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## Pigment development during ripening of the grape

by

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### Die Pigmententwicklung während der Traubenreifung

**Zusammenfassung.** — An gekennzeichneten Beeren einer einzigen Traube der Sorte Shiraz wurde über einen Zeitraum von 50 Tagen vom Weichwerden bis nach der Ernte die Zusammensetzung der extrahierbaren Traubenpigmente untersucht. Die Analyse des Gesamtextraktes der Beeren erfolgte durch Säulenchromatographie an Sephadex G25, wobei Daten über die acylierten und nichtacylierten Anthocyane und die Tanninfarbstoffe gewonnen wurden.

Die acylierten Anthocyane lagen schon in den frühesten Beerenstadien vor; acylierte und nichtacylierte Anthocyane erreichten ihre Maximalkonzentration 20—30 Tage nach dem Weichwerden der Beeren bei 21—24 °Brix. Von einem Maximum mit ca. 1,0 mg extrahierbaren Gesamtanthocyanen je Beere um den 20. Tag ging die Anthocyanmenge auf ca. 0,7 mg je Beere gegen den 50. Tag zurück; diese Abnahme war vom Schrumpfen der Beeren begleitet.

Die Tanninfraktion war anfänglich gelb und betrug am 1. Tag durchschnittlich 0,7 mg je Beere. In den ersten Tagen nach dem Weichwerden nahm der Gehalt dieser Fraktion je Beere stark zu (bis auf 2,4 mg am 4. Tag); danach schwankte er zwischen 2,1 und 4,3 mg je Beere (Mittelwert in der Zeit vom 4. bis zum 50. Tag: 3,1 mg). Auch die spektralen Kenndaten veränderten sich bis zum 30. Tag fortlaufend, wobei die Pigmentierung in den während dieser Periode isolierten Proben zunahm. Die Tanninpigmente der Trauben werden als Artefakte angesehen, die durch die Verwendung von HCl bei der Extraktion und die Messung von extrem labilem phenolischem Material entstehen; aber ihre leichte Bildung unter diesen Bedingungen erlaubt Rückschlüsse auf den normalen Ablauf der Alterungsreaktionen von Rotweinen.

### Introduction

Investigations of compositional changes in the wine grape during ripening have been conducted over many years, particularly at Bordeaux (J. RIBÉREAU-GAYON and PEYNAUD 1960). These referred mainly to organic acids, sugars, amino acids and minerals, but more recently the phenolic constituents have been the subject of similar studies. P. RIBÉREAU-GAYON (1971, 1972) has reported data on anthocyanins and tannins from the skins, and also on tannin extract from seeds of Merlot and Cabernet Sauvignon grapes in three consecutive years from the same Bordeaux vineyards. He has emphasised the difficulty of making sensible interpretations from measurements of these diverse and reactive materials. SINGLETON (1966) investigated changes in the levels of total extractable phenolics during ripening of several wine grape varieties in Davis, California. SINGLETON *et al.* (1966) then reported 'chromatographic mapping' and estimation of relative amounts of phenolic components in such extracts during ripening, with particular reference to seed extracts.

No such data have been reported for wine grapes in Australia. Because of the widely ranging climatic conditions, time of harvest varies greatly for the different wine districts, with earlier ripening in the hotter regions. Unfortunately, there has been a general tendency to delay harvest with the aim of achieving better colour as well as a higher degree of alcohol in the wine. Thus, wine pH is frequently much higher than it need be, with adverse influence upon anthocyanin equilibria and upon wine colour composition. The degree of ionisation of anthocyanins in young

red wines has been recently correlated with independent quality ratings (SOMERS and EVANS 1974) and likely benefits of earlier harvest in some Australian wine regions have been proposed (SOMERS 1975).

The purpose of this paper is to present data which indicate that the anthocyanin content of Shiraz grapes (*syn.* Petite Syrah) is optimal at a stage well in advance of the maximum Brix values. The whole experiment was based upon measurements of grapes from a single marked cluster, over a 50-day term from veraison to the post-harvest period, the aim being to examine both qualitative and quantitative changes in pigment content during the ripening period.

