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Leucoanthocyanidins of the leaves of apple-tree

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Leucoanthocyanidins belong to relatively little known flavonoid compounds and their physiological rôle has not been elucidated, although they occur commonly in higher plants. The structure of these compounds is based on polyhydroxy--flavan system (flavandiols, flavantriols, flavanolons) and thus they can be transformed on heating with acids into anthocyanidins. Leucoanthocyanidins occur as low-molecular compounds — monomers or dimers, often conjugated with catechins — or in a polymeric form.

Leucoanthocyanidin content in the leaves of *Rosaceae* plants during the vegetative period was studied by Swain and Hillis (1960) in plum-tree and by Lewak (1965) in the leaves of hawthorn. Although different methods were applied, similar patterns of the changes of the leucoanthocyanidin content in the leaves of the plants studied were found in both cases.

Structural studies of the leucoanthocyanidins occuring in the *Rosaceae* plants were carried out on the dimeric leucoanthocyanidins of the leaves and fruits of hawthorn (Freudenberg, Weinges 1961; Weinges 1961; Lewak 1964; Lewak, Radomińska 1965) and of the leaves of wild strawberry (Creasy, Swain 1965). It was found that the molecules of these compounds are built mainly from the leucocyanidin units connected among themselves through, or bound to (-) epicatechin. Leucopelargonidin presents a secondary component of the leucoanthocyanidins of hawthorn. The structure of the leucoanthocyanidins of apple-fruits has been studied by Ito and Joslyn (1964). It was found that these compounds contain leucocyanidin and leucopelargonidin moieties. No data have been available till now concerning structure of the leucoanthocyanidins of the leaves of apple-tree, nor on the content of these compounds in apple-tree tissues.

The aim of this work was to characterize leucoanthocyanidins of the leaves of apple-tree and to study changes of their content during vegetation.

RESULTS AND DISCUSSION

Colorimetric method of determination of leucoanthocyanidins elaborated by Swain and Hillis (1959) can be applied in comparative studies only since particular compounds of this group transform into anthocyanidins with different yields

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depending on their structure. However this method finds general application because of its simplicity and lack other techniques. For the same reasons this method was accepted in this work as the base of all determinations.

Macoun variety was selected for the studies on the changes of the leucoanthocyanidin content of the apple-tree leaves because of its relatively high content of the compounds studied. Some series of determinations were carried out also on the leaves of the Perkins variety in which the leucoanthocyanidin content is lower by 10%.

Preliminary experiments indicated occurence of both, low-molecular and polymeric leucoanthocyanidins in the leaves of apple -tree. Taking into consideration different solubilities of these two groups of compounds in water and alcohols the conditions ensuring solubilization of a maximal amount of low-molecular and polymeric leucoanthocyanidins have been found for extraction of the freeze-dried leaves. It was established that the best results can be obtained by extraction with 60% ethanol.

Table 1

Characteristics of anthocyanidins formed from apple-tree leucoanthocyanidins

Anthocyanidin	R_f (paper chromatography)			λ_{max} nm	λ_{max} shift after addition of $AlCl_3$ nm
	m — cresol, 5.5 N HCl, acetic acid 1 : 1 : 1	HCOOH, conc. HCl, water 5 : 2 : 3	acetic acid, conc. HCl, water 5 : 1 : 3		
Main product formed from the apple-tree leucoanthocyanidins	0.71	0.26	0.35	550	29
Other product	0.81	0.39	0.54	535	0
Cyanidin, standard	0.71	0.26	0.34	550	25
Pelargonidin, according to Hyashi (1962)	0.82	0.33	0.55	530	0

Leucoanthocyanidins of the leaves of apple-tree were transformed into anthocyanidins, which were characterized by paper chromatography and spectrophotometrically. The results obtained are presented in Table 1.

