The Determination of Anthocyanins in Aging Red Wines: Comparison of HPLC and Spectral Methods

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Total free anthocyanin contents of red table wines and port wines measured by high performance liquid chromatography (HPLC) were much lower than those estimated by the spectral method of Somers and Evans. Differences between the methods were greatest in young wines and decreased with aging. HPLC indicated that very young red table wines contained 20% to 35% polymeric pigments, and freshly-made port wines contained 17% to 69% polymeric pigments, expressed as proportions of total wine color in acid solution. A large part of the polymers were formed during skin fermentation. The findings indicate that oligomeric pigments formed during red wine aging are partially bleached by the bisulfite used in the spectral method so that anthocyanin contents calculated on this basis are too high. Progressive increases in pigment resistance to bleaching by bisulfite and decreases in color gain on polymer acidification are envisaged, as oligomeric and polymeric pigments of increasing complexity are formed during wine aging. Losses of total pigments and total free anthocyanins were logarithmic with time during port wine aging. The rate constant of anthocyanin loss as determined by HPLC is suggested as a true measure of anthocyanin aging in port wine.

The color of red wine is due principally to anthocyanins derived from the fruit (monomeric or 'free' anthocyanins) and polymeric pigments formed from the anthocyanins by condensation with other flavonoid compounds and probably aldehydes during wine aging (5,11,12,13,14,20). Somers and Evans (16,17) developed a spectral method of estimating the extent of polymeric pigment formation in young red wines of the current vintage, which has been subsequently adopted by many workers. In this study, the color of red wine at its natural pH due to polymeric pigments was considered to be the residual wine color after bleaching the anthocyanins with a large concentration of sodium metabisulfite. Total pigment was measured as the wine color when diluted with acid (1 *M* HCl). The color of the polymeric pigment in acid was estimated as 5/3 × its color at natural wine pH. Total anthocyanin color in acid was then obtained by difference (total pigment − acidified polymeric pigment) and converted to concentration using an appropriate value of the absorptivity of red wine anthocyanins (16).

The advent of high performance liquid chromatography (HPLC) now allows the direct measurement of anthocyanins in red wines, since they appear as discrete peaks without interference (in young wines, at least) from polymeric pigments which are seen as diffuse humps of long retention times. Integration of the peak areas of the individual monomeric anthocyanins determines the total monomeric anthocyanin content. Thus, it was of interest to compare the measurement of total anthocyanins, and hence the extent of polymeric pigment formation in red wines as determined by HPLC, with that estimated by the spectral method.

Materials and Methods

Wines: Red wines were made by standard methods. Port wines were made by fermentation at 28°C and fortification to 19.5% v/v ethanol with brandy at sp gr 1.045 and were stored at 15°C.

Color measurements: Wine color (WC) and polymeric pigment color (PPC) at wine pH were measured at 520 nm, and total pigment color (WCA) was measured in 1 *M* HCl at 520 nm (6). Polymeric color in 1 *M* HCl (PPCA) was assumed to be equal to 5 PPC/3, and the color of the monomeric anthocyanins in 1 *M* HCl (ACA) was obtained by difference from ACA = WCA − 5 PPC/3.

The concentration of total monomeric anthocyanins (C) in 1 *M* HCl was expressed as malvidin 3-glucoside chloride (molecular mass 529) using the molar absorptivity value (ϵ) of 28 000 L/mole/cm (9) at 520 nm. Hence, C (mg/L) = 18.9 ACA.

Other ϵ values reported are 27 000 (3) and 27 455 (8). From the absorbance value $A^{1\%}_{10\ mm}$ of 500 quoted by Somers and Evans (17), their recommended ϵ value for red wine anthocyanins can be calculated as 26 455. The values itemized are all very similar. The highest figure was chosen, as this probably signifies the purest sample of malvidin 3-glucoside.

HPLC: Two instruments were used. The Carignane and Gamay wines were measured with a Spectra-Physics SP 8000 B HPLC with built-in integration facilities operating at 520 nm under conditions described previously (10).