Pigment profiles of grape skin phenolics, from single berries after day 2, were obtained by gel column analysis (SOMERS 1968), so that a 'tannin fraction' was isolated in each analysis, and acylated anthocyanins were recorded as a separate fraction from the non-acylated pigments; this methodology has therefore provided qualitative information not previously reported. Although this 'field experiment' was conducted in the 1968 season, the data were put aside because of uncertainty about the quantitatively large tannin fraction. Much uncertainty still remains, but later work with young red wines (SOMERS 1971, 1975, SOMERS and EVANS 1974), and also the recent accounts of similar investigations in Bordeaux, have enabled tentative interpretations to be made.

#### Materials and Methods

The grapes were from a single bunch on a Shiraz vine at Magill, South Australia in the 1968 season. The berries were culled to uniform size early in January, leaving about 60 berries on the bunch. Veraison (first colour) was seen on the 18th January in several berries, and the remainder had all begun to colour by the 24th; the dates of veraison were marked by attachment of a small card to the pedicel of each berry.

Ten berries were taken for analysis at day 1, six at day 2 and then single berries through to day 50 after veraison. Pigment analysis was generally conducted on the day of sampling; the intact berry was otherwise kept at  $-10^{\circ}\text{C}$  for up to three days before analysis.

**Pigment Profiles:** The skin was removed, washed briefly in water and macerated with  $5 \times 5$  ml methanolic 0.1% HCl during 1 hour; the Brix value of the juice was measured by refractometer. The combined pigment extracts were filtered through Celite, evaporated near to dryness  $<30^{\circ}\text{C}$  and the residue then dissolved in 0.5–1.0 ml 50% aqueous acetone HCl (1.5 ml conc. HCl/1). This concentrate was clarified by brief centrifugation before application to the gel column (60 cm  $\times$  1.5 cm Sephadex G25 Fine in the same solvent mixture, flow rate 45 ml/h, 1 mm flow cell, Unicam S.P. 800A spectrophotometer, Servoscribe 2 mV recorder).

All profiles were recorded at 540 nm with appropriate signal expansion; this was  $\times 20$  for the early samples, then  $\times 10$  and  $\times 5$  for the later ones.

**The tannin fraction:** The first fraction from each run was evaporated near to dryness, and the residue redissolved in methanolic 0.1% HCl (5.0 ml). The visible spectrum was recorded (1 cm cell) and then the UV spectrum after appropriate dilution. The total solution was then evaporated and dried to constant weight in a 1 cm test tube. The ratios  $E_{280}/E_{540 \text{ nm}}$  and the values  $E_{1\text{cm}}^{1\%}$  (540 nm) were calculated and used as measures of progressive compositional changes in this fraction.

**The anthocyanin fractions:** Measures of the quantities of acylated anthocyanins and of non-acylated anthocyanins were obtained directly from the pigment profiles. The total absorbance of each fraction was integrated by weight

of a paper tracing, and the quantity of pigment per berry was then calculated by use of a factor which related to column flow rate, cell path length, signal expansion and extinction coefficient. The  $E_{1\text{cm}}^{1\%}$  (540 nm) value of 500 was used for each of the two anthocyanin fractions (SOMERS and EVANS 1974), and a 'chart unit', of dimensions 12 mm by 10 mm and weight 9.8 mg, was then equivalent to 36  $\mu\text{g}$  anthocyanins.

### Results and Discussion

It was considered that the problem of field variability, in relation to the experimental aim of investigating pigment changes during the ripening period, would be largely eliminated by sampling from one selected bunch of culled grapes (C. R. HALE, verbal advice). The observed rate of sugar accumulation, 21 to 24 °Brix being reached after 20 to 30 days, did not indicate any pathological condition in the selected bunch, nor were there any other indications that the bunch was possibly atypical. Similarly, it has been assumed that the later-sampled berries did also undergo normal maturation processes, even though the berry numbers were much depleted in the later stages of ripening; only about half of the original 60 berries on the selected bunch were actually taken for analyses.

The results obtained refer both to monomeric pigments and to the 'tannin fraction'.

