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# REDDISH PURPLE PIGMENTS IN THE SECONDARY PERIDERM TISSUES OF WESTERN NORTH AMERICAN CONIFERS*

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**Abstract**—A new class of non-anthocyanic *reddish purple pigments* has been found in secondary periderm tissues of coniferous bark by application of a thin-layer chromatographic technique developed for the purpose. Contrary to established belief, these pigments referred to also as phlobaphenes, consist not of single substances, but of mixtures of several distinct pigments. Western red cedar (*Thuja plicata* Donn.) contains at least eight of these new pigments, amabilis fir (*Abies amabilis* (Dougl.) Forbes) at least six, and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) at least nine. Moreover, contrary to the current views, the pigments in the bark of the three species studied differ from one another. Although the exact identity of these pigments remains to be ascertained, the techniques permit qualitative comparisons of the pigments in different species. It is further established that these pigments do not occur in normal primary periderm of the bark.

## INTRODUCTION

THE CHEMICAL nature of reddish purple pigments, also referred to as phlobaphenes,[1] from the bark of several conifers have been investigated extensively.[2-6] The pigment from red fir,[3] *Abies magnifica* A. Murr., was believed to be a *single* compound consisting of a low molecular weight polymer of cyanidin. That from western hemlock,[6] *Tsuga heterophylla* (Raf.) Sarg., was found to be identical or closely related to the pigment of red fir. It was also concluded[6] that the pigmentation of western hemlock was attributable neither to cyanidin as such, nor related anthocyanins. Recent investigations from this laboratory[7] have shown that a minor part of the hemlock pigmentation is due to cyanidin and pelargonidin in the free state. The reddish purple pigment of western red cedar,[5] *Thuja plicata* Donn., appeared to be a heterogeneous polyphenolic-polyester containing both an organic cation and an organic anion.

Botanists[8, 9] have paid scant attention to the pigments in the secondary periderm tissues of conifers. Some observations recorded in the literature appeared contrary to our preliminary findings. For example, it has been stated that the reddish purple pigments in the bark tissues of western hemlock are found in the phelloderm,[6] whereas in this laboratory critical examination of the secondary periderm tissues of western red cedar, western hemlock, amabilis fir and several other true firs reveals they occur only in the normal secondary phellem.

* Part I in the projected series "Studies of periderm."

1 H. L. HERGERT, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 573, MacMillan, New York, N.Y. (1962).
2 H. L. HERGERT and E. F. KURTH, *Tappi* **36**, 137 (1953).
3 E. S. BECKER and E. F. KURTH, *Tappi* **41**, 380 (1958).
4 G. L. CARLSBERG and E. F. KURTH, *Tappi* **43**, 982 (1960).
5 E. P. SWAN, *Forest Prod. J.* **13**, 195 (1963).
6 H. L. HERGERT, L. E. VAN BLARICOM, J. C. STEINBERG and K. R. GRAY, *Forest Prod. J.* **15**, 485 (1965).
7 D. B. MULLICK, *Can. J. Botany*, in press.
8 Y. CHANG, Anatomy of Common North American Pulpwood Barks, Tappi Monograph, No. 14 (1954).
9 Y. CHANG, Bark Structure of North American conifers. U.S.D.A. Tech. Bull. No. 1095 (1954).

Our interest in the reddish purple bark pigments arose during our studies of insect–host tree interactions. The reddish purple pigments occur[10] in the corky layer surrounding the feeding sites of balsam woolly aphid, *Adelges piceae* (Ratz.), in the bark tissues of several true firs. It was sought to determine whether the pigments occurring at the feeding sites and those found in the secondary phellem were chemically identical. Chromatographic methods available for the resolution of these pigments were found to be inadequate. This paper describes a thin-layer chromatographic technique for the resolution of these bark pigments from the secondary periderm tissues of the three western North American species of conifers.