The equipment used for analytical HPLC of port wines consisted of a Model 100A/101A Altex solvent pump, a Pye Unicam LC-XP gradient programmer, and a Pye Unicam LC3 UV detector fitted with a visible wavelength accessory kit to monitor at 520 nm. A Pye Unicam CDP 1 one-channel integrator was connected to integrate the visible peaks (520 nm). A Rheodyne valve (Model 7125) fitted with a 20-μL loop was used to inject 20-μL samples. A reversed-phase Spherisorb-Hexyl (5 μm) Shandon column (5 mm i.d. × 100 mm long, packed in the laboratory using a Shandon Column Packing pump) was maintained at 35°C using a Jones Chromatography heating block. Gradient elution was made with

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Table 1. Comparisons of total anthocyanins and polymeric pigments in two red wines by the spectral method and HPLC.

Cultivar	Time of analysis (days after crushing)		Total anthocyanins spectral ACA_1 (A^{10mm}_{520nm})	spectral C_1 (mg/L)	HPLC C_2 (mg/L)	HPLC ACA_2 (A^{10mm}_{520nm})	Total pigment color WCA (A^{10mm}_{520nm})	Polymeric pigment contribution to WCA (%) spectral	HPLC
Carignane	after pressing	(3)	-	-	223	11.8	16.1	-	27
	after racking	(30)	11.4	216	149	7.9	12.1	6	35
	after bottling	(213)	7.0	132	57	3.0	8.2	15	63
Gamay	after racking	(15)	15.7	296	227	12.0	17.0	8	29
	after bottling	(226)	6.3	119	59	3.1	8.3	24	62

Table 2. Comparisons of the percentages of total wine color (in 1 *M* HCl) due to polymeric pigments by the spectral method and HPLC.

Cultivar	No. of Wines	Analysis time	Polymeric pigment color (%) spectral	HPLC	Ratio HPLC/spectral Range	Mean
Carignane	5	after pressing	-	20-25	-	-
	5	after racking	4-9	31-40	4.3-7.6	5.6
	10	after bottling	12-18	41-63	3.1-5.0	3.7
Gamay	5	after racking	3-5	27-32	3.2-5.7	4.1
	11	after bottling	13-25	37-65	2.2-3.7	2.8

mixtures of 0.6% aqueous perchloric acid and methanol (2).

Standardization of HPLC: The concentration of monomeric anthocyanins in wines and ports was quantified by using an external malvidin 3-glucoside chloride standard. A suitable standard solution was made of approximately 1 mg chromatographically pure malvidin 3-glucoside chloride dissolved in 25 mL 0.1 *M* HCl. The absorbance (A) at 520 nm was measured on a 10× dilution with *M* HCl in a 10-mm cell using a SP8 100 Pye Unicam spectrophotometer, and the concentration (C) of malvidin 3-glucoside chloride was calculated using the same ϵ value as in the spectral method from C (mg/L) = 18.9 A. The total peak area at 520 nm of a red wine or port was compared with the peak area obtained from injection of a known quantity (20 μL) of standard solution and expressed as a concentration of malvidin 3-glucoside chloride.

The linear responses of the detectors were checked, and the reproducibility of the analyses were confirmed for the standard solution and for wines and ports (2). Standard analyses were made daily (two with red wines, two to four with ports). The separation and distribution of individual anthocyanins in red wines and ports has been described elsewhere (1,2); here, we are concerned only with the total anthocyanin contents obtained by integration of peak areas.

Results and Discussion

Table 1 shows typical results for two red wines (Carignane and Gamay). Total anthocyanins determined by the spectral method are given in terms of their measured color values in 1 *M* HCl (ACA_1) and also as calculated concentrations (C_1 mg/L) of malvidin 3-glucoside. Total anthocyanins determined by HPLC are given as measured concentrations (C_2) of malvidin 3-glucoside and also after conversion to the color values (ACA_2) they would exhibit in 1 *M* HCl ($C_2/18.9$). The latter is necessary to compare with total pigment values expressed as the total color in 1 *M* HCl. Table 1 shows that total anthocyanins measured by HPLC were always much lower than those estimated by the spectral method. Polymeric pigment colors derived by difference between the values of total pigment color (WCA) and anthocyanin color (ACA) were correspondingly higher by HPLC than spectrally. Table 2 summarizes similar results for many wines made by varying processing parameters as part of other experiments. Overall, the percentages of polymeric pigment color to total color of acidified wines of the two cultivars derived from HPLC measurements of monomeric anthocyanins were 4.8× greater after racking and 3.2× greater after bottling than estimated by the spectral method.

