Journal of Chromatography, 75 (1973) 151–155

Note

CHROM. 6377

Cellulose thin-layer chromatographic separation of *Rubus* fruit anthocyanins*

A simplified two-dimensional thin-layer chromatographic (TLC) procedure for the identification of fruit anthocyanins was outlined by NYBOM[1,2]. He confirmed the presence in the fruit of *R. occidentalis* of four cyanidin glycosides found by HARBORNE AND HALL[3] using paper chromatography (PC), namely the 3-glucoside, 3-rutinoside, 3-sambubioside and 3-xylosylrutinoside. The present work was undertaken to identify the anthocyanin pigments of four *Rubus* species indigenous to the Pacific Northwest: *R. ursinus*, *R. spectabilis*, *R. parviflorus* and *R. leucodermis*. Only *R. parviflorus* had been examined previously[3] and found to contain both cyanidin and pelargonidin glycosides. Detailed examination of these species is a prerequisite to their use as parents in a raspberry breeding program at this center.

Experimental

Plant materials. Fruits of the thimbleberry, *R. parviflorus* Nutt., western trailing blackberry, *R. ursinus* Cham. & Schlecht., western blackcap, *R. leucodermis* Dougl., salmonberry, *R. spectabilis* Pursh., Himalayan blackberry, *R. procerus* Muell., and the elderberry (*Sambucus* spp.) were collected from the wild. Rhubarb petioles and fruit of the boysenberry and Marion blackberry were obtained from nearby commercial plantings. Fruit of *Fragaria vesca* L. cv. Alpine, red raspberry, *R. ideaus* L. cv. Willamette and Meeker and evergreen blackberry, *R. laciniatus* Willd., were obtained from plantings at this center. All fruit was frozen and stored at −15° until examined.

Extraction. A modification of the extraction procedure of WROLSTAD *et al.*[4] was used. Fifty grams of material were homogenized for 1.5 min in a Waring blender. The homogenate was filtered through glass wool and washed with 50 ml of reagent methanol–water (1:1).

A water slurry of insoluble polyvinylpyrrolidone (PVP), Polyclar AT, was poured into a buchner fritted glass funnel containing Whatman G F/A filter paper. Suction was applied until no surface water was visible on PVP. The anthocyanin extract was poured on to the damp PVP layer. The PVP–anthocyanin adsorbate was washed with 150 ml of water followed by 30 ml of methanol.

The colored PVP–anthocyanin adsorbate layer was extracted with 0.1 % HCl in methanol and the extract concentrated to dryness on a rotary evaporator (water-bath 40°) and taken up in three 1-ml portions of 0.01 % HCl in methanol.

Chromatography. The concentrated extract was spotted on to 10 × 10 cm precoated microcrystalline cellulose glass plates (Brinkmann Instruments, Inc., Celplate-22). Four plates were cut from a standard 20 × 20 cm plate.

The plates were run in two directions using the solvent systems of NYBOM[1]: Solvent system A — first direction, *n*-butanol–conc. HCl–water (BHClW) (5:2:1); second direction, water–conc. HCl–formic acid (WHClF) (8:4:1). Solvent system

* Scientific Paper No. 3913, College of Agriculture Research Center Project 0051.

B — first direction, *n*-butanol–glacial acetic acid–water (BAW) (6:1:2); second direction, water–conc. HCl–conc. propionic acid (WHClP) (10:2:3).

The concentrated extracts were also run one-dimensionally using 1 % HCl as solvent. As many as fourteen 10 × 10 cm plates were run at one time using a plastic rack in a 21 × 15 × 13 cm polyethylene tank with a specially fabricated tightly fitting glass lid. The plates were sprayed with Neu's reagent (flavone reagent)[5]: 1 % methanolic solution of diphenylboric acid, B-aminoethyl ester, and the color change observed. Neu's reagent altered the color in visible light of cyanidin glycosides from red to blue while the color of pelargonidin 3-glucoside did not change appreciably. Neu's reagent gave the same results reported for aluminum chloride[6] but the reaction was much faster. Solvent system A was preferred and used for the illustrations as it provided greater spot definition and less diffusion of pigments near the solvent front in the second direction (WHClP).

Authentic anthocyanins isolated from *Fragaria vesca* L. cv. Alpine were cyanidin 3-glucoside and pelargonidin 3-glucoside[7,8], from rhubarb, cyanidin 3-glucoside and cyanidin 3-rutinoside[7–10], and from *Sambucus* spp., cyanidin 3-glucoside and cyanidin 3-sambubioside[2].

Results and discussion

Blackberry: *R. ursinus*, *R. laciniatus*, *R. procerus*, *and Marion*. Only two pigments were found in these species (Table I) and when co-chromatographed with rhubarb pigment 'a' was coincident with cyanidin 3-glucoside and pigment 'c' with cyanidin 3-rutinoside. Cyanidin 3-glucoside was a major pigment in the four blackberries examined while cyanidin 3-rutinoside was a major pigment only in *R. ursinus* and Marion. The pigments of these blackberry species have not been reported previously. In these four blackberries the anthocyanin pattern conforms to the generalization[3] that blackberries contain only two anthocyanins.