#### The anthocyanins

The gel column elution profiles of total grape or wine phenolics characteristically show three fractions (SOMERS 1966, 1968). In these analyses, the 'tannin' fraction, excluded from the gel, was eluted as a narrow band at 39 ml, acylated anthocyanins at 52 ml, and anthocyanins at 69 ml (Fig. 1).

No qualitative changes were seen in the composition of total anthocyanins during ripening; from the first blush of colour, at veraison (day 1), the presence of acylated anthocyanins was clearly evident (Fig. 1). Fluctuation in the relative quantities of acylated and non-acylated anthocyanins during ripening was apparently due to individual variation between berries, but the latter were consistently the major fraction.

Total anthocyanin content became maximal between 20 and 30 days after veraison, when the Brix readings were 21 to 24 degrees, and there were subsequently no evident qualitative changes in the pigment profiles to day 50 (Fig. 2). Estimates, based upon elution curve areas, of quantities of the two fractions of anthocyanins at each stage of berry growth are shown in Fig. 3. There was an evident decrease in total anthocyanins after day 30. A similar decline in the level of extractible anthocyanins during the period of 'sur-maturation' was reported by P. RIBÉREAU-GAYON (1972), whose observations were based upon samplings of 200 berries from certain Bordeaux vineyards in 1969, '70 and '71.

It seems likely that the 'extractibility' of the anthocyanins is adversely affected by shrinkage of the berries during the later stages of ripening (from day 35). In any case, sugar accumulation was more than adequate by day 30 for the making of dry red wines, and there was no indication that more deeply coloured wines might be obtained by delaying harvest.

However, it is generally well recognised that the rate of ripening of wine grapes, and also the level of pigments attained in the harvest of any particular variety, are determined by seasonal, regional and vineyard factors. By means of growth cabinet studies, HALE and BUTTROSE (personal communication) have recently demonstrated

a controlling influence of temperature upon both rate of anthocyanin accumulation and the level of pigments actually attained in Cabernet Sauvignon grapes.

#### The 'grape tannins'

Large and progressive qualitative changes were seen in the tannin fraction, as isolated by gel column analysis, during the first two weeks after veraison. Again in contrast with the anthocyanin fractions, there was no consistent pattern of variation, apart from a sharp initial increase, in the actual quantity of material in this fraction.

The mean quantity per berry isolated from the sample of ten berries taken at day 1 was 0.7 mg. The weight of this fraction increased to 2.4 mg at day 4, and varied thereafter to the post-harvest period between 2.1 and 4.3 mg/berry (mean 3.1 mg).

The qualitative changes in the tannin fraction during ripening express themselves in the increasing colour of that fraction. At day 1, this was pale yellow, becoming yellow-brown by day 6, brown-red by day 10, with progressive increase in redness through to about day 30. Spectral measures of these dramatic changes in