Findings with port wines were similar. Measurements made of 50 single-cultivar port wines (immediately after fortification with brandy) during three years gave the following percentages of polymeric pigment color to total color (in 1 *M* HCl): 3% to 24% (mean 9.7%) by the spectral method and 17% to 69% (mean 36.6%) by HPLC. Polymers derived from HPLC measurements were 2× to 8× (mean 3.8×) greater than those found spectrally. The behavior of a typical port wine during

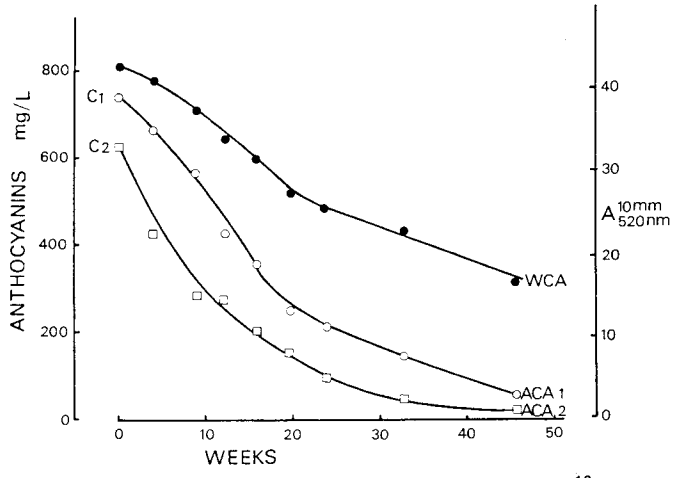

Fig. 1. Decreases in total pigment color (WCA as $A^{10\ mm}_{520\ nm}$) and total anthocyanins determined spectrally (ACA_1 as $A^{10\ mm}_{520\ nm}$ and C_{10} as mg/L) and by HPLC (C_2 as mg/L and ACA_2 as $A^{10\ mm}_{520\ nm}$) during aging of a port wine.

Table 3. Comparisons of total anthocyanins determined by the spectral method and HPLC during port wine aging.

Age (weeks after fortification)	Total anthocyanins spectral		HPLC		Total pigment color	Ratios spectral	HPLC
	ACA_1 (A^{10mm}_{520nm})	C_1 (mg/L)	C_2 (mg/L)	ACA_2 (A^{10mm}_{520nm})	WCA (A^{10mm}_{520nm})	$\frac{ACA_1}{WCA}$	$\frac{ACA_2}{WCA}$
0	39.0	737	627	33.2	42.6	0.92	0.78
4	35.0	661	424	22.4	41.4	0.85	0.54
7.8	30.1	569	286	15.1	37.4	0.80	0.40
12.3	22.4	423	279	14.8	33.9	0.66	0.43
15.8	18.8	355	204	10.8	31.8	0.59	0.34
19.8	13.0	246	152	8.0	27.4	0.47	0.29
23.8	11.2	211	96	5.1	25.7	0.43	0.20
32.8	7.6	143	45	2.4	22.8	0.33	0.10
45.8	3.3	62	24	1.3	16.7	0.20	0.07

aging is illustrated in Figure 1 based on the data in Table 3. The fall in total pigment color (WCA) is indicative mainly of the formation of pigments of progressively decreasing absorptivity values (ϵ). Total anthocyanins measured by HPLC were consistently lower throughout than when estimated by the spectral method. The difference between the two methods was greatest when the port was young and became less as it aged. Thus, the ratio of polymeric pigments (HPLC)/polymeric pigments (spectral) was 3.0 initially, but fell to 1.2 after 46 weeks. Particularly noteworthy is the considerable anthocyanin pigment polymerization occurring during skin fermentation (Table 1, 2, 3).