The results presented indicate that leucocyanidin is the main component of the leucoanthocyanidins of the leaves of apple-tree and that leucopelargonidin is an additional component. This observation is consistent with results of Ito and Joslyn (1964) for the fruits of the same plant. Similar results obtained in studies of the leucoanthocyanidins of the leaves and fruits of hawthorn (Lewak, Radomińska 1965) indicate similarities between biosynthesis of these compounds in *Rosaceae* plants. Traces of a third unidentified anthocyanidin (R_f — 0.80 in the system acetic acid, hydrochloric acid, water — 5 : 3 : 1, λ_{max} — 515 nm) were found in products of acid transformation of the mixture of apple-tree leucoanthocyanidins. It is possible that this compound is formed as a product of further degradation of leucoanthocyanidin molecule.

The content of leucoanthocyanidins was determined in 60% ethanolic extracts of the freeze-dried leaves collected at intervals between May and November. In parallel series fully developed, mature leaves from the middle sections of the branches and young leaves from the tip of the shoot were collected. The results of these determinations are presented in Fig. 1.

The fact that the content of leucoanthocyanidins is markedly higher in mature leaves than in the developing ones indicates that the biosynthesis of these compounds

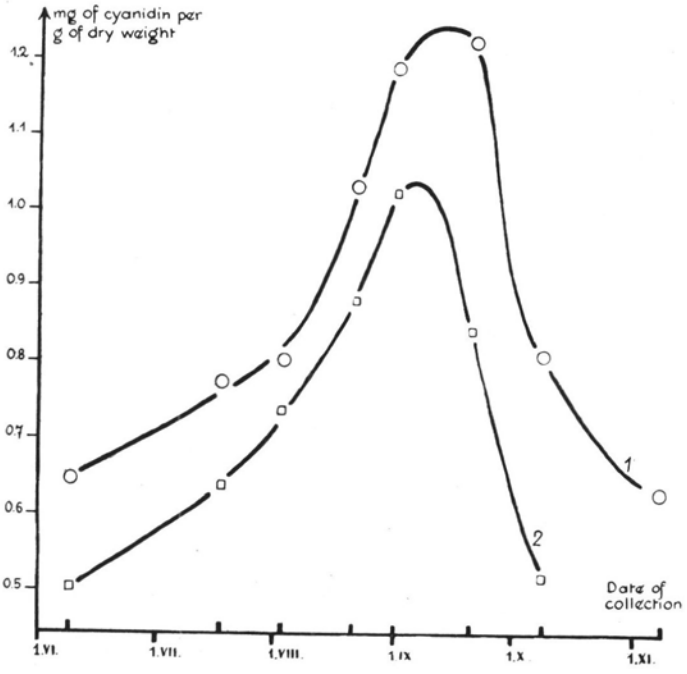

Fig. 1. Changes of total leucoanthocyanidin content in the leaves of apple-tree, 'Macaun' variety, during vegetation.

1 – leucoanthocyanidin content in mature leaves; *2* – leucoanthocyanidin content in developing leaves.

proceeds in the leaves. The curves presented in Fig. 1 have similar patterns to the curves representing changes of the leucoanthocyanidin content in the leaves of other *Rosaceae* plants (Swain, Hillis 1960; Lewak 1964). After an initial period of rapid growth, in September a maximum is observed of the content of the compounds studied and than a decrease follows.

Fractionation of the leucoanthocyanidins of the leaves of apple-tree with organic solvents allowed to isolate two fractions: a) low-molecular leucoanthocyanidins soluble in ethyl acetate and b) flavan polymers insoluble in the same solvent. This procedure has been applied for quantitative determination of both leucoanthocyanidin fractions in samples of freeze-dried leaves. Changes during vegetation of the content of low- and high-molecular leucoanthocyanidins in fully developed leaves of apple-tree, Macoun variety are presented in Fig. 2.

