CHROM. 4778

CHARACTERIZATION OF AN ADDITIONAL ANTHOCYANIN PIGMENT IN EXTRACTS OF STRAWBERRIES, *FRAGARIA**,**

R. E. WROLSTAD, K. I. HILDRUM*** AND J. F. AMOS
Department of Food Science and Technology, Oregon State University, Corvallis, Oreg. 97331 (U.S.A.)
(Received March 31st, 1970)

SUMMARY

Investigation of the anthocyanin pigments of forty lots of strawberries representing thirteen selections and five varieties revealed the presence of a third pigment in most samples not previously identified. The pigment is not a chromatographic artifact as it is unchanged upon rechromatography. It is composed of pelargonidin and glucose in a 1:1 molar ratio. During acid hydrolysis the pigment readily forms pelargonidin-3-glucoside as an intermediate before pelargonidin formation. Infrared and ultraviolet-visible spectral properties eliminate the possibility of acylation with organic acids. Radiotracer experiments eliminate the possibility of incorporation of methanol as an ether or acetal into the pigment. On the basis of spectral data, chromatographic properties, and hydrolysis experiments, it is suggested that the pigment is either the furanose form or the alpha anomer of pelargonidin-3-glucoside.

INTRODUCTION

The main anthocyanin pigment in strawberries was identified as pelargonidin-3-glucoside (pgd-3-glu) by ROBINSON AND ROBINSON[1]. LUKTON *et al.*[2] identified a second minor pigment to be cyanidin-3-glucoside (cyd-3-glu). In assaying the relative amounts of these two pigments and the total anthocyanin pigment of thirteen different selections and five varieties of strawberries[3] (forty samples), we observed the presence of a third band in most analyses. The quantity of this pigment was as much as 5 % of the total anthocyanin content. This publication reports its characterization.

EXPERIMENTAL

Isolation of pigments

Strawberries supplied by the OSU Horticulture Department were washed,

* Technical Paper No. 2783, Oregon Agricultural Experiment Station.
** Presented at the 1969 meeting of the Phytochemical Society of North America, Banff, Alberta, Canada.
*** Author is holder of a research fellowship from the Royal Norwegian Council for Scientific and Industrial Research and on temporary leave from The Research Committee for Preservation of Agricultural Food Products, Ullevålsveien 72, Oslo 4, Norway.

individually quick frozen at −35°, and stored in polyethylene bags at −26°. Pigments were isolated from 500-g samples by adsorption on insoluble polyvinylpyrrolidone (PVP) as described by WROLSTAD AND PUTNAM[4].

Chromatography

Whatman No. 3 paper was used for preparative chromatography. Descending chromatography was used for all paper chromatography excepting sugar determinations which utilized Whatman No. 1 paper in an ascending system. All runs were carried out in the dark at 21 ± 1°.

Cellulose thin-layer plates of 0.25 mm thickness were developed in an ascending manner under the same conditions.

The following solvent systems were used:

(1) glacial acetic acid–water–conc. HCl (15:82:3) (AWHCl);
(2) less dense phase of *n*-butanol–glacial acetic acid–water (4:1:5) (BAW 415); equilibrated for three days;
(3) conc. HCl–water (3:97) (1 % HCl);
(4) *n*-butanol–pyridine–water (6:3:1) (BPW 631);
(5) glacial acetic acid–water (2:98) (2 % HAC);
(6) ethyl acetate–*n*-propanol–water (5:3:2) (EPrW 532);
(7) ethyl acetate–pyridine–water (2:1:2) (EPW 212).

Chromogenic spray reagents

Partridges reagent used for visualization of sugars was formulated as described by ALBACH *et al.*[5].

Organic acids were detected using Bromocresol Green spray reagent[6] and phenolics with tetrazotized benzidine[7].

Spectroscopy

UV-visible spectra were determined with a Beckman DK-1 spectrophotometer. AWHCl-developed chromatograms were air-dried and bands were extracted with spectro-grade MeOH. After determining spectra, solutions were concentrated and their purity assayed by TLC.

