

A Method for the Quantitative Determination of Anthocyanin in Rose Petals.

By Hitoshi YASUDA

Department of Biology, Faculty of Liberal Arts and Science, Shinshu University.

(Received Dec. 26, 1964)

One of the most convenient methods for the quantitative determination of anthocyanin in plant tissues is to compare the color of extract from the tissues using water or methanol, which is acidified by hydrochloric acid respectively, with the color of standard solution of anthocyanin.

It had been needed to us to determine the quantity of anthocyanin in rose petals of horticultural variety "ENA HARKNESS". But on account of an apparent difference in color tone between the extract from material and the standard solution, the above mentioned method was found not to be applied. That is, while cyanin is probably contained in petals of ENA HARKNESS (Experiment 2) and its acidic solution should have a maximal absorption at 500 m μ , the acidic extract of petals had actually a maximal absorption at 530m μ .

As the quantity of the pigment cannot be expressed in absolute units of mg. by the said method as it is, researchers generally have been inevitably satisfied with the relative expression proposed by THIMANN⁽¹⁾ and KLEIN⁽²⁾ et al.

Under such circumstances, some methods are available by which the extract can be purified by means of solvent-method or paperchromatography, being followed by the quantitative determination of the pigment. But the author attempted to express the quantity of pigment in absolute unit without applying these purifying methods. That is, the acidic extract from material was diluted and the relation between the dilution degree and the absorbance was observed. The dilution degree within a limit following BEER's law was relatively expressed as a convenient unit (in this report pigment units are shown as in () of Fig. 1.) If C mg of anthocyanin was added to the diluted extract containing A pigment units and then the value of pigment units became B, the pigment units can be expressed by $C/(B-A)$ in mg. The author carried out experiments under this assumption and came to the conclusion that in case of rose petals this calculation was fairly acceptable within a certain range of A and C.

A part of this work was delivered at the 27th Annual Meeting of Botanical Society of Japan, 1962.

In general, on the determination of anthocyanin content in plant tissues, we often encounter the difference in color tone between the extract and the standard solution. Besides, in some cases, on account of a complex construction of anthocyanin in plant tissues, the standard solution can not be prepared with ease. The author expects that the method of calculation described may contribute to some degree to the estimation of pigment in plant tissues.

Experimental

1) The materials used in the present experiment were rose petals of a horticultural variety "ENA HARKNESS" cultured outdoors.

2) *Quantitative tests on anthocyanin* : Paperchromatography according to the BATE-SMITH'S method⁽³⁾ was performed on the crude material obtained from the petals and a spot of Rf 0.07 was observed.

A spot of Rf 0.65 was found in the aglycone sample obtained by extracting, with small quantity of isoamylalcohol, the said crude material, to which the equal volume of conc. hydrochloric acid had previously been added and which had been boiled for 3 minutes.

By these Rf values they were identified with cyanin Rf (0.07) and cyanidin Rf (0.65) respectively.

The red band of the masspaperchromatogram of the said crude material was cut out, and extracted with 0.1% hydrochloric acid. The absorption curve in the visible fraction of the extract was prepared by means of BECKMANN'S electrophotometer and the maximal absorption was observed at 500 m μ , which was identified with the wave length of that of 0.1% hydrochloric acid solution of cyanin.

The extract obtained from the masspaperchromatogram was hydrolyzed and subjected to a qualitative test on anthocyanidin according to ROBINSON et al.⁽⁴⁾ The hydrolyzed substance contained in the said chromatogram in each case was identified with cyanidin.

From the observation above mentioned, it may be concluded that the anthocyanin contained in the present material consists mainly in cyanin.

3) *Relation between the dilution degree of the extract and absorbance* : 10 g of rose petals was soaked in 50 cc of 0.1 N hydrochloric acid for 24 hours, filtered and an extract was obtained. 0.1 N KCl solution was added to the extract and pH was adjusted to 2.0.

