Phytochemistry, 1969, Vol. 8, pp. 2003 to 2008. Pergamon Press. Printed in England.

NEW TESTS FOR MICROSCALE IDENTIFICATION OF ANTHOCYANIDINS ON THIN-LAYER CHROMATOGRAMS*

D. B. MULLICK†

Faculty of Forestry, University of British Columbia, Vancouver 8, B.C.

(*Received* 27 *September* 1968, *in revised form* 18 *March* 1969)

Abstract—Five color tests, three of which are based on exposure to NH_3, one on ammonium molybdate, and the other on lead acetate for identification of the six common anthocyanidins in quantities far below 1 μg, are presented. Anthocyanidin fluorescence is intensified by an NH_3 chamber test so that concentrations well below the visible range can be detected. Each of the six anthocyanidins is identified individually by its characteristic serial fluorescent modifications produced on treatment with formic acid or sulfuric acid prior to extended NH_3 exposure. The visible and u.v. colors produced following ammonium molybdate or lead acetate sprays are distinctive, and also diagnostic of O-dihydroxylated anthocyanidins.

IN THE course of a biochemical study of the defence reactions of western conifers to insect attack, we became interested in the reddish-purple pigments occurring in the periderm tissues. It was found that the pigmentation was due to traces of anthocyanidins in the free state[1] co-occurring with several non-anthocyanic red pigments.[2] The limited amounts of periderm tissues obtainable, together with interference by non-anthocyanic red pigments, made the identification of anthocyanidins by usual visual colors and R_f values difficult. A need for tests to identify anthocyanidins on a microscale from relatively crude plant extracts had thus become imperative.

It is well known that anthocyanidins turn blue in NH_3 vapors. It was observed that anthocyanidins show vivid and distinctive color change in u.v. light after extended exposure to NH_3 vapors. The observation was exploited in development of three tests for identification of anthocyanidins. In addition, the molybdic acid test of Quastel[3] for O-dihydroxyphenols was modified and used, and the lead acetate test of Fuleki and Francis[4] for anthocyanins was extended.

The tests presented in this paper were carried out on the six common anthocyanidins, namely, delphinidin (Dp), petunidin (Pt), malvidin (Mv), cyanidin (Cy), peonidin (Pn), and pelargonidin (Pg). These tests in conjunction with the techniques of thin-layer chromatography developed in this laboratory[5] permit unambiguous identification of anthocyanidins. Moreover, they were also useful in differentiating delphinidin from a new leuco-anthocyanidin[6] which is difficult to resolve from delphinidin by chromatography.

* Presented at the 8th Annual Meeting of the Phytochemical Society of North America held at Tucson, Ariz., U.S.A., 6–8 June, 1968.

† Research Scientist, Forest Research Laboratory, Department of Forestry and Rural Development, Fredericton, New Brunswick, seconded as Assistant Professor to the Faculty of Forestry, University of British Columbia, Vancouver, B.C. Canada.

[1] D. B. MULLICK, *Can. J. Botany*, in press.
[2] D. B. MULLICK, *Phytochem.*, to be published.
[3] J. H. QUASTEL, *Analyst* **LVI**, 311 (1931).
[4] T. FULEKI and F. J. FRANCIS, *Phytochem.* **6**, 1161 (1967).
[5] D. B. MULLICK, *J. Chromatogr.* **39**, 201 (1969).
[6] D. B. MULLICK, unpublished.

1. NH_3 CHAMBER TEST

All six anthocyanidins fluoresced when irradiated with short-wave or long-wave transilluminators,[7] except delphinidin which did not glow under long-wave. Low concentrations of anthocyanidins (0·01 μg/spot) cannot be detected on chromatoplates. They were, however, readily detected after exposure for 30 sec to NH_3 vapors because this treatment markedly

TABLE 1. AMMONIA-INDUCED SEQUENTIAL VISIBLE COLOR MODIFICATIONS OF UNTREATED ANTHOCYANIDINS ON TLC PLATES

Compound†	Sequence of visible color change*
Dp	Purple $\xrightarrow{\frac{1}{2}}$ Blue violet $\xrightarrow{4}$ Greenish blue $\xrightarrow{15}$ Greenish gray $\xrightarrow{30}$ Yellowish gray
Mv	Mauve $\xrightarrow{\frac{1}{2}}$ Blue $\xrightarrow{4}$ Turquoise $\xrightarrow{30}$ Turquoise gray $\xrightarrow{60}$ Pale gray
Cy	Red $\xrightarrow{\frac{1}{2}}$ Blue $\xrightarrow{4}$ Blue $\xrightarrow{10}$ Dull bluish violet $\xrightarrow{30}$ Gray
Pn	Red $\xrightarrow{\frac{1}{2}}$ Violet blue $\xrightarrow{4}$ Blue $\xrightarrow{30}$ Dull bluish black gray
Pg	Orange red $\xrightarrow{10}$ Blackish gray $\xrightarrow{30}$ Light chocolate brown $\xrightarrow{60}$ Brownish gray

* Recorded from a chromatoplate while it was still in the chamber: the figures above each arrow give the approximate time in min.

