Leuco-Anthocyanins

1. DETECTION AND IDENTIFICATION OF ANTHOCYANIDINS FORMED FROM LEUCO-ANTHOCYANINS IN PLANT TISSUES

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A method for the detection of leuco-anthocyanins, and the chromatographic identification of the anthocyanidins formed from them by boiling with mineral acid, has been described in an earlier paper (Bate-Smith, 1953). This method has been improved and applied to the leaves and other tissues of numerous species of plants. In the present paper the results of identification of anthocyanidins produced from leuco-anthocyanins in some of these species are reported. In the paper which follows the systematic distribution of the leuco-anthocyanin reaction in leaves is discussed.

EXPERIMENTAL AND RESULTS

Paper chromatographic separation of anthocyanidins

The main problem is to obtain a solution of the anthocyanidins in sufficient concentration for paper chromatography, without at the same time concentrating irrelevant substances. This is accomplished by converting the leuco-anthocyanins into anthocyanidins in aqueous solution (rather than in methanol as described previously) and extracting the anthocyanidins with isoamyl alcohol.

Conveniently, 0.2–1.0 g. of tissue is covered with 2N-HCl (about 3 ml.) in a test tube, and heated in boiling water for 20 min. The aqueous solution is decanted (filtered if necessary) into a small narrow test tube and shaken with sufficient isoamyl alcohol (3-methylbutan-1-ol) to give a supernatant layer just deep enough to be drawn cleanly into a capillary tube, from which the solution is spotted on the starting line of the chromatogram. The applications are repeated, employing a current of hot air to accelerate drying, until the colour is deep enough to ensure visibility of the anthocyanidins on the developed chromatogram. A marker of known identity is applied on each paper.

In order to prevent the anthocyanidins from fading, it is necessary to maintain a low pH during chromatography. This was originally achieved (Bate-Smith & Westall, 1950) by using the upper phase of the mixture n-butanol–2N-HCl (1:1, v/v). A solvent brought to our notice by Forestal Laboratories has given better-defined spots and more consistent results. This ‘Forestal solvent’ consists of water–acetic acid–cone. HCl (10:30:3, v/v). Solvents containing m-cresol and HCl have also been tested, and one of these consisting of m-cresol–5N-HCl–acetic acid (1:1:1, v/v) (acetic acid is added in order to bring the aqueous and phenolic constituents into a single phase) has given promising results. As was found with the phenolic solvents employed in earlier work on the chromatography of flavonoid compounds (Bate-Smith, 1949), this solvent has the effect of suppressing the lyophilic properties of methoxyl groups to a greater extent than the aliphatic solvents, so that the order

References:

Table 1. \(E_{\text{max}}\) and \(R_p\) values of some anthocyanidins

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>(E_{\text{max}}) (m(\mu))</th>
<th>(R_p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Butanol–HCl</td>
<td>0·80</td>
<td>0·68</td>
</tr>
<tr>
<td>Acetic acid–HCl</td>
<td>0·69</td>
<td>0·72</td>
</tr>
<tr>
<td>m-Cresol–HCl–acetic acid</td>
<td>0·56</td>
<td>0·45</td>
</tr>
</tbody>
</table>

Table 2. Spectrographic examination of anthocyanidins from leaves

<table>
<thead>
<tr>
<th>Species</th>
<th>(E_{\text{max}}) (m(\mu))</th>
<th>Chromatographic identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamomum sericeum</td>
<td>543</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Erioderpa japonica</td>
<td>543</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Pseuda gracissima</td>
<td>545</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Raphiolepis indica</td>
<td>545</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Rhoicissus rhomboidea</td>
<td>547, 553</td>
<td>Cyanidin, delphinidin</td>
</tr>
<tr>
<td>Rumex lunaria</td>
<td>545</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Thea sinensis</td>
<td>543, 557</td>
<td>Cyanidin, delphinidin</td>
</tr>
</tbody>
</table>

of the \(R_p\) values of the anthocyanidins is different in this solvent from those in acetic acid–HCl and n-butanol–HCl (Table 1).