## RESULTS AND DISCUSSION

The ethyl acetate extracts from the tissues, when resolved by TLC with a solvent combination *A* (Fig. 1), showed that amabilis fir contained six (Table 1), western red cedar eight (Table 2), and western hemlock (Table 3) at least nine distinct pigments. The pigments from western hemlock are very labile as compared with those from the other two species, and their orange

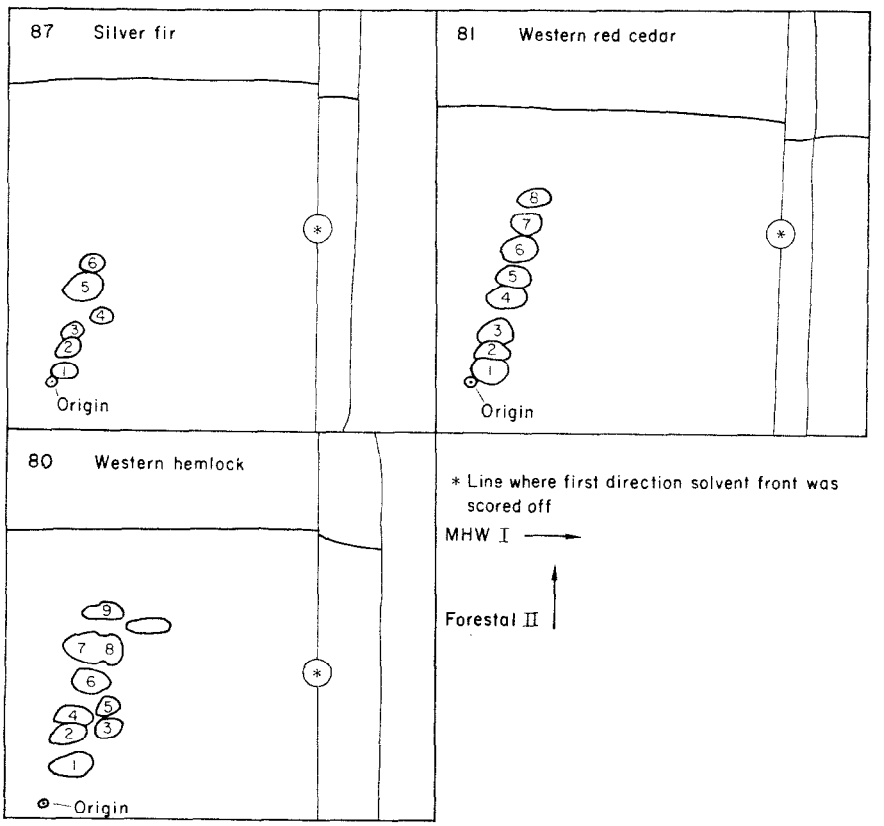


FIG. 1. THIN-LAYER CHROMATOGRAPHIC RESOLUTION OF THE REDDISH PURPLE PIGMENTS FROM SILVER FIR (ALSO KNOWN AS AMABILIS FIR), WESTERN RED CEDAR, AND WESTERN HEMLOCK ON MICROCRYSTALLINE CELLULOSE AVICEL SF WITH A SOLVENT COMBINATION A, CONSISTING OF MHW (FIRST DIRECTION) AND FORESTAL (SECOND DIRECTION).

[10] R. E. BALCH, Canada Dept. Agric. Publ. No. 867, 76 pp. (1952).

TABLE 1. VISIBILE AND U.V. COLORS, RELATIVE CONCENTRATION,[a] AVERAGE $hR_f$ VALUES AND COLOR REACTIONS OF THE REDDISH PURPLE PIGMENTS FROM THE SECONDARY PERIDERM TISSUES OF AMABILIS FIR BY TWO-DIMENSIONAL TLC ON AVICEL SF[c]

| Spot No. | U.v. colors[bdq] (sw) | Rel. conc.[a] u.v. (sw) | Average $hR_f$ values in MHW/Forestal | Color reactions[g] | |
|---|---|---|---|---|---|
| | | | | Ammonium molybdate[14] | $NH_3$ chamber test[14] u.v. (sw)[deh] |
| | | | | $v^{jp}$ | 1 min |
| 1 | Orange-red fl. | 5 | 9/6 | Red | Orange fl. |
| 2 | Dull mauve | 2 | 10/14 | Blue when wet, violet when dry | Mauve |
| 3 | Dull mauve | 1 | 11/20 | | Mauve |
| 4 | Orange-red fl. | 3 | 20/24 | | Dull orange |
| 5 | Bright red fl. | 25 | 17/36 | | Crimson fl. |
| 6 | Dull mauve | 2 | 18/43 | | Faded |

[a] Relative concentration as estimated visually using short wave transilluminator is included to indicate major and minor spots.

[b] All pigments on TLC plates are visible red to reddish purple. Although the boundaries of the strong spots can be readily demarcated, those of weak spots are diffuse, but they are distinct when viewed with a short wave transilluminator. The fluorescence intensity of the pigments is several fold greater than their visual intensity.

[c] Now available as Avicel TG-104 (FMC Corporation, American Viscose Division, Newark, Delaware, U.S.A.).