The changes of the leucoanthocyanidin content in developing leaves show si-

milar patterns but the content of the polymeric compounds is lower by 0.100 to 0.200 mg/g. This means that the fraction of the polymeric compounds is responsible for differences between total contents of leucoanthocyanidins in young and mature leaves. Polymeric leucoanthocyanidins play also a major role in differences of the total content of leucoanthocyanidins between samples collected at different stages

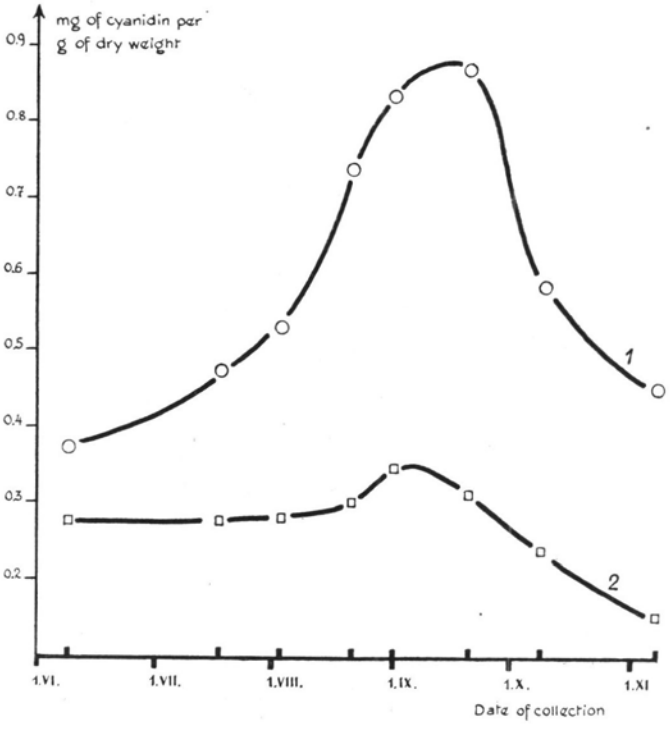

Fig. 2. Changes of the content of low-molecular and polymeric leucoanthocyanidins in the leaves of apple-tree, Macaun variety during vegetation.

1 — polymeric leucoanthocyanidins; *2* — low-molecular leucoanthocyanidins.

of vegetation, since the curve representing changes of the content of the low-molecular compounds until October is nearly flat.

Biosynthesis of polymeric flavans proceeds probably in two stages: a) biosynthesis of C_{15}-units and b) condensation of low-molecular flavans (Roux 1958, Freudenberg 1960). Our results indicate that the increase of the content of the polymeric flavans in the leaves of apple-tree during vegetation is connected with simultaneous increase of the rate of biosynthesis of C_{15}-units and with stimulation of the polymerization processes, so that the content of the low-molecular compounds remains almost unchanged. The polymerization rate increases markedly in the period preceding ripening of the fruits. Perhaps this process goes on until lignification starts and its products are further transformed.

It seemed interesting to find out whether both main structural groups of leucoanthocyanidins i.e. derivatives of leucocyanidin and derivatives of leucopelargo-

nidin participate to the same extent in the observed changes of the leucoanthocyanidin content in the leaves of apple-tree. For this purpose a method has been worked out for determination of the ratio of these two moieties in the material studied. The method consist in transformation of leucoanthocyanidins into anthocyanidins, separation of the products by thin layer chromatography on cellulose, elution and colorimetric determination. Results of the determinations are given in Table 2.

Table 2

Leucopelargonidin/leucocyanidin ratio in the leaves of apple-tree, 'Macaun' variety, during vegetation

Date of collection	In mature leaves	In developing leaves
8.VI.65	0.38	0.72
17.VII.65	0.37	0.70
3.VIII.65	0.36	0.66
21.VIII.65	0.34	0.62
1.IX.65	0.31	0.59
20.IX.65	0.30	0.51
8.X.65	0.32	0,47

The results presented show that leucopelargonidin/leucocyanidin ratio in leucoanthocyanidins of the mature apple-tree leaves does not depend on the vegetation period and its value is close to 0.3. On the other hand the value of this ratio is higher in the developing leaves than in the mature leaves over the whole vegetation period although this ratio decreases by more than 30% from May to October.

These results suggest that the nature of the processes of biosynthesis of the flavan units is to a lesser extent dependent on the stage of development of the leaf than on the physiological state of the whole plant.

The degree of hydroxylation of the B ring of the flavonoid system is determined on the earlier stages of biosynthesis, prior to the condensation of the C_6 and C_{6+3} units (Alston 1964). However, it seems that the increase of the relative leucocyanidin content during vegetation cannot be explained by activation of the hydroxylation of the phenylpropane units in the leaf at later stages of the vegetation period.

