

PHYTOCHEMISTRY OF PROANTHOCYANIDINS

E. C. BATE-SMITH

A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England

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Key Word Index—Proanthocyanidins; differences in extractability and conversion to anthocyanidins; procyanidins; prodelphinidins.

Abstract—Procyanidin A from *Aesculus hippocastanum* differs from the B type procyanidin in that it is difficultly soluble in water, gives a higher yield of cyanidin when heated in butanolic HCl and the production of cyanidin continues beyond the 2 hr period sufficient for maximum production in the case of B type procyanidin. Anthocyanidin production and tannic acid equivalent (TAE) of extracts of species in which A and D type procyanidins have been reported to occur have been studied. Evidence of their presence was also found in *Ribes laurifolium*. Preparations of prodelphinidin from leaves of red currant and sainfoin had approximately twice the anthocyanidin production and the astringency of procyanidin B. Sources especially rich in prodelphinidin were a number of *Ribes* spp., *Salix cinerea* and *Platanus acerifolia*, from all of which the tannin was easily extracted. Some other species such as *Pilea cadieri*, although rich in prodelphinidin, are not suitable as sources because of the inextractability of the tannin.

PROCYANIDINS A AND D

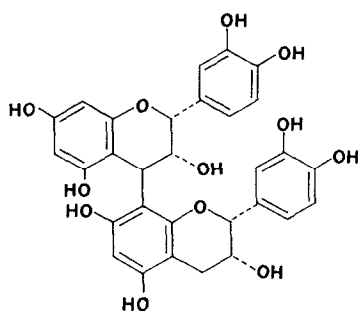
Thompson *et al.* [1] reported the presence of procyanidins (PCy) A-1 and/or A-2 in the leaves of 4 out of 24 of the species examined by them, in the fruit of *Aesculus hippocastanum*, and in the seed of *Persea gratissima*. Procyanidins D-1 and/or D-2 were also present in some species and were found in quantity without A-1 or A-2 in *Ribes sanguineum*, *Azalea indica* and *Rhododendron ponticum*. The distinctive properties of these procyanidins suggest that their occurrence in the leaves of some but not all plants may have some significance from the systematic point of view, and this is confirmed by the later report of their structure [2]. The difference between the A and D and the B and C type procyanidins (Scheme 1) is the presence of an additional oxygen function in the former, in consequence of which the conversion to anthocyanidin by heating with mineral acid, in which atmospheric oxygen is involved, is facilitated.* In the case of the A type, the product is in part of

cyanidin and in part of anthocyanidin with lower R_f in Forestal solvent [3] thought to be dimeric. Species in which the A type procyanidins were reported are not, however, closely related systematically, comprising as they do members of the Rosaceae, Lauraceae, Hippocastanaceae and Ericaceae. Nevertheless it was thought necessary to obtain further evidence for or against the homology of proanthocyanidins wherever they may occur. Consequently, leaves of all the species reported [1] to contain A type procyanidins have been examined chromatographically, spectrophotometrically, and by haemanalysis [3]. A sample of procyanidin A-2 prepared from fruits of *Aesculus hippocastanum* (kindly supplied by Dr. E. Haslam) has been available for comparison with B type procyanidins previously examined [3].

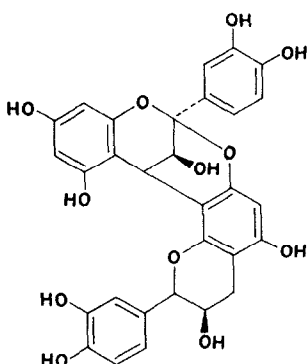
Properties of procyanidin A-2: solubility

As eluted from the Sephadex column and dried without recrystallization, procyanidin A-2 is completely soluble in hot water, but precipitates almost completely on cooling. Because of its insolubility in the cold, it has not been possible to

* Dr. Haslam (personal communication) reports that spots D1 and D2 correspond with three compounds at least which are all trimers.