The losses of anthocyanins in aging red wines as measured by HPLC are generally similar to those found by Nagel and Wulf (8). When the wines were one year old, the anthocyanin contents of the wines described in Table 2 were small and varied from 10 to 39 mg/L depending upon treatment. But, unlike our spectral data which are similar to those of Somers (15,17 - chemical age index ii), Nagel and Wulf found rapid anthocyanin losses by the spectral method. Thus, differences between their spectral and HPLC measurements were small; they found 20% more anthocyanins by HPLC than spectrally at the start of fermentation, but 16% to 34% less after 240 days of aging. In view of the latter inconsistencies and the large differences between the methods found in our analyses, we have verified the accuracy of our HPLC measurements and conclusions.

First, a freshly-made grape skin extract was analyzed quantitatively for total anthocyanins (C_2; ACA_2) by HPLC and total pigments (WCA) by the spectral method of measuring its color in 1 *M* HCl. Since both measurements were standardized using the same solution of malvidin 3-glucoside, identical values were expected (ratio ACA_2/WCA of unity). Analyses of four different concentrations of grape skin extract in the range of 13 to 535 mg/L anthocyanin gave ratios ACA_2/WCA of 0.99, 1.00, 0.96, and 0.95 (mean 0.98). Second, a test was done to check whether all the anthocyanins could be detected in a port which contained many more compounds than a grape skin extract. A five-month-old single-cultivar port containing an appreciable concentration of anthocyanins, but also a substantial concentration of polymeric pigments, was analyzed by HPLC. A second analysis was done after an addition of an accurately known quantity of grape skin extract of the same cultivar. The percentage recovery, after correction for dilutions, was 104%. Thus, there appeared to be no fault or artifact in the quantification of the HPLC method for the determination of free anthocyanins.

To further investigate whether polymerization can occur during fermentation, the early part of a laboratory scale fermentation at 25°C was monitored. As the grapes used had been stored at −20°C, the extraction of the anthocyanins was probably faster than during a fermentation of fresh grapes, due to damage of the cells, which allowed the anthocyanins to leach out easier. A sample taken immediately after crushing contained 292 mg/L total anthocyanins (ACA_2 = 15.4), and total pigment color (WCA) was 15.6, confirming the absence of polymeric pigments initially (ratio ACA_2/WCA = 0.99). As shown in Figure 2, both total pigment and total anthocyanin contents increased during fermentation, but the latter increased at the slower rate. After 24 hours of

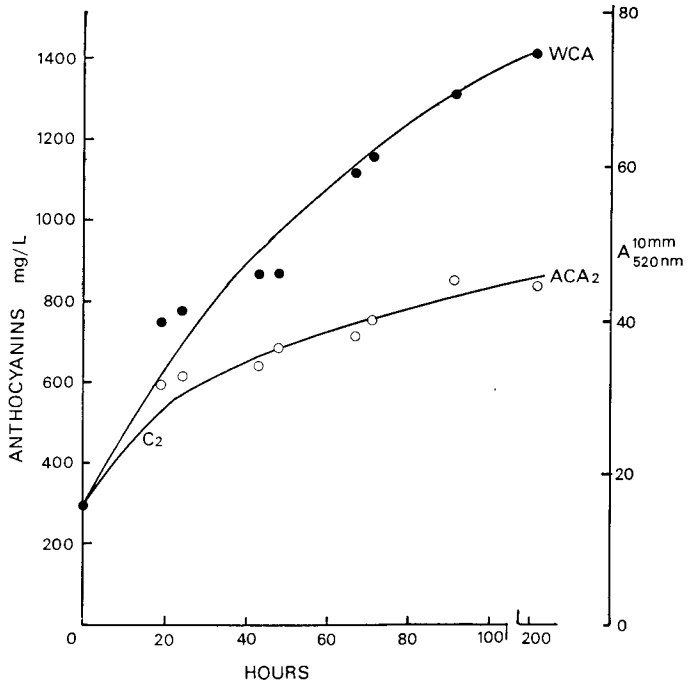

Fig. 2. Increases in total pigment color (WCA as $A^{10\ mm}_{520\ nm}$) and total anthocyanins determined by HPLC (C_2 as mg/L and ACA_2 as $A^{10\ mm}_{520\ nm}$) in must during fermentation on the skins (25°C).

extraction, the ratio of ACA_2/WCA was 0.79, indicating that 21% of color in the total pigment measurement was due to polymeric material. After three days, the ratio was 0.65; thus, a substantial amount of polymerization (at least 35%) had occurred during this short fermentation period at 25°C.

