Phytochemistry, 1966, Vol. 5, pp. 183 to 187. Pergamon Press Ltd. Printed in England

THE ANTHOCYANIN PIGMENTS OF BARLINKA GRAPES

B. H. KOEPPEN and D. S. BASSON

Department of Food Science, University of Stellenbosch, South Africa

(*Received* 8 *April* 1965)

Abstract—By means of preparative paper chromatography five crystalline anthocyanins were isolated from the skins of Barlinka grape berries. The pigments were identified as oenin (malvidin 3-glucoside), mono-*p*-coumaroyl oenin and the 3-glucosides of peonidin, petunidin and delphinidin. Oenin was by far the major pigment of the grape skins while the 3-glucosides of petunidin and delphinidin occurred only in very small concentrations. The identification of all the compounds as monoglycosides supports the classification of Barlinka as a cultivar of *Vitis vinifera*.

INTRODUCTION

BARLINKA is the most important table grape variety exported from South Africa and accounted for 48·2 per cent of the country's total grape exports during the 1963–64 season.[1] Although its retention of freshness and flavour during relatively long periods of cold storage makes it a particularly satisfactory export variety, a major problem sometimes encountered by the producer is a variable and irregular development of the characteristic black colour of the ripe grape. Perold[2] who found Barlinka growing near Algiers and brought it to South Africa 55 years ago, considered it to be a *Vitis vinifera* cultivar but no chemical evidence on the nature of its pigmentation has hitherto been available to support this classification. The present study was therefore undertaken in order to establish the identity of the pigments prior to an investigation of their biosynthesis.

RESULTS AND DISCUSSION

Examination of a methanolic 1 % (w/v) HCl extract of the grape skins by chromatography in butan-1-ol: acetic acid: water (BAW) (20:5:11, v/v) revealed the presence of five pigments (*A* to *E*). *Pigment C* was clearly the major constituent and appeared to be about twice as concentrated as *pigments A* and *B*. *Pigments D* and *E* were present in only extremely small concentrations.

Pigment A was converted to *C* by alkaline hydrolysis whereas all the other pigments remained unaltered under these conditions. This indicated that *A* was an acylated form of *C*. Controlled acid hydrolysis[3] revealed that none of the pigments was a diglycoside. Although *pigment A* did reveal an intermediate product of hydrolysis, this had a lower, and not a higher, R_f value than the original pigment in BAW. Furthermore, this intermediate was identical with *C* by chromatography and was thus formed through hydrolysis of the ester linkage in *A*.

[1] Deciduous Fruit Board, *Annual Report*, p. 22. Addendum to *Decid. Fruit Grower*, **14**, 1964).
[2] A. I. PEROLD, *Handboek oor Wynboue*, Pro Ecclesia Press, Stellenbosch (1926).
[3] B. V. CHANDLER and K. A. HARPER, *Australian J. Chem.* **14**, 586 (1961).

The aglycone liberated by acid hydrolysis of *pigments A* and *C* was shown to be malvidin whereas those liberated from *pigments B*, *D* and *E* were found to be peonidin, petunidin and delphinidin respectively.

Examination of the acid hydrolysates for the presence of sugars revealed only glucose in each case and it thus appeared that all the pigments were monoglucosides. That the sugar residues were attached at the 3-positions of the anthocyanidins was confirmed by hydrogen peroxide oxidation of the pigments and alkaline hydrolysis of the products.[3,4]

Additional evidence on the nature of the pigments was obtained by alkali fusion[5] and degradation with dilute NaOH solution[6] which confirmed that in *A* and *C* the aglycone was malvidin whereas in *B*, *D* and *E* it was peonidin, petunidin and delphinidin respectively.

Pigment A was found to be acylated with *p*-coumaric acid. Alkaline hydrolysis of peroxide-oxidized *A* resulted in the liberation of a compound possessing a considerably higher *R* value than glucose in partitioning mixtures. The substance reacted similarly to glucose with aniline hydrogen oxalate[7] but, like *p*-coumaric acid, it exhibited a blue fluorescence on exposure to ammonia vapour under u.v. light. Birkofer and Kaiser[8] and Harborne[9] who reported similar results with acylated anthocyanins, considered their products to be glucose acylated with *p*-coumaric acid. It would therefore appear that in *pigment A* also, the *p*-coumaroyl residue is attached to one of the alcoholic hydroxyl groups of the glucosyl residue. However, it was particularly striking that *pigments B*, *C*, *D* and *E* were decolorized within a few seconds by hydrogen peroxide whereas *A* maintained its colour for at least 10 min, and by comparison with *C* it is difficult to explain how acylation in the glucosyl residue could confer such extra stability on the phenolic portion of the molecule.