Procyanidin B2



Procyanidin A2

Scheme 1.

determine its tannic acid equivalent (*TAE*) by haemanalysis. It is readily soluble in methanol, and the yield of anthocyanidin (*E*) when heated in butanolic HCl has been measured both from methanolic solution and as the dry solid. Its insolubility in water in the cold requires that care has to be taken, when 50% MeOH extracts of plant material are concentrated, not to evaporate to dryness, and to use the concentrated aqueous extract immediately after preparation for determination of *E* and *TAE* values. Extracts allowed to stand at room temperature overnight, even if warmed and stirred, have lower *E* and *TAE* values, and if cooled in a refrigerator these values are even more reduced.

Anthocyanidin formation

When heated in butanolic HCl at 95°; the amount of cyanidin formed from B-2 procyanidins reach a maximum as shown by spectral measurement in 2 hr. The *E* values at 550 nm on a weight basis increase with increasing molecular wt from $E_{1\text{cm}}^{1\%}$ of 90–110 for dimers to 200 for tetramers and higher oligomers [3]. Under the same conditions

the A-2 preparation continue to produce anthocyanidin after 2 hr heating, the *E* value at that time averaging 570, reaching a constant value of about 600 after 3 hr. This difference in behaviour between the two types can be used as a diagnostic character for the presence of A type procyanidins.

A second diagnostic character is the presence of an anthocyanidin spot at a lower R_f than that of cyanidin (0.5) in Forestal solvent [3]. In the case of the PCy A-2 preparation, this second spot was at R_f 0.27, but other sources gave spots variously at R_f 0.34, and 0.4. The spots at 0.27 and 0.34 can be, and on occasion have been, mistaken for delphinidin, but they can be clearly distinguished by the position of their λ_{max} in BuOH-HCl. In this solvent cyanidin and the other products of procyanidin have λ_{max} 547 nm, while delphinidin has 558 nm. This difference is easily detectable and the relative amounts of cyanidin and delphinidin can be calculated with considerable accuracy from the visible spectrum.

The production of anthocyanidin in aqueous HCl is qualitatively the same as in BuOH-HCl, but quantitatively very much less. Paper chromatography is usually carried out with an amyl alcoholic extract of the resulting hydrolysate, which also contains the flavonoid aglycones and hydroxy acids.

Extractability

Proanthocyanidins are seldom completely extractable from plant tissues with aqueous or alcoholic solvents, and may indeed be completely inextractable. Procyanidins of type A although less soluble after separation than the B type, do not seem to be any less extractable with aqueous methanol from the fresh or dried tissues.

Extractability can be roughly evaluated by determination of the ratio of the anthocyanidin produced in the extract compared with that produced when the finely powdered unextracted tissue is heated with BuOH-HCl. Solution of the anthocyanidin formed in the BuOH-HCl in the latter case is not always complete and the solid residue can then be seen to contain undissolved red pigment. The yield can be improved by first boiling the powdered tissue with a little 50% MeOH [4].

Astringency

The protein-precipitating ability is conveniently

determined by haemanalysis [3]. For this determination it is necessary to remove MeOH from the extract by evaporation *in vacuo* and rediluted to a convenient volume with water. Astringency is expressed in terms of tannic acid equivalent (*TAE*) % of the dry wt of the tissue. A value for the *TAE* of the finely powdered tissue can also be obtained by shaking the powder vigorously with diluted blood [4].

RESULTS

The results for anthocyanidin production extractability and astringency and the λ_{\max} of the anthocyanidin produced from seven of the species reported by Thompson *et al.* [1] to contain type A and/or D procyanidins are given in Table 1 together

with the occurrence of the different procyanidins [1]. Also included in the Table 1 are results for *Ribes laurifolium*, which contains A type procyanidin and for *Rhododendron rubiginosum*, which contains prodelphinidin (PD) but not procyanidins A or D. The λ_{\max} of the anthocyanidin produced from this species and from *Ribes sanguineum* clearly shows the effect of the presence of PD.

The presence of A type procyanidins is noticeable in the lower ratios of *TAE* / *E* of the extracts containing them, reflecting the higher yield of cyanidin from these precursors; and also suggesting that the astringency of the A type (which because of its low solubility in water could not be measured directly) is probably of the same order as that of the B type.