† Dp, delphinidin; (Pt. petunidin;) Mv, malvidin; Cy, cyanidin; Pn, peonidin; Pg, pelargonidin.

TABLE 2. AMMONIA-INDUCED SEQUENTIAL MODIFICATIONS OF FLUORESCENCE[a] OF UNTREATED ANTHOCYANIDINS ON TLC PLATES

	Fluorescence			
		After exposure to NH_3 (min)[b]		
Compound*	Without NH_3 exposure†	0·5	4	35
Dp	B-R	R	G[c]	Y-Gr
Pt	B-R	B-R	O	Gr-Y
Mv	B-R	B-R	B-R	Faded O-F
Cy	R	B-R	B-R	O[d]
Pn	R	B-R	B-R	B-R
Pg	O-R	B-R	B-R	R

* See Table 1.

† Color code: B, blue; F, flesh; G, gold; Gr, green; O, orange; P, pink; R, red; V, violet; W, white; Y, yellow.

[a] Short-wave transilluminator was used for photographing fluorescence on Ektachrome-X after every 2-min exposure to NH_3.

[b] The duration of each exposure to NH_3 is measured from the time a chromatoplate is introduced in NH_3 chamber. The exposure in min is the summation of time the same chromatoplate remained in the chamber during intermittant exposures. The successive 2-min intervals during which plates were removed for photography and observations were not added.

[c] The fluorescence between 2 min to 4 min is orange.

[d] Fluorescence around 10 min is orange-red.

[7] Ultraviolet Products Inc., San Gabriel, California, U.S.A.

increased the fluorescence intensity. It should be noted, however, that certain chalcones and aurones also fluoresce red on exposure to NH_3.

A characteristic sequence of color modifications for each anthocyanidin occurred in visible and u.v. light, when a two-dimensional chromatoplate of anthocyanidins was left in NH_3 chamber over a period of time (Tables 1 and 2). These sequential color modifications are characteristic for each anthocyanidin and therefore have diagnostic value. This test, however, is useful largely for identifying Dp, and Pt within a few minutes, because the fluorescence of all anthocyanidins fades *noticeably* after extended exposure under the conditions of NH_3 chamber test.

2. FORMIC ACID–NH_3 AND SULFURIC ACID–NH_3 TESTS

The rate of NH_3-induced fluorescence modifications is markedly faster and the fading minimal when the chromatoplates are pretreated either with formic acid or sulfuric acid.

TABLE 3. AMMONIA-INDUCED SEQUENTIAL MODIFICATIONS OF FLUORESCENCE[a] OF FORMIC ACID PRETREATED ANTHOCYANIDINS ON TLC PLATES

		NH_3 exposures in min								
		Serial exposures[b]							Direct exposures	
Compound*	Fluorescence after HCOOH treatment†	0·5	2	6	10	15	25	35	10	25
Dp	B-R	O[c]	Y	Gr-Y	Gr-Y	Gr-Y	Gr-Y	Gr-Y	Gr-Y	Gr-Y
Pt	B-R	R[c]	O	Y	Y-Gr	Gr	Gr	Gr	Y-Gr	Y-Gr
Mv	B-R	B-R	B-R	O-P	F-P	F-O	F	Faded-F	F-O	F
Cy	R	B-R	B-R	O-R	O	O-Y	G	G-Y	O	G-Br
Pn	R	B-R	B-R	B-R	B-R	B-R	B-R[d]	B-R[d]	B-R	B-R
Pg	O	B-R	B-R	B-R	R	O-R	O	O	Bright R	O

* See Table 1.

† Color, see Table 2.

[a] Short-wave transilluminator was used for photographing fluorescence on Ektachrome-X.

[b] The initial exposure time or the zero time is measured from the time of disappearance of red colors of anthocyanidins.

[c] The rate of change of fluorescent modifications for Dp and Pt is too fast, and therefore the test cannot be used for detection of weak anthocyanidin spots.

[d] The bluish-red fluorescence of Pn is relatively brighter than the orange fluorescence of Pg.