Ultraviolet spectral measurements[9-12] supported the chromatographic and chemical identification of the pigments and direct determinations of the sugar aglycone ratio in *C* and the anthocyanin:cinnamic acid ratio in *A* were shown to be 1:1 in each case.

The i.r. spectra of the Barlinka pigments all exhibited a high absorption band in the 1630–1640 cm^{-1} region due to the aryl-conjugated heterocyclic oxygen atom of flavylium salts.[13] Ribéreau-Gayon and Josien[13] found the 1466 cm^{-1} band in malvidin to be characteristic of the methoxylated anthocyanins which they examined and they therefore ascribed it to $-OCH_3$ deformations. This band was, however, not apparent in the spectra of any of the grape anthocyanins although all, including *E*, showed absorption at 1457–1460 cm^{-1}, probably due to aromatic C–C stretchings.[14] A prominent band at 1335–1345 cm^{-1} in the spectra of *A*, *B*, *C* and *D* occurred only at 1300 cm^{-1} in *pigment E* providing an interesting correlation with earlier results[13] where the 1313 cm^{-1} band of delphinidin was at a significantly lower frequency than the corresponding bands of the other anthocyanidins examined. Ribéreau-Gayon and Josien[13] consider the absorption pattern between 1100 and 1000 cm^{-1} to be the most valuable for differentiating the six anthocyanidins studied but the differences

[4] P. Karrer and G. de Meuron, *Helv. Chim. Acta* **15**, 507 (1932).
[5] D. G. Roux, *J. Am. Leather Chemists' Assoc.* **53**, 384 (1958).
[6] P. Karrer and R. Widmer, *Helv. Chim. Acta* **10**, 555 (1927).
[7] R. H. Horrocks and G. B. Manning, *Lancet* **256**, 1042 (1949).
[8] L. Birkofer and C. Kaiser, *Z. Naturforsch.* **18b**, 337 (1963).
[9] J. B. Harborne, *Phytochem.* **3**, 151 (1964).
[10] L. Jurd in *The Chemistry of Flavonoid Compounds* (Edited by T. A. Geissman) p. 107. Pergamon Press, Oxford (1962).
[11] T. A. Geissman, E. C. Jorgensen and J. B. Harborne, *Chem. & Ind. (London)* 1389 (1953).
[12] J. B. Harborne, *Biochem. J.* **70**, 22 (1958).
[13] P. Ribéreau-Gayon and M. L. Josien, *Bull. Soc. Chim. France* 934 (1960).
[14] L. J. Bellamy, *The Infra-red Spectra of Complex Molecules*, Methuen, London (1958).

in the spectra of the Barlinka anthocyanins in this region were not very great. Furthermore, the introduction of glycosyl residues appears to increase the general absorption in the i.r. and individual bands were less well resolved in anthocyanins than in the corresponding anthocyanidins. *Pigment A*, however, showed a characteristic absorption band at 1690 cm^{-1} due to C=O stretching in the *p*-coumaroyl residue. The ester carbonyl band in the vicinity of 1700 cm^{-1} therefore readily distinguishes acylated from non-acylated anthocyanins.

Although previous workers[15] have reported the occurrence of a mono-*p*-coumaroyl malvidin 3-glucoside in *V. vinifera*, this pigment does not appear to have been isolated in crystalline form prior to the present study. The other Barlinka pigments have all been found in various *Vitis* species, oenin (malvidin 3-glucoside) having long been recognized as the major pigment of *V. vinifera* since its first isolation from this source 50 years ago.[16] It is, too, the major pigment of Barlinka grapes.

An exclusively monoglycosidic pattern has been found to occur in *Vitis* species other than *V. vinifera*,[17] but the nature of the pigments isolated strongly supports the botanical classification of Barlinka as a *V. vinifera* cultivar.

EXPERIMENTAL

All chromatograms were developed by downward migration of the solvent. Whatman No. 3 paper sheets (46 × 57 cm) were used for preparative work; for all other purposes Whatman No. 1 chromatography paper was employed. Solvent systems used were butan-1-ol:acetic acid:water (20:5:11, v/v) [BAW]; butan-1-ol:2 N HCl (1:1, v/v) [BuHCl]; benzene:acetic acid:water (125:72:3, v/v) [BeAW]; butan-1-ol:benzene:pyridine:water (5:1:3:3, v/v) [BBePW]; acetic acid:conc. HCl:water (30:3:10, v/v) [Forestal]; formic acid:conc. HCl:water (5:2:3, v/v) [Form-HCl]; aq. 2% (v/v) acetic acid [2% HOAc]; aq. 15% (v/v) acetic acid [15% HOAc] and phenol:water (3:1, v/v) [PhOH]. In two-phase systems (BuHCl and BBePW) the upper layer was used.