Since the HPLC method appears well founded, the possibility of deficiencies in the spectral procedure arises. The latter is based upon two assumptions: 1) bisulfite bleaches only the monomeric anthocyanins and is without effect on polymeric pigments; and 2) the color of the polymers increases ⅝× on acidification. Since the spectral anthocyanin color in acid ACA = WCA − ⅝ × PPC, ACA may be too high because PPC is too low, implying that bisulfite bleaches some polymeric pigments as well as anthocyanins. Previous work by Burroughs (4) has indicated that oligomeric anthocyanins may have some affinity for bisulfite. The following experiments showed conclusively that this was the case.

Black grape skins were extracted with methanol or methanol-2% formic acid; the extracts were dissolved in 10% ethanol/90% 0.2 *M* potassium hydrogen tartrate (pH 3.5), a few drops of chloroform were added to prevent bacterial spoilage, and the solutions were stored in stoppered flasks in the dark at about 20°C (14). At the beginning and at intervals thereafter, the degree of bleaching by sodium metabisulfite (2000 mg/L SO_2) was measured spectrally, and the disappearance of the anthocyanins was followed by HPLC. During 133 days of storage, the color of the solutions decreased ($A^{1\,mm}_{520\,nm}$ fell from 0.410 to 0.165), and there was a gradual increase in SO_2-resistant pigments ($A^{1\,mm}_{520\,nm}$ from 0.020 to 0.065), in agreement with the results of Somers (14). At the start of the experiment, 95% of the color was bleached and attributed to anthocyanins. Figure 3 shows the anthocyanins (measured by HPLC) and the bleachable pigment found in a Carignane extract at various sampling times, both expressed as percentages of the amounts present at the start of the experiment. If the anthocyanins alone were bleached by SO_2, the two curves should be superimposable. However, the bleachable pigments were always greater than the anthocyanins. This shows clearly the presence of colored components (probably oligomeric pigments) in addition to anthocyanins which are bleached by SO_2. After 68 days, for example, the solutions contained SO_2-bleachable pigment equivalent to 20% of the original anthocyanin color ($A^{1\,mm}_{520\,nm}$ = 0.078) as well as SO_2-resistant pigment ($A^{1\,mm}_{520\,nm}$ = 0.050 at the time of sampling).

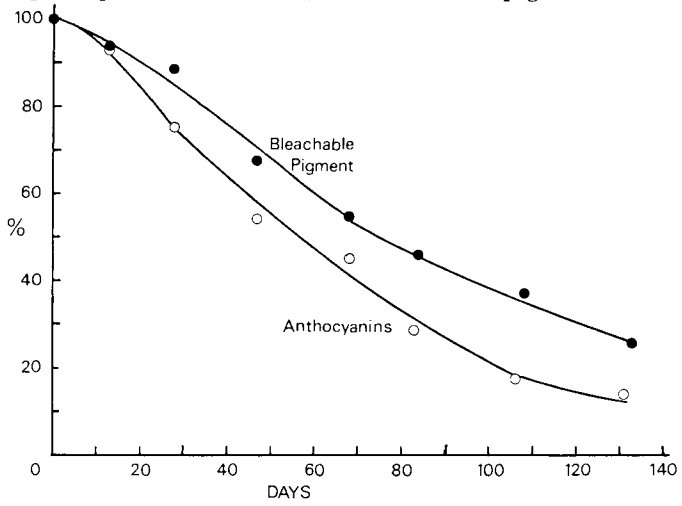

Fig. 3. Anthocyanins (by HPLC) and bleachable pigment during aging of Carignane grape-skin extract, both expressed as percentages of their initial values.

Results were similar with extracts of Cabernet Sauvignon and Gamay grape skins. In the former, pigment bleaching still occurred (10% of the original anthocyanins) when all the anthocyanins (measured by HPLC) had disappeared.

Even the more complex polymeric pigments in older wines are affected by SO_2. Thus, additions of bisulfite (to give 20 to 100 000 mg/L SO_2) to a four-year-old wine containing a large proportion of polymeric pigment caused progressive color decreases, not only with increasing SO_2 concentration but also with time (Fig. 4); increased color with time is attributed to oxidation of SO_2 and formation of ethanal.