[d] Ultraviolet colors were determined using standard u.v. filters supplied with the transilluminators.

[e] Slight fading of fluorescence occurs after 20 min exposure in the $NH_3$ chamber.[14]

[g] All major spots turned bluish with $FeCl_3$–$K_3Fe(CN)_6$ test for phenols.[10, 11]

[h] All major spots turned visibly bluish on exposure to $NH_3$.

[i] Spots 2 to 6 show dark absorptions under long wave transilluminator; spot No. 1, however, remains fluorescent red after the molybdate spray.

[p] A total of 15 ml of the reagent[16] were sprayed after intermittent drying.

[q] All spots were red visibly.

fl. fluorescence

abs. absorption

TABLE 2. VISIBLE AND U.V. COLORS,[j] RELATIVE CONCENTRATION,[a] AVERAGE $hR_f$ VALUES AND COLOR REACTIONS OF THE REDDISH PURPLE PIGMENTS FROM THE SECONDARY PERIDERM TISSUES OF WESTERN RED CEDAR BY TWO-DIMENSIONAL TLC ON AVICEL SF

| Spot No. | U.v. colors[bdq] (sw) | Rel. conc.[a] u.v. (sw) | Average $hR_f$ values in MHW/Forestal | Color reactions[g] | |
|---|---|---|---|---|---|
| | | | | Ammonium molybdate[14] $v^{kp}$ | $NH_3$ chamber test[14] u.v. (sw)[de] 1 min |
| 1 | Dull red | 1 | 11/10 | Blue | Faded |
| 2 | Dull mauve | 2 | 10/16 | Blue | Mauve |
| 3 | Dull orange | 3 | 12/23 | Blue | Faded red |
| 4 | Bright scarlet fl. | 15 | 16/34 | Indigo | Crimson fl. |
| 5 | Red fl. | 3 | 17/41 | Turquoise | Red fl. |
| 6 | Bright orange fl. | 7 | 19/52 | Purple | Bluish red fl. |
| 7 | Bright orange fl. | 4 | 21/59 | Purple | Bluish red fl. |
| 8 | Weak red | 1 | 23/70 | Weak | Faded |

See footnotes in Table 1 for explanations of superscripts and abbreviations.

[k] All spots show dark absorption under long wave transilluminator.[14]

fluorescence (Table 3) is similar to spots 6 and 7 of western red cedar (Table 2); amabilis fir pigments do not yield orange fluorescent spots.

The pigments from all three species, and western hemlock in particular, fade and diffuse in the solvent combination *B* (see Experimental). Although solvent combination B was not investigated extensively because of poor chromatographic resolution, it was useful for delineating that most of the pigments from the three species differ from one another. Chromatographic characteristics in this combination suggest that Spots Nos. 1 and 5 of amabilis fir (Fig. 1) may be a mixture of two spots each. Thus, amabilis fir may contain up to eight reddish purple pigments.

TABLE 3. VISIBLE AND U.V. COLORS,[j] RELATIVE CONCENTRATION,[a] AVERAGE $hR_f$ VALUES AND COLOR REACTIONS[ghlm] OF THE REDDISH PURPLE PIGMENTS FROM THE SECONDARY PERIDERM TISSUES OF WESTERN HEMLOCK BY TWO-DIMENSIONAL TLC ON AVICEL SF[c]

| Spot No. | U.v. colors[bdoq] (sw) | Rel. conc.[a] u.v. (sw) | Average $hR_f$ values in MHW/Forestal |
|---|---|---|---|
| 1 | Orange fl. | 5 | 12/18 |
| 2 | Dull orange | 3 | 11/29 |
| 3 | Dull orange | 1 | 22/30 |
| 4 | Dull orange | 3 | 14/35 |
| 5 | Orange yellow fl. | 1 | 23/38 |
| 6 | Dull orange | 4 | 18/50 |
| 7 | Bright orange fl. | 50 | 24/60 |
| 8 | Orange fl. | 15 | 18/61 |
| 9 | Orange fl. | 6 | 23/73 |
| 10[n] | Orange fl. | | 38/68 |

[j] See footnotes in Table 1 for explanation of superscripts and abbreviations.

[l] All spots except No. 7 gave faint red fluorescence after 1 min which disappeared within 3 min in the $NH_3$ chamber test;[14] Spot No. 7 gave crimson red fluorescence which disappeared within 8 min.

[m] All major spots turned violet with ammonium molybdate and showed dark absorption under u.v. (long wave).[14]

[n] This spot was observed only occasionally. It is left as an unnumbered spot in Fig. 1.