Malvin and delphinidin chlorides were obtained commercially (Fluka AG.), peonidin was prepared by reductive acetylation of isorhamnetin,[18] petunidin 3-glucoside was kindly supplied by Mr. H. Malan, Chemistry Department, Stellenbosch University, and 3-*O*-methylgallic acid was prepared by the method of Fischer and Freudenberg.[19]

Infrared spectra were recorded on a Perkin–Elmer model 21 spectrometer by the KBr-disk method. Sample concentrations were 1 mg/250 mg KBr for *pigments A*, *B* and *E*, and 0·6 mg/250 mg KBr for *pigments C*, *D* and malvidin. Spectral measurements in the visible and u.v. region were made with a Zeiss model PM QII spectrophotometer.

Isolation of Pigments from Barlinka Grape Berries

The skins (300 g) from fresh, ripe Barlinka grape berries were extracted with methanolic 1% (w/v) HCl (1·2 l.) and methanol (5 × 1 l.), the combined extracts were vacuum concentrated at 35°, streaked on Whatman No. 3 paper sheets, and the chromatograms developed in aq. 1% (w/v) HCl for 18–24 hr to wash water-soluble constituents from the anthocyanin

[15] Leading references. O. COLAGRANDE and G. GRANDI, *Ann. Sper. Agrar. (Rome)* **14**, 325 (1960); F. DRAWERT, *Vitis* **2**, 288 (1961); H. MALAN, M.Sc. (Agric.) Thesis, Stellenbosch University, South Africa (1963); R. F. ALBACH, R. E. KEPNER and A. D. WEBB, *J. Food Sci.* **30**, 69 (1965).

[16] R. WILLSTÄTTER and E. H. ZOLLINGER, *Ann. Chem. Liebigs* **408**, 83 (1915).

[17] J. RIBÉREAU-GAYON and P. RIBÉREAU-GAYON, *Am. J. Enol.* **9**, 1 (1958).

[18] H. G. KRISHNAMURTY, V. KRISHNAMOORTHY and T. R. SESHADRI, *Phytochem.* **2**, 47 (1963).

[19] E. FISCHER and K. FREUDENBERG, *Ber. Deut. Chem. Ges.* **46**, 1116 (1913).

pigments. The chromatograms were partially dried and the pigments eluted with aq. 75% (v/v) methanol. The combined eluates were concentrated under reduced pressure reapplied to Whatman No. 3 paper and developed in BAW. A total of five clearly distinguishable pigment bands was obtained (*A*, *B*, *C*, *D* and *E* in order of decreasing R_f value). The individual pigments were eluted from the chromatograms with aq. 80% (v/v) methanol.

Pigment A. The combined, concentrated eluates from two experiments were left in a vacuum desiccator until a thick precipitate had formed. The solid was filtered off and dissolved in ethanolic 5% (w/v) HCl. After 2 days the fine, dark-red needles were filtered off, washed with ethanol at 0° and dried. Yield: 16·3 mg; λ_{max} 283 and 536 mμ in methanolic 0·1% (w/v) HCl (log ε 4·42 and 4·48 respectively).

Pigment B. The combined, concentrated eluates from two experiments yielded thick, brown-red needles on storage at 5°. The solid was recrystallized from ethanolic HCl as described for *pigment A*. Yield: 14·0 mg; λ_{max} 281 and 529 mμ in methanolic 0·1% (w/v) HCl.

Pigment C. The concentrated eluate yielded a crystalline solid which was filtered off and recrystallized twice from ethanolic 5% (w/v) HCl. Yield: 154·5 mg; λ_{max} 279 and 538 mμ in methanolic 0·1% (w/v) HCl (log ε 4·13 and 4·47 respectively).

Pigment D. The combined, concentrated eluates from three experiments were evaporated to near dryness in a vacuum desiccator, aq. 1% (w/v) HCl (1·2 ml) was added and the solution was kept at 5°. Small, dark-blue crystalline globules separated after several days and these were filtered off and recrystallized from ethanolic 5% (w/v) HCl. Yield: 2·0 mg; λ_{max} 276 and 540 mμ in methanolic 0·1% (w/v) HCl. The higher wavelength maximum was shifted to 558 mμ in 30 mM $AlCl_3$ in methanolic 0·1% (w/v) HCl.