As mentioned earlier, anthocyanin contents derived from the spectral method also depend upon the extent to which polymeric pigment color is increased on acidification. Factor ⅝ may be too low, as well as PPC. If young oligomeric pigments are sensitive to SO_2, they may be more sensitive to pH than by factor ⅝, since bisulfite and hydroxyl ions are both nucleophiles and react with anthocyanins in similar ways (19). In young wines, variation in this factor is likely to be less important than the bleach-

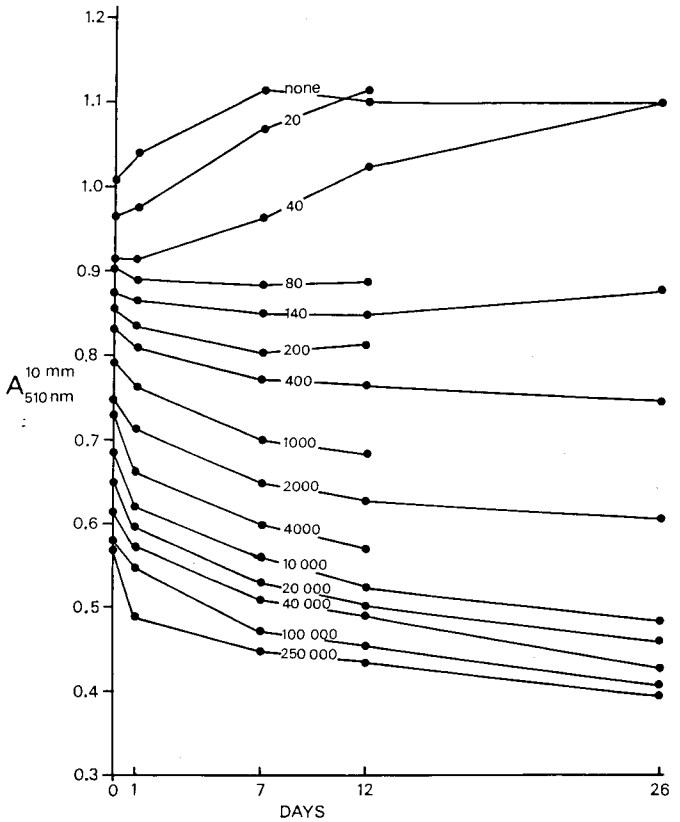

Fig. 4. Bleaching of a four-year-old red wine (Santenay) by varying concentrations of SO_2 (mg/L).

ing of oligomeric pigments, since the absorbance error produced is small in relation to the total absorbance in 1 *M* HCl (17). The polymeric pigments in very old wines, which are very resistant to SO_2, are also very resistant to acidification. For these, factor 5/6 (recommended only for young wines) is then far too high, since its use gives negative values of ACA.

The results presented here indicate that the assumptions inherent in the spectral method are over-simplifications and that some polymers, particularly oligomeric pigments formed during the early life of red wine, can be bleached by bisulfite and may be also more pH-responsive (*i.e.*, PPCA > 5 PPC/3) than assumed previously. Rather than the abrupt change from monomers to polymers implicit in the spectral method, the course of anthocyanin condensation and polymerization during red wine aging may be represented better by a gradual transition from monomeric anthocyanins through oligomers to polymeric pigments. A progressive increase in pigment resistance to bleaching by bisulfite and a progressive decrease in color gain on acidification is envisaged as oligomeric pigments of increasing complexity are formed. This explains why the difference between the methods is greatest when wines are young and least when older. It is unlikely that any one single concentration of bisulfite and any one invariant polymer acidification factor can be applied to a red wine whatever its age, even those of the current vintage. The existence of "intermediate pigment forms" in young wines with uncertain response to SO_2 and pH change was foreseen by Somers and Evans (17). HPLC now indicates that they are of greater significance than previously supposed. However, it is not possible to measure the concentrations of free or monomeric anthocyanins in red wines at wine pH values by HPLC since wine has to be acidified (to approx. pH 1.5) in order to give sufficiently sharp peaks (21). Thus, bleaching a proportion of wine color at its own pH with bisulfite (12,16,17), although requiring reinterpretation, remains a valuable technique particularly when derived parameters, such as degree of ionization, can be correlated with wine quality (16,18).