The sequential fluorescent modifications obtained with the formic–NH_3 and sulfuric–NH_3 tests are shown in Tables 3 and 4. Delphinidin was the first compound whose fluorescence changed and Pt was the next, and these two can be identified by their characteristic fluorescence changes within 5 sec after H_2SO_4 pretreatment (Table 4) and within 2 min after HCOOH pretreatment (Table 3). Mv and Cy can be identified similarly within 2 min (Table 4), and ten min (Table 3). All six anthocyanidins may be identified by a single exposure of 10–15 min in sulfuric–NH_3, or of 25 min in formic–NH_3 tests. Pn and Pg are differentiated easily from the other anthocyanidins because they stay reddish in all NH_3 tests. Pn and Pg can be differentiated readily, however, because Pg changes after about 15 min to orange-red and then to orange fluorescence, whereas Pn remained bluish-red throughout in the formic-NH_3 test; the orange-red fluorescence of Pg, however, faded significantly in relation to the bluish-red fluorescence of Pn. In sulfuric–NH_3 test, Pg showed brighter fluorescence than Pn; moreover, the colors following extended exposure were distinct.

Although the overall sequence of the fluorescence changes for each anthocyanidin is reproducible, the time at which sequential modification occurs is not, owing to variations in room temperature and leakage of NH_3 from the tank. A delay or an enhancement in the rate of fluorescence modifications can be recognized readily by using standard anthocyanidins chromatographed on the same plate.

The six anthocyanidins could not be resolved on TLC plates in non-alcoholic solvents,[5] so that only three spots, one consisting of Dp, the second a mixture of Cy and Pt and third, a mixture of Mv, Pn and Pg, were obtained. The ammonia test when applied to such a mixture of unresolved anthocyanidins permitted a dependable characterization of the components of the mixture.

TABLE 4. AMMONIA-INDUCED SEQUENTIAL MODIFICATIONS OF FLUORESCENCE[a] OF SULFURIC ACID—PRETREATED ANTHOCYANDIINS ON TLC PLATES

		NH_3 exposures in min						
		Serial exposures[b]					Direct exposures	
Compound*	Fluorescence after H_2SO_4 treatment†	0·1	0·5	2	6	15	10	45
Dp	B-R	O[c]	Y	G	G-Y	Gr-Y	Y	Y
Pt	B-R	Y[c]	Lime-Y	Lime	Pale-lime	Pale-lime	Gr-Y	W-Y
Mv	B-R	B-R	B-R	O-P	F-O	F	W-F	Faded
Cy	R	R	O-R	O	G-O	G	G[d]	Rusty-Br
Pn	R	B-R	B-R	B-R	B-R	B-R	Faded-P	Faded-F
Pg	O	B-R	B-R	B-R	B-R	B-R[e]	R	R

* See Table 1.
† See Table 2.
[a] Short-wave transilluminator was used for photographing fluorescence on Ektachrome X.
[b] Same as footnote b in Table 3.
[c] Same as footnote c in Table 3.
[d] Sometimes yellowish-green and sometimes khaki.
[e] The bluish-red fluorescence of Pg is relatively brighter than that of Pn.

3. AMMONIUM MOLYBDATE TEST

The colors produced by the reagent (Table 5) were highly reproducible and markedly stable even after months at room temperature. Anthocyanidins with a free catechol group became visibly bluish, while those without a free catechol group remained essentially red following the spray. Moreover, examination with a long-wave transilluminator allowed a clear-cut differentiation of the two classes of pigments: the O-dihydroxy anthocyanidins showing dark absorptions, while the non-O-dihydroxy anthocyanidins retained their red fluorescence. The test is excellent for differentiating Dp, Pt and Mv because their blue, turquoise and violet colors stand out vividly.

4. LEAD ACETATE TEST

The colors produced immediately after spraying by this reagent were reproducible, but were unstable,[4] and underwent changes on standing (Table 5). When the chromatoplates were irradiated with long-wave u.v. after the spray, the O-dihydroxylated anthocyanidins

showed absorptions, while the non-O-dihydroxylated anthocyanidins showed bright red fluorescence which faded on standing. The test is of value in differentiating pelargonidin from peonidin (Table 5).

TABLE 5. VISIBLE AND ULTRA VIOLET‡ COLORS OF ANTHOCYANIDINS ON TLC PLATE FOLLOWING AMMONIUM MOLYBDATE ANS LEAD ACETATE SPRAYS

	Ammonium molybdate		Lead acetate			
	Colors after spraying†		Colors immediately after spraying		Colors after 20 hr	
Compounds*	V.	u.v. (long-wave)	V.	u.v. (long-wave)§	V.	u.v. (long-wave)
Dp	B	Dull dark abs.	B	Dark Gr-B abs.	B-black	Dark Gr-B abs.
Pt	Turquoise	Dull dark abs.	Turquoise	Dull abs.?	Grey-B	Faded dull abs.
Cy	V	Dull dark abs.	B	Rust-Br abs.	Grey	Br abs.
Mv	Mauve R	R fl	B-V	Bright R fl	Grey V	Faded B-P‖ abs.
Pn	B-R	R fl	V-R	Bright R fl	Faded V-R	O-R fl‖
Pg	B-R	R fl	B-R	Bright R fl	Mustard¶	Y O fl‖

* See Table 1. fl = fluorescence. abs. = absorption.
† See Table 2.
‡ Long-wave transilluminator was used for photographing fluorescence on Ektachrome X.
§ U.v. colors of Dp, Pt and Cy have better diagnostic value than the visible colors.
‖ Fading of fluorescence occurs on standing.
¶ Mustard yellow color of Pg is very distinct. Note that Pn remains reddish. The difference can be used for characterization.