Methanol pigment solutions were concentrated in the tip of prepressed KBr "wick-sticks"* by ascending development at 4°. Concentration time was shortened by drying wick-sticks in a vacuum oven (25°, 25 mm pressure) between repeated MeOH developments. The tip containing concentrated pigment was dried, ground in an agate mortar and pestle, and pressed. IR spectra were run on KBr–pigment pellets using a Beckman IR 5 double beam spectrophotometer fitted with a beam condensor. Purity of material was checked by MeOH extraction of the pellet and subsequent chromatography.

Partial acid hydrolysis

Less than 1-mg samples of purified pigments were hydrolyzed in 2 ml of 1 *N* HCl at 97° using the procedure of LYNN AND LUH[8]. Aliquots were withdrawn at 5-min intervals, spotted on cellulose TLC plates, and developed in AWHCl.

* Harshaw Chemical Co., Division of Kewanee Oil Co., Cleveland, Ohio, U.S.A.

Pigment: aglycone ratio

The molar ratio of anthocyanin pigment and sugars yielded after complete hydrolysis wer. determined using a modified procedure of CHANDLER AND HARPER[9]. The molar quantity of anthocyanin was assayed spectrophotometrically on an aliquot of the pigment solution using the extinction coefficient of pgd-3-glu (E = 36, 600, 510 nm). From 0.1–1 μmole of pigment was hydrolyzed (1 N HCl solution at 100° for 60 min in the dark under N_2). The molar quantity of sugars in the hydrolysate was assayed using anthrone reagent[10].

Distribution number

Purified pigment (0.2 mg) in absolute MeOH was evaporated to dryness and dissolved in 15 ml 0.01 N HCl in n-butanol. The concentration of pigment was determined by measuring the absorbance of an aliquot diluted with the same solvent. Five milliliters of the concentrated solution were shaken with 5 ml of 0.01 N HCl in water for 2 min and the absorbance of the organic layer determined. The distribution number is expressed as the per cent pigment remaining in the butanolic phase.

Measurement of radioactivity

A sample of fruit (ORE-US 2931, 2.29 g) was extracted with $^{14}CH_3OH$ having an activity of 0.120 μCi/ml (1.05×10^7 d.p.m./mole). A total of 30 ml was used in the extraction process. The extract was concentrated to 0.5 ml and stored for 10 days at −5°. Quantities of 1 μl were spotted on TLC plates, free MeOH was allowed to evaporate and the plates were developed in AWHCl. The developed plates were dried and scanned using a Packard Model 7200 Radiochromatogram Scanner. Efficiency was determined by scanning a plate spotted with 21,078 d.p.m. of [1-^{14}C]tyrosine and found to be 19.1 %. Peak areas were calculated by triangulation.

RESULTS AND DISCUSSION

A third pigment could be resolved from pgd-3-glu and cyd-3-glu when anthocyanin extracts of *Fragaria* were chromatographed in AWHCl on paper or cellulose thin-layer. It had a higher R_F value than pgd-3-glu in this system and in 1 % HCl but could not be resolved using BAW 415 (Table I). There appeared to be a varietal relationship in that some selections consistently contained substantial amounts of this pigment (band 3) and in others it was absent or barely detectable. Quantities of band 3 tended to increase with storage at −5°. No band 3 was detectable in a sample of *Fragaria ananassa* Duch. cv. "Northwest" when chromatographed immediately after isolation; however, after two weeks subsequent storage at −5° the same sample contained band 3.

Two-dimensional thin-layer chromatography (TLC) using AWHCl in both directions resulted in only three spots, which lay along a 45° angle, indicating that band 3 was not a chromatographic artifact[11]. Band 3, extracted from preparative paper chromatograms with 0.01 % HCl in methanol (MeOH), had the same R_F value as pgd-3-glu when concentrated and rechromatographed. When extracted with absolute MeOH, however, band 3 could be isolated in pure form, retaining its original R_F value.

