solvent mixture. Chlorophyll spots in samples stored by the method (D) are uniform.

3. During all the types of storage some phaeophytins are formed, which sometimes (sunflower, tobacco) separate into two spots of phaeophytin *a* and *b*. In clover and sugar beet not even a three-month storage by the method (A) and (B) gave rise to any detectable amount of phaeophytin. Drying of samples brings about a marked formation of phaeophytins, particularly from chlorophyll *a*. The decrease in chlorophyll content on drying is least marked with sugar beet.

4. Methods (A) and particularly (B) preserve the carotenoids well (1 spot of carotene and 2—3 spots of xanthophylls). Methods (C) and (D) bring about a frequent decrease in the amount of carotenoids, particularly of xanthophylls with more oxygen atoms in the molecule.

5. In red fodder kale (var. Coulet de Flandre) and particularly in begonia, a high amount of phaeophytins could be detected, which increases sharply on heating in acetone [method (B)] until chlorophylls disappear completely. Similarly carotenoids are broken down.

6. Results of the chromatographic study are in complete agreement with the values obtained by colorimetry and they confirm the advantages of the method (B) and of storage in cold.

Discussion

A review of literature alone displays a high variability of results. In addition to the various types of chlorophyll determination it is also very likely caused by the difference in material. Thus very acid leaves (begonia, red fodder kale of the Coulet de Flandre variety) must be excluded from storage. The pH of begonia cell juice is reported to be 1·3—1·6 (Drawert 1955, Table 5), as contrasted with the pH of 5·6—7·5 as given by various authors for clover, tobacco, sunflower and pumpkin. The exact relationship between the pH of the cell juice and the destruction of chlorophyll on storage cannot be derived as the leaf pH changes in the course of ontogenesis according to the above author and at the same time the stability of the chlorophyll-protein bond decreases (Osipova 1947; Maslova 1958) thus rendering chlorophyll more vulnerable. The effect of the acid cell juice depends very probably on cell properties, particularly on permeability and on cell structure changes due to acetone, drying etc. These assumptions are in full agreement with the irregular decrease in chlorophyll content during storage of red fodder kale as well as with the process of pigment destruction in begonia. Phaeophytinization can also be observed during grinding and extraction of fresh begonia samples and not even very high amounts of $MgCO_3$ can prevent it. The unexpected increase in chlorophyll content in the method (D) (fresh discs of begonia kept in a test-tube in the cold) can be explained by a decrease in acidity of cells by resorption of acids, their loss due to respiration etc. The problem of extraction and storage of acid plant tissues is more complex and requires a detailed independent study. However, these extremely acid types occur only rarely among the commonly analyzed plants.

Shortcomings of the drying method consist not only in the formation of phaeophytin but also in the low extractability of chlorophyll from dried ground material. It is probable that irreversible changes in chloroplast structure took place, even at temperatures recommended by Harriman (1930).

In agreement with the authors cited above in the introduction, room temperature has an unfavourable effect in the described experiments, as it varies considerably (from +10° to +25° C), even during storage in acetone. However, conservation at temperatures just above 0° C proved to be suitable even for fresh material [method (D)]. Enzymatic activity is apparently very much restricted and the decomposition of pigments by heat is prevented.

It follows from the results shown in Table 1 and 2 that the best method is (B) with storage in cold. After 3 months less than 10% chlorophyll was broken down and after 1 month less than 5%, which is permissible in serial analyses working with an error of ± 5%. Heating in acetone (boiling at +56·5° C) inhibits enzymatic activity and acetone penetrates well into the tissues. Acetone vapour displaces most of the air from above the sample and from the acetone itself; air oxygen could effect oxidation changes of the pigments. Acetone, recommended already by ZSCHEILE et al. (1944), does not bring about an allomerization of the chlorophylls which changes their spectral properties (in contrast to alcohol). The formation of chlorophyllides (STRAIN 1954) is of no consequence in the given case. Contact with the surrounding atmosphere and evaporation of the solvent are prevented by the paraffin stopper. Paraffin prevents furthermore the pigments from soaking into the cork stopper. Acids liberated from the vacuoles are partly neutralized by $MgCO_3$. Conservation in dark and in cold diminishes the possibility of other destructive processes.

According to Table 1 only the procedure (D) approximates to this method but the decrease in chlorophyll content is somewhat higher, particularly after 28 days of storage; carotenoids are also decomposed.

For serial determinations the method (B) appears to be most suitable because the samples can be preserved with very primitive equipment even under field conditions and transferred to the low-output refrigerator only on the following day. The procedure is rapid—one worker can prepare 50 samples for storage within 30 minutes (not counting the removal of discs from leaves).

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Метод хранения листьев для анализа хлорофилла

ЗДЕНЕК ШЕСТАК

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Резюме

Был испытан метод, при помощи которого можно сохранить образцы листьев в течение 1—3 месяцев, без снижения количества хлорофилла (a + b) больше чем на 5—10%.

Диски диаметром в 8 мм, вырезанные из листьев по 10—15 штук помещались в пробирки, размером 10—12 × 85—110 мм. Прибавлялось немного $MgCO_3$ и 2 мл безводного дистиллированного ацетона. Пробирка погружается в воду, нагретую примерно до 80 °C. Как только ацетон начнет кипеть, пробирка быстро затыкается пробкой, насыщенной теплым парафином, и охлаждается погружением в холодную воду. Пробирки хранятся в темноте при температуре около 0 °C.

Происходящие изменения в составе пигментов не снижают данные колориметрических измерений, за исключением кислых тканей (бегония, красные сорта кормовой капусты). Этот метод быстрее и лучше, чем остальные испытанные простые приемы. Он оказался очень удобным для работы с тыквой, подсолнечником, кормовой капустой (некоторые сорта), сахарной свеклой, клевером и табаком. Его можно успешно использовать для анализа хлорофилла при сельскохозяйственных опытах широкого масштаба.