Table 1. Analytical data on procyanidins

Procyanidins* reported	<i>Malus sylvestris</i>	<i>Aesculus hippo- castanum</i>	<i>Vaccinium myrtillus</i>	<i>Vaccinium vitis-idaea</i>	<i>Persea gratissima</i>	<i>Rhodo- dendron ponticum</i>	<i>Rhodo- dendron rubiginosum</i>	<i>Ribes san- guineum</i>	<i>Ribes laurifolium</i>
B1	++	++	++	++	++	—		+	
B2	++	++	++	+	++	—		+	
B3	—	+	—	++	+	+		—	
B4	+	+	(+)	+	++	—		—	
B5	+	+	—	(+)	+	—		—	
B6	—	—	—	+	—	++		—	
B8	—	—	—	+	—	+		—	
C1	+	+	+	—	+	—		—	
C2	—	—	—	+	—	+		+	
A1	—	+	—	—	+	—		—	
A2	+	++	—	+	++	—		—	
D1	(+)	+	—	—	++	++		++	
D2	—	+	—	+	++	—		—	
E	+	+	—	—	+	—		—	
Anthocyanidins produced									
Cyanidin	+	+	+	+	+	+	+	+	+
Delphinidin	—	—	—	—	—	—	+	+	—
Others (<i>R_f</i>)	—	0.27	0.65	0.4	—	0.27	—	—	0.27
λ_{\max} nm	547	548	548	548	548	548	553	555	550
<i>E</i> _{1cm} ^{1%}									
Powder	19.5	54.4	19.6	36	20	14.6	25	29.6	13.3
Extract	7.9	53	16.8	26	9.5	11	13	11.5	13
<i>TAE</i>									
Powder	6.3	16	8.3	10	4.6	9.5	3.2	7.3	(18.5)
Extract	5.1	15	8	11	6	12	7.5	7.9	(13.3)
<i>TAE/E</i>									
Powder	0.33	0.30	0.42	0.28	0.23	0.65	0.13	0.25	
Extract	0.65	0.29	0.48	0.42	0.63	1.1	0.58	0.7	
Extractability									
<i>E</i> extract									
<i>E</i> powder	0.40	0.98	0.86	0.72	0.48	0.75	0.52	0.39	1.0

* By Thompson *et al.* [1].

The data for the occurrence of procyanidins are taken from Thompson *et al.* [1]. ++ = Major component; *TAE* = tannic acid equivalent % of dry leaf. The *TAE* of *Ribes laurifolium* includes a contribution from ellagitannins also present. For this reason the ratio *TAE/E* is omitted.

The absence of A type procyanidins from *Malus sylvestris* and *Persea gratissima* does not necessarily mean that they were absent in the samples examined by Thompson *et al.* [1], because neither the phytochemical nor the systematic significance of the different types of proanthocyanidins is at present understood. Their report of the presence of D type procyanidin in *Ribes sanguineum* is, however, open to doubt, because none of the closely related species of *Ribes* contains them, and all, like *R. sanguineum*, contain prodelphinidin. *R. laurifolium*, in Section Davidia, is quite unlike any of these: it is evergreen, its leaf form quite distinct, and it contains a considerable amount of ellagitannins, otherwise found in small amounts in only three other species.

In view of the evolutionary significance attaching to the presence of the trihydroxyflavonoids, the authenticity of records of the occurrence of prodelphinidin is now open to question on account of possible confusion with the lower anthocyanidin arising from A and D type procyanidins. An extensive survey is therefore being made of species reported [5] as containing PD. Up to the present date *Ribes laurifolium* is the only species in respect of which a correction has to be made. *Aesculus hippocastanum* was not recorded as containing PD [5]. Many of the species previously recorded as exceptionally rich in PD have now been re-examined with a view to their suitability as sources of prodelphinidin for chemical studies.

RICH SOURCES OF PRODELPHINIDIN

Prodelphinidins are much less common than procyanidins and seldom occur alone or in greater quantity than PCy. It will be helpful, therefore, to select species unusually rich in PD and poor in PCy as especially promising sources for the chemical examination of the former group. Of the several thousand species of vascular plants which have been surveyed, about 20 appear to be possible candidates in this respect, having PD exclusively or in great excess over PCy, and having no hydrolysable tannins. The most promising of these are reported here, ready availability and extractability also being taken into consideration.