The UV-visible spectra of band 3 and pgd-3-glu are shown in Fig. 1. The $A_{440}/A_{vis\,max}$ value indicates that the 5 position is free[12]. The visible absorption maxi-

TABLE I

CHROMATOGRAPHIC INVESTIGATION OF PIGMENTS AND THEIR HYDROLYSIS PRODUCTS

Compound	R_F *values*				*Color (Partridges reagent)*
	AWHCl	*1% HCl*	*BAW 415*	*BPW 631*	
Band 1 (cyd-3-glu)	0.35	0.12	0.34		
Band 2 (pgd-3-glu)	0.52	0.22	0.48		
Band 3	0.68	0.38	0.48		
Pelargonidin	0.18	0.06	1.00		
Band 2 hydrolysis pigment					
original	0.51				
aglycone	0.19		1.00		
Band 3 hydrolysis pigments					
original	0.67				
intermediate	0.49				
aglycone	0.18		1.00		
Band 2 sugars				0.23/0.31	olive/red-brown
Band 2 sugars (repurified)				0.23	olive
Band 3 sugars				0.24/0.31	olive/red-brown
Band 3 sugar (repurified)				0.24	olive
Authentic sugar standards					
glucose				0.24	olive
galactose				0.21	brown
arabinose				0.31	red-brown
xylose				0.38	red-brown
rhamnose				0.49	olive

mum eliminates the possibility of substitution in the 7 position[13,14]. The absence of absorption peaks in the 300–330 nm range eliminates the possibility of acylation with cinnamic acids[15]. The intensity differences in the UV range were similar to those reported by SUN AND FRANCIS[16] for cyanidin-3-arabinoside and cyanidin-7-arabinoside; however they also reported a difference in the visible maximum of 3 nm. As the spectra in our study were not determined on crystalline compounds we question whether the intensity differences in the UV region are meaningful.

A compound having the same R_F value as pgd-3-glu was formed as an intermediate in the partial hydrolysis of band 3 to pelargonidin (pgd), the hydrolysis of

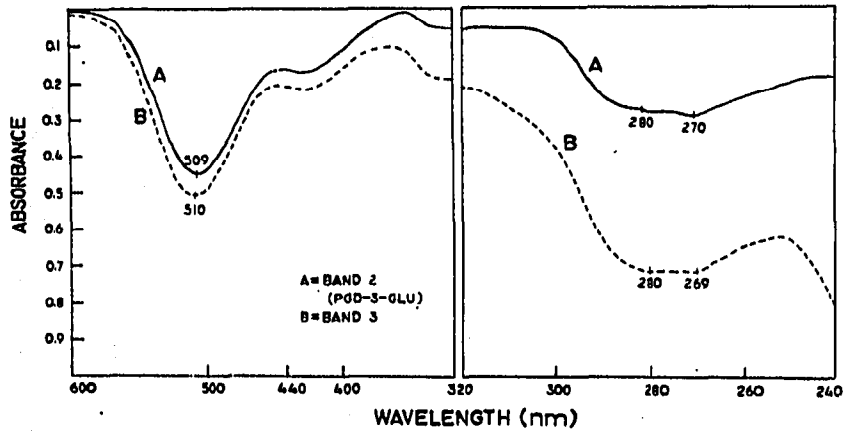

Fig. 1. UV-visible spectra of band 2 (pgd-3-glu) and band 3. Solvent: Spectro-grade MeOH.

band 3 to pgd-3-glu being complete after 5 min. After the developing solvent had evaporated from the thin-layer chromatogram of the band 3 hydrolysis samples, the plate was sprayed with tetrazotized benzidine; there was no indication of phenolic compounds (other than pelargonidin) being liberated with hydrolysis. BOCKIAN *et al.*[17] suggested a minor pigment of *Cabernet sauvignon* grapes with similar chromatographic properties to be a complex of malic acid and malvin. An ether extract of the band 3 hydrolysate was chromatographed in AWHCl along with malic, benzoic, ascorbic, *p*-hydroxybenzoic, caffeic, ferulic, and *p*-coumaric acids. The developed plate was sprayed with Bromocresol Green visualization reagent; no organic acids were detected in the band 3 ether extract.

The IR spectra of pgd-3-glu and band 3 are shown in Fig. 2. The absence of an absorption band at 1725–1750 cm^{-1} eliminates the possibility of esterification with an organic acid. Differences in the O–H stretching region (3100–3500 cm^{-1}) are due to the presence of MeOH, the "wick-stick" developing solvent. Band 3 shows additional bands at 794 and 1143 cm^{-1}, which are due to contamination with free arabinose, an artifact arising from preparative paper chromatography. Thus no obvious differences between the two spectra exist.