Our results show that early stages of the biosynthesis of aromatic compounds in the developed leaf do not depend on the vegetation stage. On the other hand, the hydroxylation of the flavan precursors in the young leaf could be delayed on the early stages of the vegetation.

Accumulation in the leaves of apple-tree of considerable amounts of polymeric leucoanthocyanidins could be connected with the observed by various authors (Hulme, Jones 1963; Hulme, Jones, Wooltorton 1964; Williams 1963) inhibitory function of the macromolecular phenolic compounds in relation to various enzymatic systems. Leucoanthocyanidins contained in vacuoles and cell membranes can bind enzymatic proteins thus acting as regulators of the metabolic processes in the leaf.

EXPERIMENTAL

Material

The experiments were carried out on the leaves of apple-tree, Macoun variety collected in the period between June and November, 1965 from six-year old trees in the garden of the Institute of Pomology in Skierniewice. For parallel series of experiments the samples were collected of the developed, mature leaves growing at the middle section of the branch and of the young leaves from the tip of the shoots. In some series of experiments the samples were used of the leaves of apple-tree collected from six-year old trees of Perkins variety in the period from June to October, 1964.

Ca 10 g samples of leaves were frozen in solid CO_2 immediately after the collection, freeze-dried and stored in paraffin wax sealed vessels.

Methods

Paper chromatography was carried out on Watman No 1 paper by ascending technique.

Thin-layer chromatography was carried out on 11×18 cm glass plates covered with 0.3 mm layer of MN 300 G cellulose (Macherey u. Nagel, Düren) by ascending technique.

Concentration of the solutions was carried out using vacuum rotatory evaporator at bath temperatures below 40°.

Colorimetric determinations of anthocyanidins were carried out on a Hilger photocolorimeter using filter No. 55 in Pyrex glass 10 ml tubes, dia. 15 mm.

The absorption curves for anthocyanidins were obtained using VSU 1 Spectrophotometer (Zeiss, Jena) using glass cells, $1 = 10$ mm.

All quantitative results are arithmetic mean values of three parallel determinations.

Determination of total leucoanthocyanidins in freeze-dried leaves of apple-tree

8 ml of 60% ethanol was added to 500 mg of ground, freeze-dried leaves. After 3 h the extract was filtered to the distillation flask, another 8 ml portion of 60% ethanol was added to the residue and the mixture was left for 16 h. The extract was filtered and the residue was washed with 8 ml of the solvent. Combined extracts were evaporated to dryness.

2 ml of water and 2 ml of ethyl ether were added to the residue, the flask was shaken and the ether phase was separated. The extractions with 2 ml portions of ether were repeated 10 times. Combined ether extracts were washed with 1 ml of water wich was then added to the remaining water phase. Ether extract was discarded and the water phase was made up to 10 ml in a volumetric flask (solution A).

Determination of total leucoanthocyanidins was carried out in 0.5 ml samples of the solution A by the method of Swain and Hillis (1959) using a calibration curve prepared for solutions of cyanidin in n-butanol containing 5% of conc. hydrochloric acid.

Determination of low- and high-molecular leucoanthocyanidins in freeze-dried leaves of apple-tree

5 ml of solution A was extracted 10 times with 2 ml portions of ethyl acetate. The extract was washed with 2 ml of water which was then added to the remaining water phase. The water phase was made up to 5 ml (solution B).

The ethyl acetate extract was evaporated to dryness. The residue was dissolved in 5 ml of water (solution C).

The content of the polymeric leucoanthocyanidins was determined by the Swain and Hillis method in the solution B and that of the low-molecular leucoanthocyanidins — in the solution C.

Determination of leucocyanidin and leucopelargonidin in freeze-dried leaves of apple-tree

1 g sample of ground, freeze-dried leaves was heated with 30 ml of ethanol containing 40% of conc. hydrochloric acid on boiling water bath for 30 min. Cool mixture was filtered, the residue was washed on the filter with 30 ml of water and the filtrate was concentrated to 10 ml. Then, 15 ml of water was added and the mixture was extracted five times with 10 ml portions of ethyl ether and two times with 10 ml portions of ethyl acetate. The water solution remaining after the extraction was evaporated to dryness, dissolved in 10 ml of water and extracted five times with 5 ml portions of amyl alcohol.