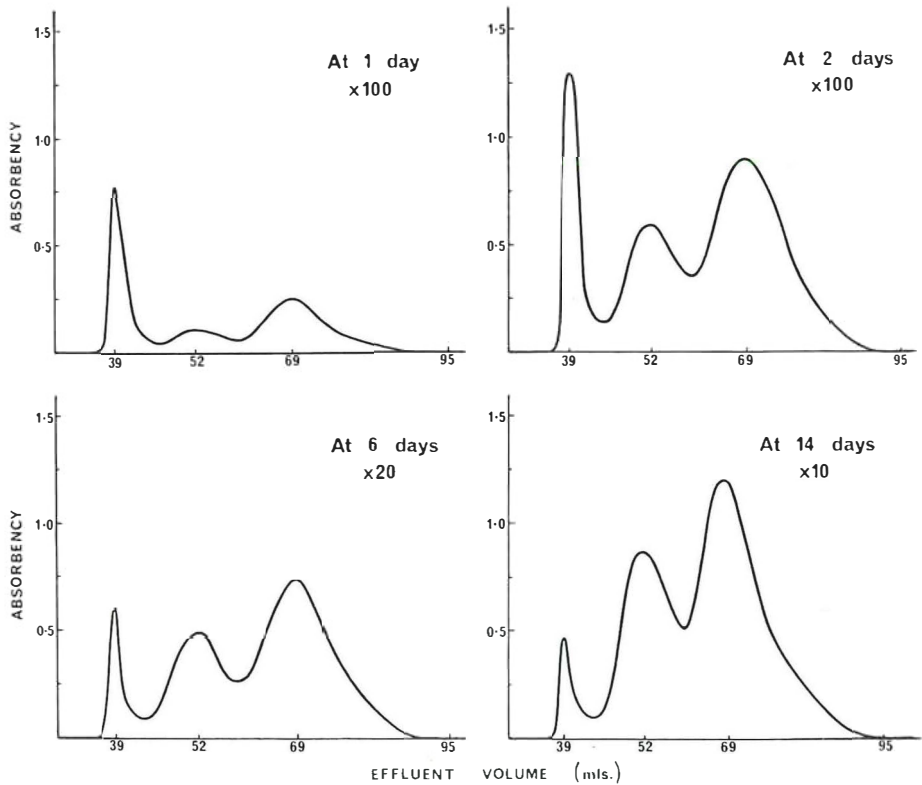


Fig. 1: Pigment profiles of Shiraz skin extracts during early stages of ripening. Each profile refers to the extract of a single berry; the different expansion factors are indicated.

Pigmentprofile von Beerenhautextrakten der Sorte Shiraz; frühe Reifungsstadien. Jedem Profil liegt der Extrakt einer einzigen Beere zugrunde; die verschiedenen Faktoren der Anzeigerverstärkung sind angegeben.

'tannin composition', which relate to rapid increase in the chromophoric content of this fraction, are presented in Fig. 4.

From about four weeks after veraison, at a Brix reading of about 23 degrees, the spectral characteristics of this fraction became fairly constant (Fig. 4). Spectral differences between the yellow-brown fractions isolated soon after veraison and the red materials seen near grape maturity are illustrated in Fig. 5.

Before considering what these observations may mean, it can be said that the method used for extraction of total phenolics from the washed skins, the easy solubility of the materials isolated in organic solvents, the spectral properties and the absence of nitrogen, all support the opinion that the 'tannin fractions' isolated were entirely phenolic. Their polymeric state was indicated both by exclusion from the gel and their immobility in the usual chromatographic solvent mixtures. The pro-anthocyanidin nature of the early samples was shown by the identification of cyanidin and delphinidin as products of mild acid hydrolysis, though the main product was a dark red polymer.

It is noteworthy that the range of tannins seen during ripening of Shiraz from a few days after veraison (2.1—4.3 mg/berry) is similar to the mean quantities of the tannin fractions from ripe berries of Shiraz and from five other red wine grape