Properties of prodelphinidin

Since pure preparations of prodelphinidin frac-

tions similar to the procyanidins A2 and B are not yet available, it has not been possible to make a comparable study of the anthocyanidin yield from polymers of known molecular size. The only samples available (both separated on Sephadex columns and kindly supplied by Dr W. T. Jones) were from sainfoin and red currant leaves and were estimated by analytical centrifugation to be of MW of at least 7000. Each produced a single anthocyanidin spot, R_f 0.33 in Forestal solvent, the λ_{\max} in BuOH-HCl being 558, and they therefore consisted entirely of prodelphinidin. Their E values, which reached a peak in 2–3 hr at 95°, averaged 300 and 350, respectively, compared with the average value of 150 for the procyanidins B2 [3].

Spectra of anthocyanidin produced

The λ_{\max} of the peak of absorption of the anthocyanidin produced by heating the powdered leaf or extract is sometimes displaced by several nm due to contributions in the region of 450–550 nm from other constituents, e.g. chlorophyll. These can be compensated in part by means of an unheated sample of appropriate dilution, and the position of the peak then gives an approximate idea of the relative proportions of PCy and PD in the sample. The proportions so indicated broadly agree with the proportions judged from the chromatographic results included in Table 2. The highest λ_{\max} observed was 558, corresponding with that of delphinidin itself, the lowest 554, corresponding with about 60% delphinidin.

Astringency

Both samples of PD tended to form fine dispersions with the blood proteins, requiring longer times of centrifuging than usual. The red currant sample, which was readily soluble in water, was extremely astringent, the TAE values ranging from 100 to 110%. The sainfoin sample did not dissolve completely in water, its astringency being correspondingly lower, TAE values ranging from 46 to 52%. Since these samples cannot be considered to be representative of the prodelphinidins as they are likely to occur in leaf tissue, the above values cannot at present be used to interpret the results obtained with leaf extracts, but they indicate that prodelphinidins not only have twice the E values of procyanidins, but are also likely to have twice the TAE values.

Table 2. Analytical data for prodelpinidins

	PD	PCy	powder	<i>E</i> extract	<i>E</i> extract <i>E</i> powder
<i>Ribes diacanthum</i> Pall	++	(+)	54	23.5	0.44
Red currant "Laxton's No. 1"	+++	(+)	48	33	0.69
<i>R. sativum</i> Syme	++	+	42	25	0.60
<i>R. petraeum</i> Wulf	++	(+)	45	23.5	0.53
<i>R. alpinum</i> L.	++	(+)	44	23	0.52
<i>R. cynoshati</i> L.	++	(+)	42	31.5	0.75
<i>R. missouriense</i> Nutt.	++	(+)	39	27.5	0.70
<i>R. distans</i> Jancz.	+	—	37.5	20	0.52
<i>R. rubrum</i> L.	+	(+)	37.5	15	0.38
<i>R. sanguineum</i> Pursh.	++	(+)	29.5	11.5	0.39
<i>R. reclinatum</i> L.	++	(+)	26	6.3	0.24
<i>Platanus acerifolia</i> (Ait.) Willd.	+	(+)	45	24	0.55
<i>Salix cinerea</i> L.	++	+	42	32.5	0.77
<i>Crassula argentea</i> Thunb. (<i>C. portulacae</i> Lam.)	++	(+)	44	21	0.48
<i>Grevillea robusta</i> A. Cunn.	++	—	20	14	0.70
<i>Onobrychis sativa</i> Lam. (<i>O. viciaefolia</i> Scop.)	++	+	24	34	0.13
<i>Pilea cadieri</i> Gagnep. and Guill.	+	(+)	17.5	nil	0.0
<i>Parrotia persica</i> C. A. Mey.	++	—	23	15	0.65
<i>Jamesia americana</i> Torr. and Gray	++	+	39	32.5	0.83
<i>Trifolium repens</i> L. (flower)	++	(+)	33.5	19	0.57

PD = Prodelpinidin, PCy = prococyanidin. +++ very strong; ++ strong; + moderate; (+) weak anthocyanidin spots on chromatogram in Forestal solvent. *E* values calculated from absorbance at peak of anthocyanidin produced by heating in 5% butanolic HCl.