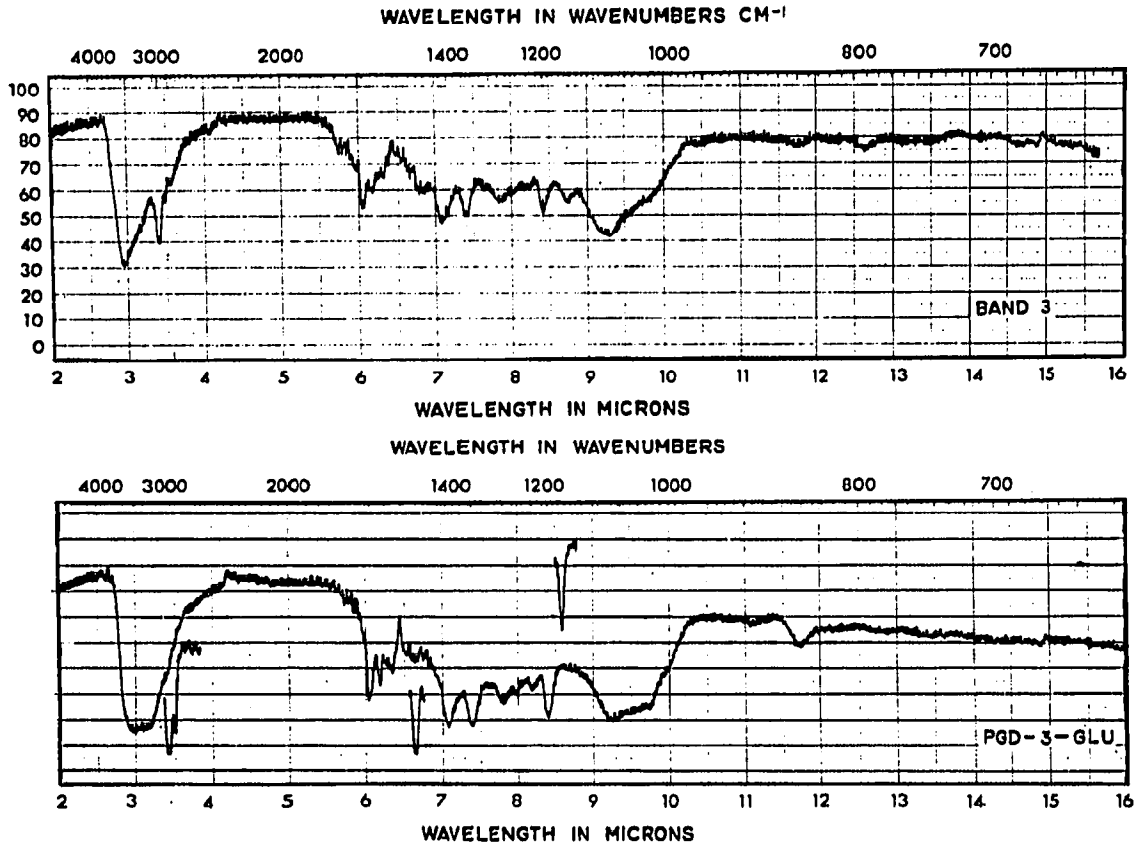

Fig. 2. IR spectra of band 3 and pgd-3-glu (KBr pellet).

After removal of acid from the hydrolysate by extracting with 10 % di-*n*-octylmethylamine in chloroform, the sugars were chromatographed. Only glucose was detected in hydrolysates of pgd-3-glu and band 3. In a second experiment, however,

chromatography of the sugar fraction obtained from the sugar:pigment molar ratio determination showed substantial quantities of pentose sugar in band 3 hydrolysate and trace amounts in the pgd-3-glu sample. The sugar:pigment molar ratio and the partition behavior between butanol and water (Table II) showed that a large quantity of sugar was present in band 3. Subsequent investigation showed the pentose sugar was an artifact arising from development of Whatman No. 3 paper in AWHCl. The contaminating sugar could be removed by rechromatographing the isolated bands in 2 % acetic acid. The sugar:pigment molar ratios and the partition values (Table II) of band 3 thus purified showed it to be a monoglycoside. Chromatography of the sugar fraction of this hydrolysate showed only glucose present. MATHEW[18], in studying the anthocyanidins formed from leucoanthocyanins by treatment with alcoholic HCl,