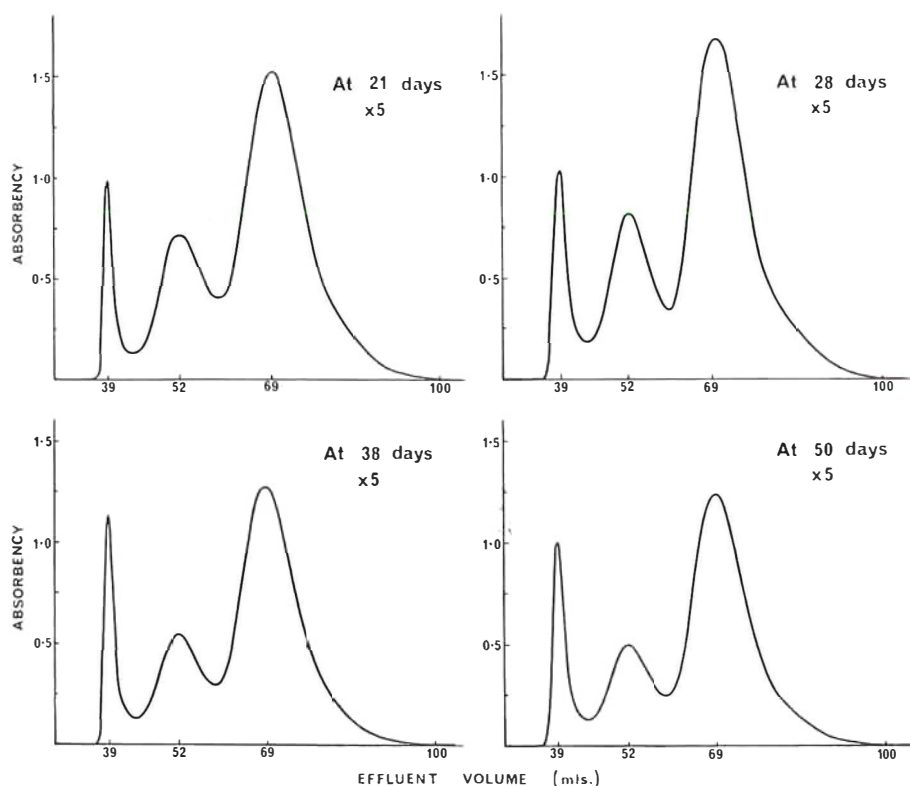


Fig. 2: Pigment profiles of Shiraz skin extracts during later stages of ripening. Each profile refers to the extract of a single berry with  $\times 5$  signal expansion.

Pigmentprofile von Beerenhautextrakten der Sorte Shiraz; spätere Reifungsstadien. Jedem Profil liegt der Extrakt einer einzigen Beere zugrunde; Anzeigeverstärkung  $5 \times$ .

cultivars examined in previous seasons (SOMERS 1968), though there was a 12-fold range in anthocyanin content per berry for the various cultivars. The tannin levels per berry were somewhat lower than RIBÉREAU-GAYON'S (1972) estimates of extractible tannin in the skin of Merlot and Cabernet-Sauvignon grapes; the Bordeaux data were obtained by a colorimetric analysis of total extract, with reference to a proanthocyanidin standard derived from pine bark. They show an initial increase in skin tannin content after veraison, and then fairly uniform levels per berry during ripening, much as seen in the present Shiraz experiment. SINGLETON (1966), measuring total grape phenolics by the Folin-Denis procedure, found that typically, for all varieties examined at Davis, there was a steady increase in the average content per berry for about a month after veraison; subsequently, the levels became fairly constant, being about 6 mg/berry (gallic acid standard) for Petite Syrah.

However, in the Shiraz experiment at Magill, there was also the qualitative feature of rapidly changing spectral characteristics in the 'tannin fraction' during ripening (Figs. 4, 5). Later observations (SOMERS 1971) indicated that the tannin pigments isolated from grape extracts should be regarded as artefacts of the procedures employed, rather than materials actually occurring *in vivo*. The