RESULTS

In Table 2 the *E* values of the powdered leaves and their 50% aq. MeOH extracts are recorded. The richest sources of PDs to date are currants, *Ribes* spp. It was remarked above that *R. sanguineum*, the flowering currant, thought to contain procyanidin D1 as a main constituent [1], had in fact so much PD in its leaves that it could well be regarded as a rich source of prodelpinidin. Other spp. of *Ribes* have even more PD; so far the richest is *R. diacanthum*, a North Asian sp. The cultivated red currant, *R. sativum* Syme and its progenitors *R. rubrum* and *R. sylvestre* (Lam) Mert. and Koch are nearly as rich, but black currants and gooseberries have only about half as much. The red currant and some of the N. American species have a high extractability, which makes them exceedingly favourable sources of prodelpinidins.

Nearly equal in *E* value to the red currant is the willow, *Salix cinerea*. Common throughout the British Isles, the PDs which it contains have even higher extractability and the plant of course is easily available. Sainfoin, *Onobrychis sativa*, cultivated in drier areas of Britain and therefore also

readily available, has less PD and its extractability is very low. Several other herbaceous legumes have high PD [4] e.g. *Hedysarum multijugum* Maxim., but they are not cultivated in this country. However, the flowers of white clover (*Trifolium repens*) (and only the flowers) contain a large amount of PD which is readily extractable.

The other rich sources listed are plants which are frequently or occasionally cultivated. *Crassula argentea* is a succulent with a high content of LD which is readily extractable from the fresh leaf, but not from the dried. *Pilea cadieri*, a much-cultivated indoor plant, has PD which is completely inextractable. *Parrotia persica* is a rather large tree, not readily available; *Grevillea robusta* is also a large tree, but now frequently grown as a "spot" plant for ornament; both of these gave highly extractable LD and no LCy.

DISCUSSION

Calculation of "tannin" content of tissues containing proanthocyanidins

In view of the wide differences in the yield of

anthocyanidin from the different types of proanthocyanidins, it is unlikely that an analytical method of determining tannin content can be based on this property. For there to be any chance of this, it would first be necessary to know the relative amounts of procyanidin and prodelphinidin, and then to assume an absorption coefficient for each which would apply to the particular case under examination. Thus an E value of 30 assuming $E = 150$ for procyanidin (λ_{\max} 547 nm) would indicate 20% tannin; assuming $E = 300$ for prodelphinidin (λ_{\max} 558 nm) would indicate only 10%. Accurate determination of λ_{\max} intermediate between 547 and 558 would enable the relative proportions of LCy and LD to be calculated and an appropriate conversion factor to be applied.

Jay [6] in a survey of flavonoids in the leaves of Saxifragaceae and related families calculated the % PC + PD (LA) from the E values of the solution of anthocyanidin obtained by heating in aqueous HCl, the conversion to anthocyanidin being assumed to be one-sixth the theoretical. The values he gives for leaves of *Ribes* spp. are near to those calculated from the E values in the present work assuming a conversion factor based on procyanidin B2, but since the LA is mostly prodelphinidin, this results in values now estimated to be twice too high. Nevertheless, Jay's data are valuable in indicating other rich sources of prodelphinidin. In Saxifragaceae, for instance, there are several species of *Saxifraga* and *Deutzia* rich in LA consisting entirely of PD, and an exceptionally rich source in *Jamesia americana* (33% LA by his calculation consisting of 70% PD and therefore more likely to be about 16%). In related families, *Platanus orientalis* is credited with 21% LA (70–80% PD) and *Parrotia persica* with 6% (90% PD).

When procyanidins A and D are present, however, their much higher conversion factors make it difficult to specify which types of procyanidin are present, and there may well be a similar difficulty with prodelphinidin. However, it is only when the question of a balance sheet of the composition of a particular plant tissue is involved that the actual percent "tannin" that it contains is important. What is more important is the effect that the presence of the tannin will have in the use to which the source will be put. It is the means of measuring this effect which is important.

This is most often either the property of astrin-

gency—the precipitation of protein or the inhibition of enzymes, properties which are themselves likely to be closely related if not identical. It is obviously better to measure the effect directly rather than by way of some analytical figure which has only an indeterminate relationship with the effect in question. In the case of astringency, haemanalysis provides one such direct measurement. The inactivation of an enzyme such as β -glucosidase would provide a similar direct measurement of an effect on enzymes. In either case it is useful to express the result in a generalized form such as the "tannic acid equivalent"—if only because tannic acid is a readily available defined compound having the required properties.