TABLE I

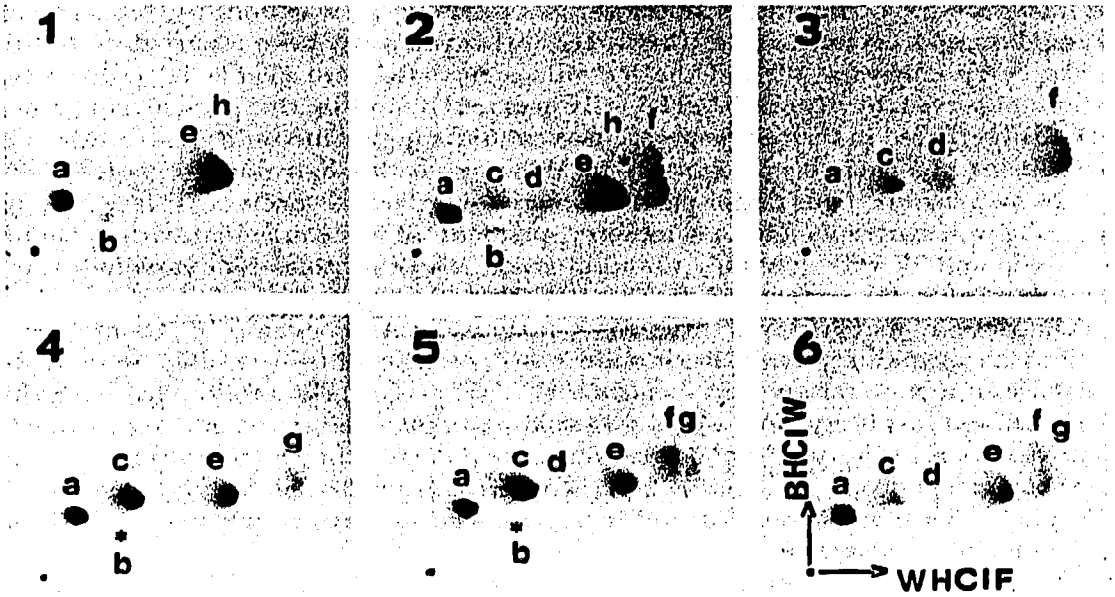
RELATIVE QUANTITIES OF *Rubus* FRUIT ANTHOCYANINS

Relative quantities were estimated visually as follows: +++ major, ++ intermediate, + minor, and T trace quantities.

Species	*Pigment reference letter*[a]									
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*j*	*k*
R. ursinus	+++		+++							
Marion blackberry	+++		++							
R. procerus	+++		T							
R. laciniatus	+++		T							
Boysenberry	++		+		+++		+			
R. ideaus										
cv. Willamette	++	+			+++			+		
cv. Meeker	++		+		+++		++	+		
R. spectabilis	+++	++	+++		+++		++			
R. leucodermis	+		++	++		+++				
R. parviflorus	+		T						+++	++

[a] Letters refer to anthocyanins tentatively identified in Table II and found in Figs. 1–6.

R. ideaus L. cv. Willamette and Meeker. Four anthocyanins were found in the fruit of Willamette and five in Meeker (Table I, Fig. 1). In both cultivars pigment 'h' was an unidentified minor pigment. Pigment 'a', when chromatogrammed with *F. vesca* and rhubarb, was coincident with cyanidin 3-glucoside. Pigment 'b' has not been previously reported in red raspberry, and is tentatively identified as cyanidin 3,5-



Figs. 1–6. Photographs of two-dimensional thin-layer chromatograms using solvent system A. The letters correspond to pigment designation in Table II and the asterisks denote spots present on the TLC plates but not visible on photographic prints. Fig. 1. Extract of *R. ideaus* cv. Willamette. Fig. 2. Extracts of *R. ideaus* cv. Willamette and *R. leucodermis* co-chromatographed. Fig. 3. Extract of *R. leucodermis*. Fig. 4. Extract of *R. spectabilis*. Fig. 5. Extracts of *R. spectabilis* and *R. leucodermis* co-chromatographed. Fig. 6. Extracts of boysenberry and *R. leucodermis* co-chromatographed.

TABLE II

CHROMATOGRAPHIC DATA FOR ANTHOCYANINS FOUND IN *Rubus* SPECIES

Pigment identification reference letter		*Color*		*R_F values × 100 in solvents*[a]				
		Untreated	*Treated with Neu's reagent*	*BHCIW (5:2:1)*	*WHCIF (8:4:1)*	*BAW (6:1:2)*	*WHCIP (10:2:3)*	*HCl (1%)*
a	Cyanidin 3-glucoside	magenta	blue	27	9	23	26	3
b	Cyanidin 3,5-diglucoside	magneta[b]	blue	17	26	13	42	
c	Cyanidin 3-rutinoside	magenta	blue	36	26	25	49	11
d	Cyanidin 3-sambubioside	magenta	blue	35	43	22	62	17
e	Cyanidin 3-sophoroside	magenta	blue	38	59	21	71	29
f	Cyanidin 3-xylosylrutinoside	magenta	blue	43	75	22	83	43
g	Cyanidin 3-glucosylrutinoside	magenta	blue	42	81	20	86	50
h	Pelardonidin glycoside	orange-red	red	53	65	29	78	—
j	Pelargonidin 3-glucoside	orange-red	red	49	25	34	47	11
k	Pelargonidin 3-rutinoside	orange-red	red	50	41	35	64	21