Most of the results recorded below have been obtained with the Forestal solvent. In every instance so far tested, however, the chromatographic results with the other solvents have agreed with those obtained with the Forestal solvent. Valuable further confirmation that the substances produced are, in fact, anthocyanidins lies in the observation that in all solvents other than those containing strong acid the substances are decolorized.

Spectrographic examination

Anthocyanidins have well-defined peaks in the visible region, either in ethanolic HCl solution or when examined directly as a spot on a paper chromatogram by the method of Bradfield & Flood (1953). By the latter method the values are accurate to no more than ± 2 m\(\mu\). Values for six anthocyanidins are given in Table 1.

The anthocyanin spots from digests of leaves were examined spectrographically on paper chromatograms and gave values of \(E_{\text{max}}\) shown in Table 2. It will be seen that the spectrographic data in every case confirmed the chromatographic identification. In the case of leuco-anthocyanin prepared from wood of *Pinus maritima*, and supplied by Professor J. Maquclier, the anthocyanidin having the \(R_p\) value of cyanidin gave a maximum of 545 m\(\mu\).

Application to various plant tissues

The earlier work (Bate-Smith, 1953) was confined to a study of white flowers, in which interference from other substances present is reduced to a minimum. No difficulty has been experienced in extending the study to leaves, in spite of the presence of chlorophyll, since this is largely destroyed in the process of heating with HCl. Any traces of chlorophyll which may remain are extracted by the isooamyl alcohol, and appear on the chromatogram near the solvent front. Leaves and other tissues pigmented with anthocyanin are, however, not easy to work with and have so far been avoided. The leaf tissues examined were from mature leaves not evidently pigmented with anthocyanins, freed from petioles and larger veins. Both immature and senescent leaves may contain anthocyanin pigment which may not be visible on mere inspection, and were therefore avoided in a survey confined to the incidence of leuco-anthocyanins. If anthocyanin pigmentation is not excessive, leuco-anthocyanins might still be detected in such instances by comparison with a control prepared from an aqueous extract of the tissue hydrolysed with dilute acid. Robinson & Robinson (1933) suggested such a procedure, selectively extracting the hydrolysed anthocyanin with isooamyl alcohol and developing the leuco-anthocyanin in the underlying aqueous layer. This would of course only apply if the leuco-anthocyanin were itself insoluble in isooamyl alcohol. Rarely, tissues may contain preformed anthocyanin in colourless form which is converted into the normal coloured form on acidification; these also have been excluded from the present study.

Catechins, when heated with hydrochloric acid, are converted into ‘phlobaphenes’ which dissolve in isooamyl alcohol to give deep golden (catechins proper) or brown (gallocatechins) solutions.

The tissues of species belonging to certain families give strong colour reactions which might interfere with the detection and identification of anthocyanidins. Such species are *Caryopteris tangutica* and *Tectona grandis* in Verbenaceae,
Table 3. Anthocyanidins formed from leuco-anthocyanins in leaves

The following results record the detection, by paper chromatography, of cyanidin (C) and delphinidin (D) formed from leuco-anthocyanins by treatment with hydrochloric acid (see text). Where the concentration of anthocyanidin was appreciably different from the average this is indicated by the letters s (strong) and w (weak).