EXPERIMENTAL

Plant material. Mature leaves from healthy plants were dried at 40° for at least 24 hr. As a rule, the dried leaves were readily powdered in a mortar, and the powder sieved through a 100-mesh sieve. Exceptionally (see below) this treatment was not possible.

Extraction. 100-Mesh powder (0.1 g) was boiled for a few minutes in 5–10 ml of 50% aq MeOH and the extract decanted through cotton wool or fibreglass. The residue was re-extracted twice with 50% MeOH and the combined extracts concentrated *in vacuo* and diluted with H₂O to 10 ml.

Determination of anthocyanidin. Extract (0.5 ml) was heated for 2 hr at 95° with 4 ml of 5% conc HCl in *n*-BuOH. The visible spectrum was recorded and absorptivity measured at the peak, which lay between 547 nm and 558 nm depending on the relative amounts of cyanidin and delphinidin present. Results were recorded as $E_{1\%}^{1\text{cm}}$ of the powdered dried leaf. Anthocyanidin production in the solid powder was determined in the same way, after first boiling the sample of powder for 4–5 min in 0.5 ml of 50% MeOH.

Determination of astringency (haemanalysis). Finger blood (0.1 ml) was diluted with 5 ml of dist H₂O [3]. To 1 ml of dil. blood was added 1 ml of the extract (or a lesser vol. dil. to 1 ml) and rapidly mixed, and centrifuged at 3000 rpm. The % pptn. was calculated from the difference between the absorptions at 578 nm of the experimental and a control sample consisting of 1 ml of dil. blood plus 1 ml of dist. H₂O. The equivalent tannic acid content of the extract was calculated from the relation % tannic acid = $0.015 + 0.00024 \times \% \text{ pptn.}$; and that of the leaf sample from the amount of leaf powder extracted. The astringency of the solid powder was determined by vigorously shaking 10 mg of powder with 3 ml (or more as necessary) of 1:100 dil. blood, centrifuging, and measuring the difference of the absorptions at 578 nm as above.

Species requiring modification of the above procedures: *Crasula argentea*. This typical succulent species required several days to dry to constant wt, and the tannin was then not extractable. The fresh leaves were therefore macerated and boiled with solvent, filtered and re-extracted as usual. The dried leaves when powdered in a Moulinex mill and sieved were used for determination of E value and astringency, behaving in these respects quite normally. *Pilea cadieri*. When dried and powdered no tannin could be extracted, but in this case maceration of the

fresh leaf and boiling with H₂O, MeOH or 50% MeOH proved equally ineffective. Some tannin, about 30% of that estimated by heating the solid powder, was extracted with 70% aq Me₂CO. The development of anthocyanidin in the solid powder was far from complete, since the residue remained deeply coloured. This was an extreme case of a difficulty frequently encountered in extracting tannin from plant tissues; another such case is that of sainfoin. Other exceptionally difficult cases have been encountered in the monocots, e.g. *Typha* spp. It was suggested earlier [4] that this difficulty is associated with the presence of prodelphinidin, but this does not now seem to be correct, since the tannins of many species containing PD are just as easily extractable as those containing procyanidins. One of the preparations of PD examined was prepared by extracting sainfoin with 70% Me₂CO, and it seems likely therefore that this solvent may prove to be a generally more suitable one than aq MeOH. *Jamesia americana*. This is typical of species with downy leaves which cannot be powdered (another example is *Potentilla anserina*). The dried leaves, milled as finely as possible but not sieved, were treated in the usual way. Other spp. leaving a flocculent residue on the sieve were *Salix cinerea* and *Ribes gayanum*, but this did not interfere with sieving to the same extent as in the case of *Jamesia* and could be disregarded. *Persea gratissima* (seed). Thin slices of tissue were dried to constant wt (4 days) at 60°. They were then easily milled to a fine

orange-brown powder which could be sieved and treated in the usual way. *Trifolium repens* (flowers). The florets were separated from the inflorescence complete with calyces, dried and powdered in the usual way. Since the green tissues do not contain proanthocyanidins, the tannin content of the petals will be higher than that recorded for the whole florets.

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