TABLE II

SUGAR:PIGMENT MOLAR RATIO AND DISTRIBUTION NUMBERS OF PIGMENTS

	Sugar:pigment molar ratio	*Distribution number (% pigment remaining in butanolic phase)*
Pgd-3-glu	1.18, 1.31	50, 47
Pgd-3-glu (repurified)	0.97	
Band 3	4.0	39
Band 3 (repurified)	0.82	45, 49

reported that additional pigments were formed which were ethers of the anthocyanidin and the alcohol. The facile hydrolysis of band 3 would not be characteristic of ethers. An acetal of MeOH and pgd-3-glu (Fig. 3) could conceivably be formed, and its hydrolysis rate would be anticipated to be in accord with band 3. Anthocyanins were isolated from ORE-US 2931, a selection which consistently contained substantial quantities of band 3, using $^{14}CH_3OH$ as the extracting solvent. Radiochromatogram scanning of an AWHCl-developed thin-layer chromatogram of the anthocyanins showed slight incorporation of $^{14}CH_3OH$ at the solvent front. A typical experiment had 600 d.p.m. at the front. The background across the plate was 200 d.p.m. Regions of the plate containing pgd-3-glu, cyd-3-glu, and band 3 were indistinguishable from

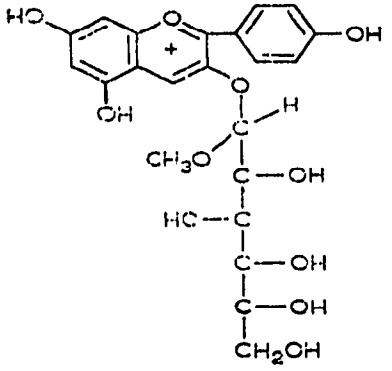

Fig. 3. Pelargonidin-3-β-methylglucose.

the background. Incorporation of $^{14}CH_3OH$ into band 3 in a 1:1 molar ratio would have resulted in a count of 750 d.p.m. It was concluded that there was no incorporation of $^{14}CH_3OH$ into the anthocyanins.

Evidence that the transformation of band 3 to pgd-3-glu was reversible was shown in our preparation of pgd. Pure pgd-3-glu was hydrolyzed in 1 *N* HCl. After the reaction was approximately 50 % complete, the pigments were chromatographed. The solution contained not only pgd-3-glu and pgd, but also band 3.

We are confident that band 3 is composed of pgd and glucose in a 1:1 molar ratio and that it does not contain MeOH, organic acids, or additional phenolics. The existence of different glycosidic forms is an interesting possibility. Several workers (ISHERWOOD AND JERMYN[19], AVIGAD AND BAUER[20] and GEE[21]) have established that α- and β-anomers and pyranose and furanose isomers of glycosides can be separated by paper and thin-layer chromatographic techniques. Furanoside linkages are considerably more labile than pyranosides, furanose compounds having hydrolysis rates in 0.01 *N* HCl 50–200 times greater than the corresponding pyranosides[22]. Hydrolysis rates of α- and β-anomers differ, which anomer having the faster rate being dependent on the sugar derivative[23].

We were interested in learning whether band 3 was an artifact of isolation or storage. Chromatography of an amyl alcohol extract of juice obtained by pressing fresh fruit (ORE-US 2786, *Fragaria ananassa* Duch. cv. "Tioga") through cheesecloth showed the presence of band 3. This would indicate that cold storage is not necessary for formation of band 3 and it is highly unlikely that its formation depended on the extremely mild isolation procedure.

Numerous workers have described minor pigments in anthocyanin extracts whose chromatographic properties are similar to our observations. ALBACH *et al.*[5] reported that a minor band (band 1) having a high R_F value in BAW 612 was interconvertible with band 2, (*p*-coumaric acid derivatives of the 3-monoglucosides of malvidin, peonidin, petunidin, and delphinidin) and speculated that band 1 was the quinoidal anhydro base and band 2 the oxonium salt form separated because of a sharp pH transition on the developed chromatogram. FRANCIS AND HARBORNE[24] observed that in chromatography of huckleberry extracts, band 2 (98 % petanin and 2 % negretein) would separate into two bands which in subsequent chromatography would recombine. The separation was not a resolution of the two pigments but rather two bands each containing both pigments; they attributed its cause to the acid-front phenomenon described by ALBACH *et al.*[5]. HETMANSKI AND NYBOM[25], in studying the anthocyanin pigments of rhubarb, reported the pigments were particularly labile, forming two intermediate pigments in their destruction which had higher R_F values in the solvent systems used. VON ELBE AND SCHALLER[26] isolated pigments not naturally occurring in Montmorency cherries and attributed their formation to ion-exchange resins and HCl. Cyanidin, one of the artifact pigments, and increasing quantities of naturally occurring cyanidin-3-glucoside, were formed from hydrolysis of cyanidin-3-gentiobioside and cyanidin-3-rhamnoglucoside. An additional pigment, however, was a cyanidin-3-diglucoside whose complete identity was not proposed but could not have arisen by partial hydrolysis.