A 1/2, 1/4, 1/8, 1/16, and 1/32 solution of this were prepared by an addition of CLARK-LUBBS' buffer solution (pH 2.0).

The absorbance of each dilution was measured by BECKMANN'S electrophotometer using the wave length of 510 m μ . The relation between the dilution degree and absorbance is shown in Fig. 1. From this figure it may be seen that BEER'S

law is precisely applicable in dilution degrees less than 1/8 dilution of the extract. Supposing that the 1/8 dilution of the extract contains 12 pigment units per cc, it is written as (12) in the figure. The application of this pigment unit is quite tentative and will be converted into mg in the following article.

4) *Conversion of the pigment unit into mg* : On the basis of Fig. 1, certain mg of cyanin was added into the diluted extract containing certain pigment units of anthocyanin, pigment units of the mixture were determined at pH 2.0 and the results are shown in Table 1.

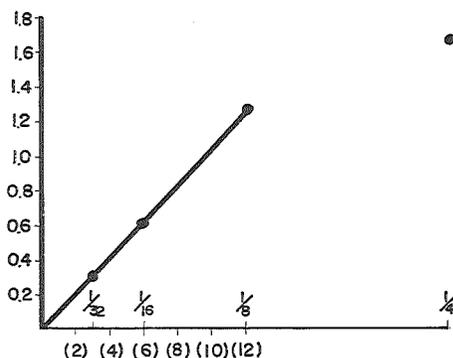


Fig. 1. The relation of the dilution degrees to the absorbance.
Vertical axis : absorbance.
Horizontal axis : dilution degree.
The numerals in () on this axis mean "pigment unit/cc".

Table 1 The changes of pigment unit when cyanin was added to the extracts.

The pigment units of extracts	The amount of cyanin added to extracts (mg)	The pigment units of mixtures
6.00	—	6.00
6.00	0.005	8.30
6.00	0.010	10.60
6.00	0.015	12.40
4.00	—	4.00
4.00	0.001	4.46
4.00	0.002	4.92
4.00	0.003	5.38
4.00	0.004	5.53

As shown in this table, 2.3 pigment units were observed to increase in the mixture of cyanin and the extract containing 6 pigment units by the addition of every 0.005 mg of cyanin until the quantity of cyanin amounted to 0.01 mg. And 0.46 pigment units were observed to increase by the addition of every 0.001 mg of cyanin into 4 pigment units extract until cyanin content amounted 0.003 mg.

From these relations one pigment unit is converted into 0.00217 mg.

Summary

On account of difference in color tone between the tissue extract and the standard solution, cyanin content of petals of "ENA HARKNESS", a horticultural variety of rose, could not be expressed in absolute units of "mg" after the quantitative determination. Accordingly a following method for expressing the pigment content was tried. That is, the acidic extract from the material was diluted and a relation between the dilution degree and the absorbance was observed. The dilution degree within the limit following BEER'S law was relatively expressed as a convenient unit. (In this report "pigment unit" was used.) If C mg of crystals of anthocyanin is added to a diluted extract containing A pigment units and pigment units of the mixture become B the pigment units can be expressed in "mg" by $C/(B-A)$. On this base experiments were carried out and it was noticed that this method could be put in practice within a certain range of A and C .

The writer wishes to thank Prof. K. NAKAYAMA for his kind revision of the manuscript.

References

- 1) THIMANN, K. V. and EDMUNDSON, Y. H. (1949) : *Arch. Biochem.* 22, 33.
- 2) KLEIN, W. H., WITHROW, R. B., ELSTAD, V. and PRICE, L. (1957) : *Amer. Jour. Bot.* 44, 15.
- 3) BATE-SMITH, B. C. (1950) : *Biochem. Soc. Scymposia* 3, 62.
- 4) ROBINSON, G. M. and ROBINSON, R. (1949) : *Arch. Biochem.* 22, 33.