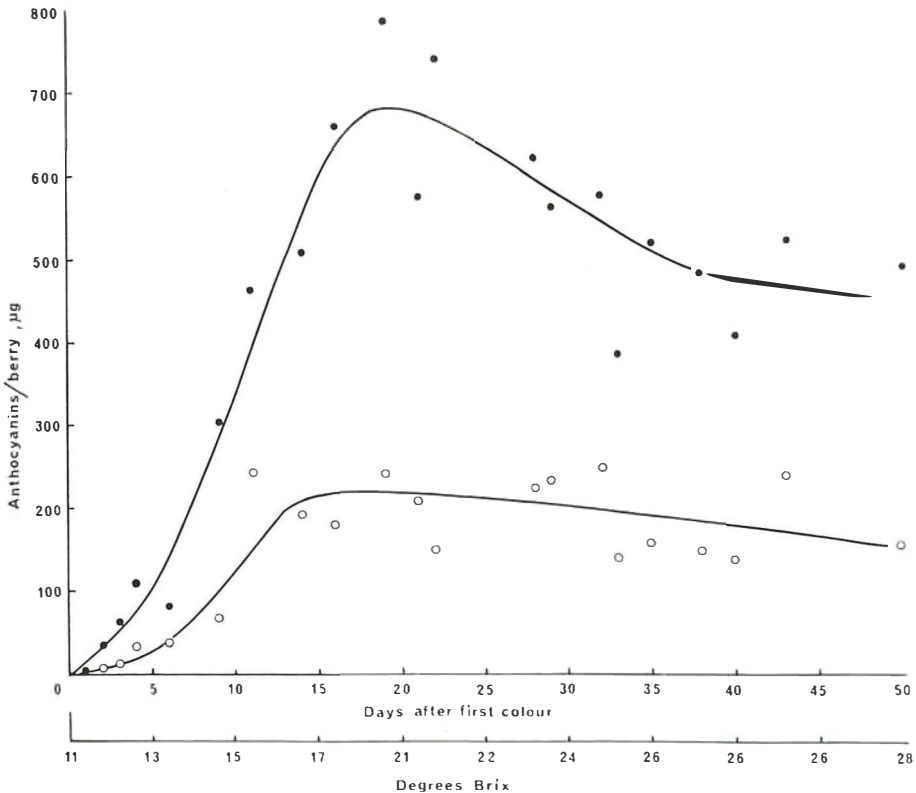


Fig. 3: Estimates of anthocyanin contents per berry during ripening. ○ = acylated anthocyanidin-3-glucosides; ● = anthocyanidin-3-glucosides.

Anthocyangehalte je Beere während des Reifungsverlaufes. ○ = acylierte Anthocyanidin-3-glucoside; ● = Anthocyanidin-3-glucoside.

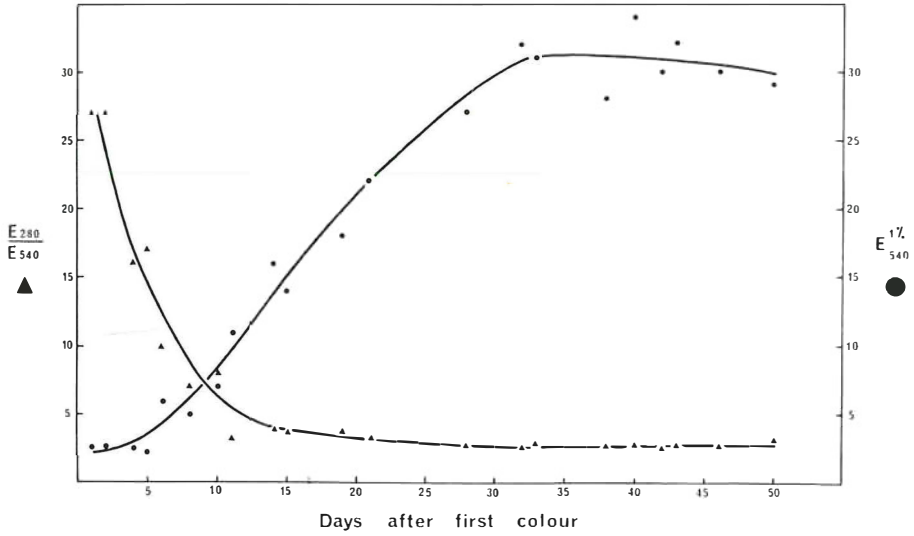


Fig. 4: Progressive changes in the tannin fractions are illustrated by plots of the spectral measures  $E_{280\text{ nm}}/E_{520\text{ nm}}$  and the extinction values  $E_{540\text{ nm}}^{1\%}$ .

Fortschreitende Veränderung der Tanninfraktionen, dargestellt als Relation  $E_{280\text{ nm}}/E_{520\text{ nm}}$  und als Werte von  $E_{540\text{ nm}}^{1\%}$ .