The chromatographic characteristics and facile transformations of the above pigments are similar to what we have observed with band 3. Further study of these pigments should, in our opinion, consider the possibility of different glycosidic forms.

ACKNOWLEDGEMENTS

The authors express their appreciation to F. L. LAWRENCE and R. M. BULLOCK of the OSU Horticulture Department for supplying the fruit and to Mrs. T. P. PUTNAM, who prepared many of the extracts. Acknowledgement is given to D. J. REED for suggestions concerning the radiotracer experiment and for use of radiotracer equipment; TOM FARR assisted in that experiment.

REFERENCES

1 G. M. ROBINSON AND R. ROBINSON, *Biochem. J.*, 26 (1932) 1650.
2 A. LUKTON, C. O. CHICHESTER AND G. MACKINNEY, *Nature*, 176 (1955) 790.
3 R. E. WROLSTAD, T. P. PUTNAM AND G. W. VARSEVELD, *Food Technol.*, in press.
4 R. E. WROLSTAD AND T. P. PUTNAM, *J. Food Sci.*, 34 (1969) 154.
5 R. F. ALBACH, R. E. KEPNER AND A. D. WEBB, *J. Food Sci.*, 30 (1965) 69.
6 Anonymous, *TLC Visualization Reagents and Chromatographic Solvents*, Eastman Kodak Company, Rochester, New York, 1969, p. 7.
7 K. RANDERATH, *Thin-layer Chromatography*, Academic Press, New York, 1964, p. 176.
8 D. Y. C. LYN AND B. S. LUH, *J. Food Sci.*, 29 (1964) 735.
9 B. V. CHANDLER AND K. A. HARPER, *Australian J. Chem.*, 15 (1962) 114.
10 D. L. MORRIS, *Science*, 107 (1948) 254.
11 B. M. LAWRENCE, *J. Inst. Can. Technol. Aliment.*, 1 (1968) 136.
12 J. B. HARBORNE, *Biochem. J.*, 70 (1958) 22.
13 J. B. HARBORNE, *Phenolics in Plants in Health and Disease*, Pergamon, New York, 1960, p. 114.
14 J. B. HARBORNE, *Methods in Polyphenol Chemistry*, MacMillan, New York, 1964, p. 23.
15 L. JURD, in T. A. GEISSMAN (Editor), *The Chemistry of Flavonoid Compounds*, MacMillan, New York, 1962, p. 135.
16 B. H. SUN AND F. J. FRANCIS, *J. Food Sci.*, 32 (1968) 647.
17 A. H. BOCKIAN, R. E. KEPNER AND A. D. WEBB, *J. Agr. Food Chem.*, 3 (1955) 695.
18 A. G. MATHEW, *Phytochemistry*, 8 (1969) 677.
19 F. A. ISHERWOOD AND M. A. JERMYN, *Biochem. J.*, 48 (1951) 515.
20 G. AVIGAD AND S. BAUER, *Carbohyd. Res.*, 5 (1967) 417.
21 M. GEE, *Anal. Chem.*, 33 (1963) 350.
22 W. W. PIGMAN AND R. M. GOEPP, Jr., *Chemistry of the Carbohydrates*, Academic Press, New York, 1948, p. 206.
23 B. CAPON, *Chem. Rev.*, 69 (1969) 407.
24 F. J. FRANCIS AND J. B. HARBORNE, *J. Food Sci.*, 31 (1966) 524.
25 W. HETMANSKI AND N. NYBOM, *Fruchtsaft-Ind.*, 13 (1968) 256.
26 J. H. VON ELBE AND D. R. SCHALLER, *J. Food Sci.*, 33 (1968) 439.