The amyl alcohol extract was evaporated to dryness and the residue was dissolved in 2.5 ml of ethyl alcohol. The ethanolic solution was subjected to thin-layer chromatography on cellulose in acetic acid, conc. hydrochloric acid, water 3 : 1 : 6. Under these conditions cyanidin has Rf — 0.30 and pelargonidin — 0.44. The cellulose bands containing separated dyes were eluted with 5 ml portions of ethanol containing 1% of hydrochloric acid. The contents of the dyes in eluates were determined colorimetricaly using the calibration curve set up for solutions of cyanidin in the same solvent.

SUMMARY

It was found that leucoanthocyanidins of the leaves of apple-tree consist of a fraction of low-molecular compounds and a fraction of polymeric ones. Leucocyanidin is the main structural unit of the compounds studied. Leucopelargonidin occurs as additional basic unit.

Studies have been carried out on changes of the total leucoanthocyanidin content, of the contents of high- and low-molecular compounds and of the leucopelargonidin/leucocyanidin ratio

in the leucoanthocyanidins of apple-tree leaves. The experiments were carried out in two parallel series on young, developing and mature leaves.

The results obtained allowed to conclude that:

a) leaves present a site of the biosynthesis of leucoanthocyanidins,

b) polymerization of low-molecular leucoanthocyanidins occurs in leaves,

c) the structure of the leucoanthocyanidins of the leaves of apple-tree depends to a lesser extent on the leaf's development stage than on the physiological state of the plant.

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STRESZCZENIE

Praca niniejsza jest kontynuacją badań nad leukoantocyjanami roślin rodziny *Rosaceae* (Lewak 1964, 1965; Lewak, Radomińska 1965). Stwierdzono, że podobnie jak leukoantocyjany owoców jabłoni (Ito, Joslyn 1964) również i leukoantocyjany liści tej rośliny są zbudowane głównie z jednostek leukocyjanidyny, a w mniejszym stopniu — leukopelargonidyny. Zwraca uwagę podobieństwo struktury badanych związków i leukoantocyjanów innych roślin rodziny *Rosaceae* (Lewak, Radomińska 1965; Creasy, Swain 1965).

Stosując metodę Swaina i Hillisa (1959) przebadano zmiany zawartości leukoantocyjanów w liściach jabłoni w okresie wegetacji. Zbierano próbki liści rozwiniętych, dojrzałych z środkowych części gałęzi, a równolegle próbki młodych, rozwijających się liści z wierzchołków pędów. Stwier-

dzono zmiany całkowitej zawartości leukoantocyjanów analogiczne do obserwowanych w liściach innych roślin rodziny *Rosaceae* (Swain, Hillis 1960; Lewak 1965). Liście młode zawierały o ok. 10% mniej leukoantocyjanów niż liście rozwinięte co świadczy o biosyntezie badanych związków w liściu.

Opracowano metodę równoległego oznaczania leukoantocyjanów niskocząsteczkowych i polimerycznych w wyciągach z liści jabłoni. Stwierdzono, że zarówno za różnice zawartości leukoantocyjanów w liściach rozwiniętych i młodych, jak i za zmiany zawartości tych związków w trakcie wegetacji odpowiedzialna jest głównie frakcja leukoantocyjanów wielkocząsteczkowych. Dane te świadczą o polimeryzacji badanych związków w liściu.

Opracowano metodę oznaczania stosunku elementów leukopelargonidyny do leukocyjanidyny w leukoantocyjanach liści jabłoni. Stwierdzono niemal niezmieniający się stosunek tych jednostek w trakcie wegetacji w leukoantocyjanach liści rozwiniętych natomiast wyraźne zmiany w leukoantocyjanach zawartych w liściach młodych. Świadczy to, że charakter procesu biosyntezy jednostek flawanowych jest w mniejszym stopniu zależny od wieku liścia niż od stanu fizjologicznego całej rośliny.

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