Dicotyledons

Actinidiaceae, Actinidia chinensis, C; Aizoaceae, Carpobrotus acinaciformis, C; Anacardiaceae, Cotinus coggyria, C, D; Annonaceae, Asimina triloba, C; Aselepidiaceae, Periploca graeca, C; Betulaceae, Corylus avellana, D; Caprifoliaceae, Viburnum lantanum, Cw; Celastraceae, Euonymus fortunei 'Silver Queen', C, Ds; E. japonicus, C, D; E. radicans 'minimus', C, D; Convulvaceae, Callicoma serratifolia, C; Ebenaceae, Diospyros lotus, Cw, Dw; Ericaceae, Arbutus unedo, C, Dw; Euphorbiaceae, Euphorbia emerus, C; Fagaceae, Fagus sylvatica, Cs, Ds; F. sylvestris*, C; Juglandaceae, Jugla regia, Cs; Lauraceae, Cinnamomum zeylanicum, C; Pterocarya fraxinifolia, C; Leguminosae, Bauhinia purpurea, C; Lamiaceae, Salvia officinalis, C; Lonicera caprifolium, C; L. perennis, C; Magnoliaceae, Magnolia denudata (=M. conspicua), Cw; Melastomaceae, Tibouchina semidecandra, C, Ds; Moraceae, Ficus sycomorus, C; Humulus lupulus, C, D; Myricaceae, Myrica gale, C, Ds; Oxalidaceae, Oxalis buphurensis, D; Platanaceae, Platanus orientalis, C, Ds; Plumbaginaceae, Limonium latifolium, C; L. sinuatum, D; Polygonaceae, Poppygymnium tataricum, C; Polygonum aviculare, D; Rheum rhabdoticum, C; Rumex lunaria, C; Proteaceae, Grevelea robusta, D; Rhamnaceae, Rhamnus cathartica, C, D; R. purshiana, C, Dw; Rosaceae, Agrimonia eupatoria*, C; Chaenomeles speciosa (=Cydonia japonica aucl.), C; Eriobotrya japonica*, Cw; Fragaria (strawberry) 'Auchincruive Climax', C; Kerria japonica*, C; Malus sargentii*, C; Meipilius germanica*, Cs; Neillia longipedunculosa*, C; Osteomeles fragilis, C; Potentilla fruticosa, C; P. montana, C; Prunus spinosissima*, C; Raphiolepis indica*, C; Sorbaria aitchisonii, C; Sorbus aucuparia, C; Spiraea salicifolia*, C; Salicaceae, Populus canescens, C; Salix dasyclados (=S. sicula), C; Sapindaceae, Koelreuteria paniculata, C, D; Xanthoceras sorbifolia, C, Dw; Saxifragaceae, Astilbe rosea 'Queen Alexandra', C; Boykinia aconitifolia, C; Hydrangea hortensis*, C; Ribes grossularia, C; R. sanguineum 'splendens', C; Tellima grandiflora, C, D; Theaceae Camellia japonica, C; Thea sinensis, Cw, Dw; Tiliaceae, Tilia americana, C; Ulmaceae, Ulmus procera, C; Vitaceae, Parthenocissus quinquefolia, C; Rhocissus rhomboidea, C; Vitis vinifera†, C.

Monocotyledons

Iridaceae, Iris pseudacorus, C, Dw; Liliaceae, Smilax rotundifolia, C.

Gymnosperms

Ginkgoaceae, Ginkgo biloba, D; Gnetaceae, Ephedra americana, Ds; Nuxvoueraceae, Arascaria bidwillii, Cw; D; Metasequoia glyptostroboids, C; Sequoiadendron giganteum, D; Taxaceae, Taxus baccata, C, Dw.

Pteridophyta

Equisetaceae, Equisetum telmateia, C; Polypodiaceae, Blechnum occidentale, C, D; Dryopteris elongata, C, D; Pellia rotundifolia, Cw, D; Pteridium aquilinum, C; Pteris chilidi, C.

* Paecnidin also present.
† Results recorded by Robinson & Robinson (1933, 1934).

Table 4. Anthocyanidins formed from leuco-anthocyanins in fruits, etc.