[a] All R_F values determined from two-dimensional chromatograms except for 1% HCl.
[b] Weak fluorescence under UV light.

diglucoside. When sprayed with flavone reagent, its color changed from red to blue and its R_F values (Table II) correspond to those for cyanidin 3,5-diglucoside[1,2]. It showed a weak red fluorescence under UV light. Anthocyanin 'e' has R_F values (Table II) that correspond to cyanidin 3-sophoroside[1], a common anthocyanin in red raspberry[2,3,8]. The Meeker cultivar contained two anthocyanins not found in Willamette. One was cyanidin 3-rutinoside and the second, pigment 'g', was tentatively identified as cyanidin 3-glucosylrutinoside. This latter anthocyanin has been reported in several red raspberry cultivars[2,3,8] and the R_F values reported here (Table II) correspond to those observed by NYBOM[1,2]. The only pigment found in the present study not previously reported in the red raspberry was cyanidin 3,5-diglucoside. WROLSTAD AND STRUTHERS[11] reported seven pigments in Willamette although none was identified.

R. leucodermis. Four major pigments were found in this species (Table I, Fig. 3). When co-chromatographed with elderberry, pigment 'a' was coincident with cyanidin 3-glucoside and 'd' with cyanidin 3-sambubioside. The R_F values for cyanidin 3-sambubioside (Table II) correspond to those of NYBOM[1,2]. When co-chromatographed with rhubarb, 'c' matched with cyanidin 3-rutinoside. Anthocyanin 'f' is tentatively identified as cyanidin 3-xylosylrutinoside based on R_F values[1,2] (Table II) and its occurrence in a close relative, *R. occidentalis*[1,3]. When co-chromatographed with the Willamette red raspberry (Fig. 2), only cyanidin 3-glucoside was a common pigment. *R. leucodermis* is phenotypically close to *R. occidentalis*, but has a separate geographic range, being found only in western North America from British Columbia south to California[12]. The four pigments found in *R. leucodermis* correspond exactly to those reported for *R. occidentalis*[1,3].

R. spectabilis. Five anthocyanin pigments were found in red fruit of this species (Table I, Fig. 4). When co-chromatographed with *R. leucodermis* (Fig. 5), only cyanidin 3-glucoside and cyanidin 3-rutinoside were coincident. When co-chromatographed with Willamette red raspberry, pigments 'a', 'b' and 'e' were coincident and identified as cyanidin 3-glucoside, cyanidin 3,5-diglucoside and cyanidin 3-sophoroside, respectively. When co-chromatographed with the Meeker red raspberry, pigment 'g' was coincident with cyanidin 3-glucosylrutinoside. The anthocyanin pigments of *R. Spectabilis* do not appear to be very different from those found in the red raspberry, *R. ideaus*.

Boysenberry. Four major pigments were found in this species (Table I), and when co-chromatographed with *R. spectabilis* the four were coincident and identified as cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside and cyanidin 3-glucosylrutinoside. When co-chromatographed with *R. leucodermis* (Fig. 6), only cyanidin 3-glucoside and cyanidin 3-rutinoside were common to both species, cyanidin 3-sambubioside and cyanidin 3-xylosylrutinoside were found only in *R. leucodermis* and cyanidin 3-sophoroside and cyanidin 3-glucosylrutinoside were found only in boysenberry. These findings confirm earlier reports on boysenberry anthocyanins[1,3].

R. parviflorus. In this species the major pigments were 'j' and 'k' while 'a' and 'c' were detected in lesser amounts (Table I). When co-chromatographed with *F. vesca* pigment 'a' matched with cyanidin 3-glucoside and 'j' with pelargonidin 3-glucoside. Pigments 'j' and 'k' did not show an appreciable color change after spraying with Neu's reagent. R_F values for pigment 'k' (Table II) correspond to those reported for pelargonidin 3-rutinoside with PC and cellulose TLC[2]. The 3-glucoside of cyanidin and pelargonidin were reported in this species, but pelar-

gonidin 3-rutinoside was not, although it was present in a closely related species, *R. odoratus*[3].

The fruits of *R. ursinus*, *R. spectabilis*, *R. leucodermis* and *R. parviflorus*, all indigenous to the Pacific Northwest, contained cyanidin 3-glucoside and cyanidin 3-rutinoside. In addition *R. spectabilis* contained the 3-sophoroside, 3-glucosylrutinoside and 3,5-diglucoside of cyanidin, *R. leucodermis* contained the 3-sambubioside and 3-xylosylrutinoside of cyanidin, and *R. parviflorus* contained two pelargonidin glycosides.

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Received August 17th, 1972