

# THE ANTHOCYANINS OF *EPACRIS IMPRESSA* LABILL.

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## ABSTRACT

Cyanidin-3-xyloside and cyanidin-3-glucoside have been identified as the major anthocyanin pigments in *Epacris impressa* Labill. It has been shown that the colour gradation evident in flowers in natural populations of *E. impressa* is due to parallel quantitative differences in both pigments. Investigation of other parts of the plant has shown that the same two pigments are present though in varying proportion.

## INTRODUCTION

Natural populations of *Epacris impressa* show a conspicuous gradation in colour of the corolla, from deep red through pink to pure white. As well as the corolla, the anthers, style, bracts and leaves may each contain a varying amount of red pigment. This gradation in colour intensity could result simply from a quantitative difference in gross pigment content. Alternatively there may be both qualitative and quantitative variation involving two or more separate pigments. A certain degree of patterning is also evident. Thus in pink flowers, in particular, the corolla pigment is not always uniformly distributed. Moreover, there appears to be no obvious correlation of the degree to which the separate parts of the plant may be pigmented.

In this investigation, leaves, bracts, corollas, styles and anthers of *E. impressa* have been separately examined and both qualitative and quantitative differences have been observed. The results are summarized in the table.

## IDENTIFICATION OF THE PIGMENTS

In their survey of anthocyanins in the Australian Flora, Gascoigne, Ritchie and White (1948), reported the occurrence of cyanidin-3-monoside in the flowers of five species of *Epacris*, and cyanidin-3-pentoseglycoside in the bracts of a sixth. (*E. impressa* was not examined). The methods used by these workers, the colour and distribution tests of Robinson and Robinson (1932), do not readily permit the definitive identification of the glycoside. Further, these tests generally overlook the possible co-occurrence of minor pigments. For these present investigations we have adopted the more sensitive analytical procedures of paper chromatography and spectrophotometry as developed for the anthocyanins by Bate-Smith (1948) and Harborne (1958a, b).

Plants used in this investigation were obtained from two localities, viz. Ridgeway and Cambridge, both near Hobart, and an independent examination was made of the different parts of the plants as listed in the table.

The fresh plant material was soaked in methanol 3% HCl for 3-12 hours to extract the pigment. After filtration, the extract was centrifuged and streaked as a line near one edge onto several sheets of Whatman 3MM chromatography paper. When dry the chromatograms were developed with the upper layer of butan-1-ol:2N HCl (1.1, by volume). Twelve to fifteen hours gave adequate development, and in all samples two distinct anthocyanin bands were evident, (E1 and E2 respectively). The papers were air-dried, the bands separately cut out and eluted with methanol:water:acetic acid (70:25:5, by volume, freshly prepared), and the eluates concentrated under vacuum as previously. E1 and E2 were further purified by successively rechromatographing in (i), 1% HCl and (ii), butan-1-ol:acetic acid:water (100:22:50 by volume, aged three days), until completely homogeneous anthocyanin fractions were obtained. Chromatography of E2 in solvent (ii), revealed the presence of two components in this fraction, viz. E2/a and E2/b, when red corollas were examined. E2/a, however, was present in very small amount, insufficient in fact, to permit of its complete identification. After the final purification, the eluates containing the anthocyanins were evaporated to dryness in vacuo, and stored pending further tests.

For spectrophotometric examination, the pigments were dissolved in methanol:0.01% HCl to give a solution with an optical density of about 1.300 at 530m $\mu$  (Harborne 1958b). The spectra of all three pigments were very similar. Each had a maximum absorption at 528-530m $\mu$ , with a bathochromic shift of 16-20m $\mu$  caused by the addition of AlCl<sub>3</sub> (5% solution in ethanol), indicating the presence of free ortho-hydroxyl groups in the B-ring of the anthocyanin. The ratio of the absorbancies at 440m $\mu$  and 530m $\mu$  was 22%-24%, suggesting that the 5-hydroxyl position (ring A) was free of glycoside in each case. Finally, the absorption spectrum in the U.V. region showed no evidence of the anthocyanins being acylated.

Partial hydrolysis of E1 and E2/b with 1N HCl for 12 minutes at 100° C under xylene, (Harborne 1958a), yielded aglycone only. Thus these pigments were each 3-monosides. The nature of the aglycones was determined after hydrolysis of a sample of each pigment with 5N HCl at 100° C for 2 minutes. The aglycones were extracted with pentan-1-ol and chromatographed in acetic acid conc.HCl:water (30:3:10 by volume), and formic acid:conc.HCl:water (5:2:3 by volume), alongside reference samples of cyanidin (obtained from roses), and delphinidin (obtained by boiling leuco-delphinidin from broad bean in dil.HCl). At the same time the Robinson colour tests for distin-

guishing aglycones were applied. These confirmed that each of the *E. impressa* pigments was a cyanidin derivative.

Identification of the sugar engaged in the glycosidic link with the aglycone, was achieved by neutralizing the hydrolysate after pentan-1-ol extraction, with N,N-di-n-octylmethylamine (10% solution in chloroform), and evaporating the aqueous layer to dryness in vacuo. The sugar residue was then dissolved in the minimum quantity of water and applied as a spot to a sheet of chromatography paper (Whatman No. 1), alongside reference samples of the pure sugars. Chromatograms were developed in (i), ethyl acetate:pyridine:water (2:1:2 by volume), and (ii) ethyl acetate:acetic acid:water (3:1:3 by volume, upper layer). Visualization was achieved by spraying the dried papers with aniline hydrogen phthallate (2.5% solution in water-saturated butan-1-ol), and heating for 5 minutes at 110° C. By this means, the glycosidic moiety of E1 was shown to be xylose, and that of E2/b glucose.

Thus the two principal anthocyanin pigments of *Epacris impressa* have been identified as cyanidin-3-xyloside and cyanidin-3-glucoside respectively. In addition the minor pigment E2/a, has been partially identified as a cyanidin derivative with glycosidic substitution at position 3 only.

#### DISCUSSION

Estimation of the relative proportion of the two major pigments in the respective plant organs (see table), was made simply by visual comparison of

the intensity of the coloured bands on the chromatograms of the extracts.

The conspicuous colour gradation expressed by the corollas of *E. impressa* appears to be a quantitative difference in the total pigment content, as the relative proportion of the two major pigments is the same in both red and pink flowers. However, in other organs although the same two pigments are present, they occur in quite different proportion to the corolla.

The identification of two cyanidin-3-monoside pigments, supports and extends the earlier observation of Gascoigne et al. (1948), in respect of the flower pigments of other *Epacris* species, but no evidence was obtained in these experiments that cyanidin-3-pentose-glycoside occurred in any organ of *E. impressa*. The possibility that the minor pigment, E2/a, may be cyanidin-3-pentoseglycoside has not been overlooked, although the chromatographic behaviour of E2/a makes this appear unlikely.

#### REFERENCES

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TABLE showing the Distribution of and relative abundance of the Anthocyanin pigments in *Epacris impressa* Labill.

Plant organ	E1	E2	Notes
	Cyanidin-3-xyloside	Cyanidin-3-E2/a-?. E2/b-glucoside	
<i>Corolla</i> (anthers red)—	red	+	} including anther pigments. anther pigment only.
	pink	+	
	white	+	
<i>Bracts</i> —	red	+	veinal regions coloured.
	"white"	+	
<i>Styles</i>	red	+	Colour intensifies after corolla withers.
	red	+	
<i>Leaves</i>	+	+	