<table>
<thead>
<tr>
<th>Fruits and nuts</th>
<th>Plant part</th>
<th>Antocyanidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple 'Bramley Seedling'</td>
<td>Flesh</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Betel nut</td>
<td></td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Cacao, Criollo</td>
<td>Seed</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Grape, purple</td>
<td>Skin</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Groundnut</td>
<td>Testa</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Hazel nut</td>
<td></td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Pear 'Conference'</td>
<td>Flesh</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Plum 'Victoria'</td>
<td>Skin</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Walnut</td>
<td>Seedcoat</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Roots, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Bergenia crassifolia</td>
<td>Rhizome</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Fagopyrum esculentum</td>
<td>Root</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Pinus maritima*</td>
<td>Wood</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>Flowers (Bate-Smith, 1953)</td>
<td>White petals</td>
<td>Cyanidin</td>
</tr>
</tbody>
</table>

* Material as named and supplied by Professor J. Masquelier, Faculté de Médecine et de Pharmacie, Bordeaux, France.
Carvillea olgae

Vol.

responsible for

Garrya

fragaceae, Cornaceae. In some instances the substances

necessary for these interfering reactions are known

(cf. Trim & Hill, 1952).

Sugars in exceptionally high concentration, as in

some ripe fruits, when heated with hydrochloric

acid give a golden-brown colour, soluble in isoamyl

alcohol to give a red-brown solution. On the

chromatogram a brown zone due to this pigment,

which appears cream-coloured when viewed in

ultraviolet light, is visible near the solvent front.

Tentative identifications, based on measurements

of $R_f$ values, of the anthocyanidins formed from

leuco-anthocyanins present in leaves, seeds and

fruits, are recorded in Tables 3 and 4.

DISCUSSION

A remarkable feature of the results obtained is that,

with only a few exceptions, the leuco-anthocyanins

present in the tissues examined yielded only

cyanidin and delphinidin when digested with

hydrochloric acid. The exceptions were confined to

Rosaceae and Leguminosae, where, occasionally,

an anthocyanin corresponding in $R_f$ value with

paeonidin was present on the chromatogram. These

areas on the chromatogram were usually confused

with a pinkish brown trail, which occurred in most

species of Rosaceae, and also in those of Fagopyrum,

Vitis, Fagus, Populus, Boykinia, Lespedeza and

Desmodium.

The near relationship, from the chemical point of

view, of the leuco-anthocyanins and the catechins

has been discussed previously (Robinson & Robinson,

1933; Bate-Smith, 1953). Up to the present no

hydroxyflavans other than catechin (I) and gallo-

catechin (II) have been found in nature (cyanom-

auchurin, occurring uniquely in Artocarpus integ-

grifolia, is excepted). Their pattern of hydroxy-

ylation is the same as that found in cyanidin (III) and

delphinidin (IV), respectively, and it is therefore

a matter of some importance, in connexion with the

origin and function in the plant of the leuco-

anthocyanins and catechins, that the leuco-

anthocyanins should be restricted to the patterns of

hydroxylation found in the catechins. The system-

matic significance of these results is discussed in the

following paper.

SUMMARY

1. The leuco-anthocyanins present in leaves, fruits and other tissues are converted into antho-

cyanidins by heating the tissues with $2N$ aqueous

hydrochloric acid. The anthocyanidins are identified

by paper chromatography using the three solvent

systems, n-butanol–$2N$ hydrochloric acid (1:1,

v/v), water–acetic acid–conc. hydrochloric acid

(10:30:1, v/v), and $m$-cresol–acetic acid–$5N$

hydrochloric acid (1:1:1, v/v).

2. Except in Rosaceae and a few Leguminosae

(which appear to contain leuco-paeonidin) the leuco-

anthocyanins appear to be restricted to leuco-

cyanidin and leuco-delphinidin.

The author wishes to thank Dr T. White of the Forestal

Land, Timber and Railways Company Ltd., Harpenden,

Herts, for supplying the details of the acetic acid–HCl

solvent, and Dr T. Swain for the spectrographic data in-

corporated in Tables 1 and 2. The work described in this

paper was carried out as part of the programme of the Food

Investigation Organization of the Department of Scientific

and Industrial Research.

REFERENCES

Acta, 4, 417.