5. SENSITIVITY OF THE TESTS

Anthocyanidin concentrations of 0·01 μg per spot, which are undetectable in visible and u.v. light, are not only detected readily by the NH_3 chamber test, but Dp, Pt, Mv and Cy are also identified by the formic–NH_3 test. The color changes of Pn and Pg cannot be ascertained at such concentrations because of fading. Although no detailed studies were carried out to determine the precise limits of sensitivity, it was found that the NH_3 chamber test can detect anthocyanidin concentrations below 0·01 μg. However, concentrations of 0·002 μg cannot be detected. The ammonium molybdate and lead acetate tests are less sensitive; they can identify anthocyanidins when their concentration is 0·1 μg. The anthocyanidin concentrations of 0·02 μg can be detected in visible and u.v. light without any treatment. At this concentration only the violet color of cyanidin produced by the ammonium molybdate spray can be detected.

EXPERIMENTAL

A. Anthocyanidin Chromatography

45 μl of the stock solution[5] of the six common anthocyanidins were resolved on micro-crystalline cellulose Avicel SF by two-dimensional thin-layer chromatography.[5] Approximate concentrations of Dp, Pt, Mv, Cy, Pn and Pg per 45 μl of the solution were 0·8, 0·5, 0·8, 1·0, 0·9 and 1·1 μg, respectively.

B. Photography

Excellent photographs with Ektachrome-X are obtained using transilluminators,[7] but the Chromatovue model C-5 gives poorer results.

C. NH_3 Chamber Test

A freshly developed chromatoplate after drying was placed for 2 min in a TLC tank containing two 25-ml short-height beakers containing 20 ml Conc. NH_4OH. The tank was sealed and the lid weighted. After each 2-min exposure (Table 2), the chromatoplate was removed, observed and photographed using short-wave transilluminator, and then returned to the chamber after exactly 2 min from the time it was taken out.

D. Formic Acid–NH_3 and Sulfuric Acid–NH_3 Tests

The chromatoplates were sprayed prior to NH_3 exposure either with 5 ml HCOOH or 2 ml of 20% H_2SO_4. For the formic–NH_3 tests the plate was placed horizontally in an efficient fume hood until just dry (*ca.* 4 min). For the sulfuric–NH_3 test the plate was dried horizontally using hot air. The plates were immediately covered with a clean glass plate. Exposure to NH_3 was begun as soon as the plates were dry. The cover plate was removed, and the chromatoplate exposed in the NH_3 chamber as before. Fresh ammonia was used at the beginning of each intermittant exposure to improve upon the reproducibility and intensity of the NH_3-induced fluorescence. At the end of each exposure, the chromatoplate was covered at once after removal from the chamber. The cover plate was only removed for photography and the plate was returned to the chamber in less than 1 min. Many phenolic compounds, including anthocyanidins, undergo reversible changes after brief exposure to NH_3. Covering the plate maintains the NH_3 environment and reduces exposure to the air, and thus markedly minimizes fading of fluorescence. Reproducible results can *only* be obtained when all the operations are carried out in a uniform manner.

E. Ammonium Molybdate Test

The chromatoplate was sprayed with 5 ml of a freshly prepared 4% solution of $(NH_4)_6 Mo_7O_{24}.4H_2O$(w/v) and dried in a stream of hot air. Refrigeration prolongs the life of the molybdate solution. The u.v. fluorescence is observable only under long-wave transilluminator.

F. Identification of Anthocyanidins by Lead Acetate

The chromatoplate was sprayed with 15 ml of neutral lead acetate solution[4] (1% lead acetate in 75% aqueous EtOH w/v) and dried in a stream of hot air. The u.v. fluorescence is observable only under long-wave transilluminator.

Acknowledgements—This work was made possible by the financial support of the National Research Council of Canada and the advice and interests of Dean J. A. F. Gardner, Faculty of Forestry, University of British Columbia, Vancouver. The painstaking technical assistance of Mrs. M. T. Heinrich and Mr. G. D. Jensen is gratefully acknowledged.