[o] Spots Nos. 1 to 6 and 9 were visibly weak.

Spots Nos. 2 and 5 of amabilis fir (Table 1) seem to be similar to Nos. 2 and 4 of western red cedar. It is likely that No. 9 of western hemlock is identical to No. 8 of western red cedar. All other pigments from the three species seem to be new and unique.

All major spots (Fig. 1) turn blue with a slight greenish hue with $FeCl_3$–$K_3Fe(CN)_6$ test and are, therefore, phenols.[11] It became apparent during the concurrent studies on leucoanthocyanins[12] from the secondary periderm tissues, that the new reddish purple pigments degrade during acidic hydrolysis, and thus are not anthocyanin-like glycosides. The pigments from the three species (Table 4) are bluish purple in anhydrous ethyl acetate, and reddish in

[11] G. M. BARTON, R. S. EVANS and J. A. F. GARDNER, *Nature* **170**, 249 (1952). The assistance of the inventors of the $FeCl_3$–$K_3Fe(CN)_6$ test in confirming our conclusion is gratefully acknowledged.

[12] To be published.

isoamyl alcohol. For example, color of the amabilis fir pigment is bluish purple ($\lambda_{max}$ 584 nm) with a distinct shoulder at 540 nm (Table 4). Following mild acidification, the bluish peak at 584 nm almost disappears, and the reddish shoulder at 540 nm becomes a distinct peak (with a slight increase in absorbance), and another peak appears simultaneously at 450 nm. The transformations of the bluish (solution) to reddish colored solution following acidification, as well as the conversion of the shoulder at 540 nm to a distinct peak without appreciable increase in absorbance signifies that the pigments are transformed to another state following acidification (probably salt formation). It is noteworthy that the pigments in unacidified, as well as acidified, iso-amyl alcohol show only the reddish peak at 547 nm (Table 4). The absence of the blue peak (584 nm) in unacidified iso-amyl alcohol, and its presence in the unacidified ethyl acetate solutions appears to be related to an acid–base reaction: iso-amyl alcohol and anhydrous ethyl acetate (see footnote b, Table 4) were found to be slightly acidic and basic respectively. The acid–base reactions of the pigments from the other two species

TABLE 4. QUALITATIVE VISIBLE SPECTRAL[a] CHARACTERISTICS OF THE PARTIALLY PURIFIED REDDISH PURPLE PIGMENTS IN ACIDIFIED AS WELL AS UNACIDIFIED ANHYDROUS ETHYL ACETATE AND ISO-AMYL ALCOHOL

| | Spectral peaks (nm) | | | |
|---|---|---|---|---|
| | Anhydrous ethyl acetate[b] | | Iso-amyl alcohol[b] | |
| Species | Unacidified | Acidified[c] | Unacidified | Acidified[c] |
| Amabilis fir | 540,[d] 584, | 450, 540, 584[e] | 453, 547, | 453, 547, |
| Western red cedar | 452[e] 540, 580, | 452, 535, | 456, 543, | 456, 543, |
| Western hemlock | 536,[d] 576, | 447, 534, | 452, 538, | 452, 538, |

[a] Spectra were determined using a Unicam SP800 spectrophotometer.
[b] Allied Chemical's reagent grade anhydrous ethyl acetate and iso-amyl alcohol were used.
[c] Five drops of 1 per cent methanolic HCl were added to 1 ml cuvettes.
[d] Distinct shoulder only.
[e] Weak inflection only.

are similar to those of the amabilis fir pigments. Unlike anthocyanins and anthocyanidins, the absorbance of the pigments does not decrease on standing in the unacidified solvents. Therefore the pigments do not undergo pseudo base transformation. Like anthocyanins, the pigments turn blue on exposure to ammonia, and red on exposure to hydrochloric acid vapors. Whereas the bluish colors of anthocyanins, produced on chromatoplates following brief exposure to ammonia vapors revert to their original reddish state, those of the new pigments do not. Moreover, the new pigments are soluble in ethyl acetate, anthocyanins and anthocyanidins are not. It is thus clear that these reddish pigments are neither anthocyanins nor anthocyanidins.