Pigment E. This was recovered from the combined, concentrated eluates of three experiments exactly as described for *pigment D*. Yield: 2·0 mg; λ_{max} 274 and 540 mμ in methanolic 0·1% (w/v) HCl. The higher wavelength maximum was shifted to 569 mμ in 30 mM $AlCl_3$ in methanolic 0·1% (w/v) HCl.

Identification Procedures

Acid hydrolysis (cf.[3]). The pigment (1 mg) was refluxed in a mixture of ethanol (1 ml) and aq. 4 N HCl (1 ml.). After 5 min samples were removed and examined directly by chromatography in BAW, Forestal and Form-HCl. The remaining solution was refluxed for 20 min, diluted with water and the aglycones were extracted with pentan-2-ol and examined by chromatography.

The colourless aqueous hydrolysate was neutralized with di-*n*-octylmethylamine[20] and examined by chromatography in BAW, BBePW and PhOH with authentic pentoses and hexoses as markers. Aniline hydrogen oxalate[7] was employed as the chromogenic spray reagent.

Alkaline hydrolysis. The pigment (1 mg) in aq. 2 N NaOH (2 ml) was kept at 20° for 3 hr. The solution was acidified and the pigment was extracted with pentan-2-ol and examined by chromatography in BAW and BuHCl. As only *pigment A* underwent any permanent change by this treatment, a further sample was hydrolysed as described and the acidified hydrolysate was extracted with ether. The ethereal extract was examined by chromatography in BAW and 2% HOAc with *p*-coumaric acid as marker. The pigment was extracted from the acidified hydrolysate with pentan-2-ol and compared with *pigment C* by chromatography in BAW, BuHCl and 2% HOAc.

[20] J. B. HARBORNE and H. S. A. SHERRATT, *Biochem. J.* **65**, 23P (1957).

Determination of glycosylation in the 3-position of the anthocyanin molecule. The hydrogen peroxide technique[4] as modified by Chandler and Harper[3] was employed. However, as hesperetin 7-glucoside was also found to liberate glucose, the hydrolysis time at 100° was reduced from 5 min to 1 min. Under these conditions no glucose was liberated from hesperetin 7-glucoside but satisfactory results were still obtained with 1 mg samples of the pigments.

Alkali fusion. Alkali fusions were performed by the method of Roux.[5] The products were examined by chromatography in BAW, BeAW and 15% HOAc using phloroglucinol, gallic, protocatechuic and *p*-hydroxybenzoic acids as markers. Bis-diazotized benzidine[21] was found to be the most satisfactory chromogenic spray reagent.

Degradation with dilute sodium hydroxide solution. The method of Karrer and Widmer[6] was adapted for use on a microscale. The pigment (1 mg) in 2 N NaOH (1·5 ml) was heated in a nitrogen atmosphere at 100° for 1 hr. The solution was cooled, acidified and extracted with ether. The ethereal extract was concentrated and examined by chromatography in BAW, BeAW and 15% HOAc using syringic, vanillic, gallic, 3-*O*-methylgallic and *p*-hydroxybenzoic acids as markers.

Determination of the molecular ratio sugar/aglycone in pigment C. The compound (4·62 mg) in ethanolic 5 N HCl (2 ml) was refluxed for 20 min in the absence of oxygen and light. The solution was cooled, diluted with methanol to 250 ml and the pigment concentration was determined by measurement of the extinction at 547 mμ ($\log \epsilon$ 4·50 for malvidin). The ratio, sugar: aglycone in *C*, was found to be 1·2:1.

Determination of the molecular ratio anthocyanin/acyl residue in pigment A. The compound (5·09 mg) was hydrolysed with 2 N NaOH as previously described. The solution was acidified, saturated with NaCl and extracted with ether (3 × 3 ml). The ethereal extract was taken to dryness under reduced pressure at 30°, the residue was dissolved in methanolic 0·1% (w/v) HCl and the extinction was measured at 311 mμ ($\log \epsilon$ 4·32 for *p*-coumaric acid). The aq. salt-saturated hydrolysate containing the anthocyanin was stored under reduced pressure for 12 hr, diluted to 250 ml with methanolic 0·1% (w/v) HCl and the extinction was measured at 538 mμ ($\log \epsilon$ 4·47 for *pigment C*). The anthocyanin: acyl ratio for *pigment A* was found to be 1:1·1.

[21] J. E. Koch and W. Krieg, *Chemiker Ztg.* **62**, 140 (1938).