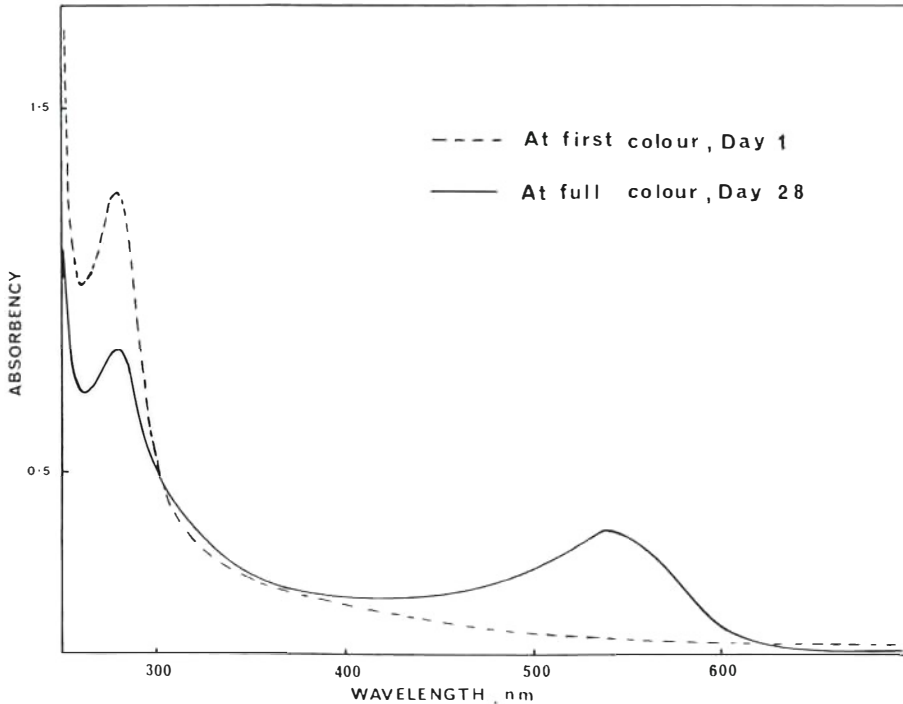


Fig. 5: Spectral characteristics of tannin fractions from unripe Shiraz and from Shiraz at full colour development.

Spektrale Charakterisierung der Tanninfraktionen aus unreifen und aus voll ausgefärbten Shiraz-Beeren.

samples obtained immediately after veraison were noted as being extremely unstable, changing to insoluble brown-red resins within a few weeks of cold storage and darkening rapidly with mild heating. The use of HCl in the extraction procedure, and in the gel column analysis, is thought to have induced formation of the polymeric pigments, these being similar to those which progressively displace the anthocyanins as the dominant pigment forms during conservation and ageing of red wines (SOMERS 1966, 1968, 1971).

Thus, the reactive phenolic precursors, which are generally accepted as being catechins, flavan-3,4-diols and related dimers, may give rise to polymeric pigments by acid-catalysed interaction with the anthocyanins *in vitro* as soon as the latter are present in any appreciable amount; this supposition helps to account for the progressive change in spectral characteristics of this fraction during the first four weeks after veraison (Figs. 4, 5).

There has been much speculation about such polymeric pigment structures in wine (SOMERS 1966, 1971, JURD 1969, RIBÉREAU-GAYON 1973), but no model condensation reactions between flavans and anthocyanins have yet been demonstrated. However, JURD (1967) has isolated a dimeric flavylum-flavan pigment by reaction of a synthetic flavylum salt with catechin, and RIBÉREAU-GAYON (1973) has shown interactions between malvidin-3,5-diglucoside and pine bark tannin.

It now seems that closer study of the 'tannin fraction' at veraison, and of its likely reaction *in vitro* with malvidin-3-glucoside will assist in interpretation of the fate of such phenolics during ageing of *Vitis vinifera* wines.

### Summary

Using marked berries from a single Shiraz grape cluster in a vineyard at Magill, South Australia, the composition of extractible grape pigment was examined over a 50-day period from veraison to post-harvest.

The analyses of total skin extract were made by gel column chromatography, from which measures of acylated anthocyanins, anthocyanins and tannin pigments were obtained.

Acylated anthocyanins were present from the earliest stages, and the levels of both acylated and non-acylated anthocyanins became maximal at 20 to 30 days after veraison, when the Brix value was 21 to 24 degrees. There was a decline from a maximum of about 1.0 mg total extractible anthocyanins per berry around day 20 to about 0.7 mg per berry approaching day 50; this decrease was associated with shrinkage of the berries.

The tannin fraction was initially yellow and averaged 0.7 mg/berry at day 1. In the first few days after veraison, there was sharp increase in the quantity of this fraction per berry (to 2.4 mg at day 4) with fluctuation thereafter between 2.1 and 4.3 mg/berry (mean 3.1 mg from day 4 to day 50). There was also progressive change in spectral characteristics up to about day 30, with increasing pigmentation in the samples isolated during this period.

The grape tannin pigments are considered to be artefacts arising from the use of HCl in the extraction and measurement of extremely labile phenolic materials, but their facile formation in such conditions is relevant to interpretations of the normal course of ageing reactions in red wines.

### Acknowledgements

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