The new pigments are resolved in the solvents which were also found to be excellent for thin-layer chromatography of anthocyanidins.[13] Inasmuch as the chromatographic resolution characteristics are comparable to those of anthocyanidins, the pigments do not appear to be low molecular weight polymeric substances as was assumed in earlier studies.[3, 5, 6] The new tests for microscale identification of anthocyanidins (particularly the $NH_3$ chamber test,

[13] D. B. MULLICK, *J. Chromatog.* **39**, 291 (1969).

and the molybdate test) developed recently in this laboratory,[14] were found to be of value in differentiating these new pigments from anthocyanidins as such,[7] which also co-occur in the secondary periderm tissues. Whereas anthocyanidins studied thus far[14] undergo diagnostic sequential modification of fluorescence in the $NH_3$ chamber test,[14] these new pigments either do not show any fluorescence modification (Tables 1 and 2), or fluorescence disappears within minutes (Table 3). The $NH_3$ chamber test thus indicates that the reddish purple pigment of western hemlock differ from those of western red cedar and amabilis fir. The molybdate test[14] indicates that all pigments may be *o*-hydroxylated phenols, except Spot No. 1 from amabilis fir which may be lacking *o*-hydroxyl grouping. The test also gives distinctive visible colors for the western red cedar pigments (Table 2). Most of the spray reagents[15, 16] used selectively for flavonoid compounds, including the vanillin-hydrochloric acid test, as well as a test for anthraquinone-type compounds,[17] failed to reveal structural features of these new pigments.

These pigments are extremely photolabile, but are relatively stable under subdued light conditions in neutral or mildly alkaline solvents, such as anhydrous ethyl acetate. They are very sparingly soluble in water at room temperatures and are relatively unstable in mildly acidic solutions. Although the pigments from amabilis fir and western red cedar are sparingly soluble, those from western hemlock are somewhat readily soluble in diethyl ether.

The bark pigments discussed here are commonly referred to as phlobaphenes,[1, 5, 6] a term derived from the Greek root words phloios = bark + baphe = dye. The term therefore connotes only their origin and colored nature and does not carry specific chemical meaning. The bark pigmentations, particularly the periderm pigmentation, is varied,[8, 9, 18] and most of the pigments are water-insoluble. Confusion arises by inclusion of such bark pigments under definition of phlobaphenes, which also include the water-insoluble reddish brown products obtained upon treatment of condensed tannins with mineral acids. The discovery that these pigments are a large family of new compounds suggests that they should be referred to as *reddish purple pigments* until their chemistry is delineated.

## EXPERIMENTAL

This investigation was carried out on a microscale because of the limited availability of the secondary periderm tissues. The reddish purple pigments were mechanically scraped from freshly exposed areas of the last formed periderm tissues (rhytidomal region) under a stereomicroscope. The scrapings (100 mg each) were extracted with 1 ml cold (−25°) ethyl acetate on a Centrifugal Homogenizer[19] (homogenization at 1500 rev/min and centrifugation in the same tube at 2300 rev/min each for 3 min). The pigments thus obtained were precipitated with 10 ml cold (−25°) light petroleum (b.p. 30–60°). The procedure was repeated on the same tissue if necessary to obtain more pigments. The precipitate, after decanting the petroleum, was freeze-dried, washed with water, diethyl ether (except western hemlock pigments which dissolved readily in ether) and benezene with freeze-drying after every washing. The washed extract, after final freeze-drying, was either taken up in a few drops of iso-amyl alcohol for TLC or for spectral analysis. The washing procedure removes the bulk of contaminants which otherwise seriously affect resolution.

Compositions of solvents used for TLC were: MHW [MeOH:conc. HCl:$H_2O$ (190:1:10)], BHAW [t-BuOH:2 N HCl:HAc:$H_2O$ (6:1:1:2)], and Forestal [HAc:conc. HCl:$H_2O$ (30:3:10)]. Solvent combination *A* consisted of MHW (first direction) and Forestal (second direction). Combination *B* consisted of BHAW

[14] D. B. MULLICK, *Phytochem.* **8**, 2003 (1969).
[15] D. G. ROUX and A. E. MAIHS, *J. Chromatog.* **4**, 65 (1960).
[16] H. L. HERGERT, *Forest Prod. J.* **10**, 610 (1960).
[17] F. FEIGL and V. ANGR, *Spot Tests in Organic Analysis*, p. 336, Elsevier, New York (1966).
[18] D. P. PROTSENKO, K. I. BOGOMAZ and D. P. KORSHUK, *Visn. Kiivs'k. Univ.* No. 3, Ser. Biol. No. 1, 56 (1960). *C.A.* **60**, 4459e.
[19] Virtis Research Equipment, Gardiner, New York.

(first direction) and Forestal (second direction). The TLC on Avicel SF was carried out by the techniques developed in this laboratory.[13]

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