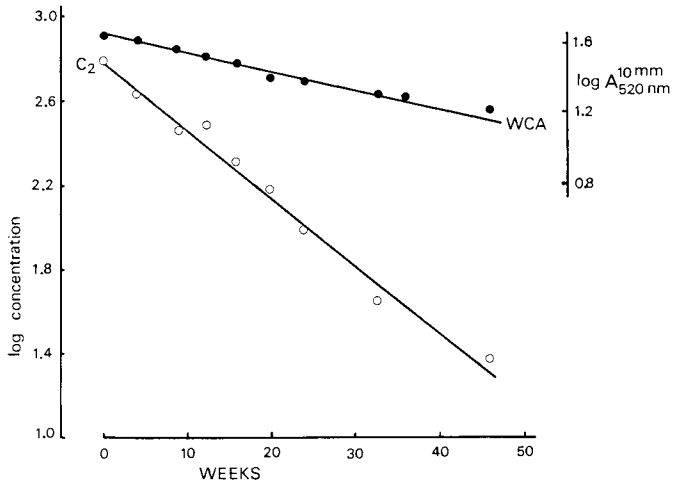

Fig. 5. Logarithmic changes of total pigment color (WCA as $A^{10\,mm}_{520\,nm}$) and total anthocyanins determined by HPLC (C_2 as mg/L) of a port wine. The scales are such that log concentration = log 18.9 $A^{10\,mm}_{520\,nm}$.

Linear regressions determined as follows:
intercepts: 2.772 (C_2), 1.640 (WCA)
slopes: −0.032 (C_2), −0.009 (WCA)
correlations: −0.993 (C_2), −0.995 (WCA)

Several workers have reported a logarithmic loss of anthocyanins in food products with either arithmetic increase in temperature or time of heating at constant temperature (7). Figure 5 shows that in the aging port wine, the changes in total pigment color and total anthocyanins were also logarithmic with time at constant temperature (15°C). Extrapolation of both lines backwards in time gives an intersection point (-6.4 weeks) at which all the pigments present would consist of monomeric anthocyanins (WCA = ACA at antilog 2.974 or 944 mg/L anthocyanins). This point is hypothetical and can never be realized since it assumes instant extraction of anthocyanins from grape skins; in practice of course, extraction is slow and is accompanied by polymerization (Fig. 2). But it serves to emphasize that the logarithm of the amount of anthocyanin lost by polymerization reactions during skin fermentation (doubtless partly due to enzymic oxidation at 28°C) of this particular port wine may be equivalent to the logarithm of the loss during 6.4 weeks of storage at 15°C. Of course, anthocyanins may be lost by reactions other than polymerization during skin fermentation, but any such loss would also be reflected in the total pigment value.

The proportions of polymeric pigment color in the wines given in the tables will be even greater when expressed as percentages of the original total pigment color at time 0 than as percentages of the total pigment colors at the times of sampling. But even the total pigment color at time 0 already contains some contribution from polymeric pigment (22% in port wine, Table 3). It is evident that the true percentage of polymeric pigment color, *i.e.*, the percentage of the original total pigment which is entirely anthocyanin cannot be realized because, as shown in Figure 2, pigment polymerization begins at the moment of crushing the grape so that even when total pigment color reaches its maximum value it will inevitably contain some color due to polymeric pigment.

Thus, the true rate of aging of anthocyanins in red wines may be represented better in terms of the rate of anthocyanin loss than by percentages of polymeric pigment formed. For the particular port wine discussed here, the rate constant (k) of anthocyanin loss is given by the slope of the straight line obtained on plotting $\log_{10} C_2$ against time according to the following equation: $k = 2.303\ (\Delta \log_{10} C_2/\Delta \text{ time})$. The rate constant was thus found to be 0.073 per week (as shown in Fig. 5). Further work is necessary to ascertain whether similar rate constants can be measured with red table wines.

Conclusions

HPLC gives a true measure of the free anthocyanin content of red wines. The higher anthocyanin contents obtained by the spectral method of Somers and Evans (16,17) are attributed to partial bleaching of polymeric pigments (anthocyanins condensed with other flavonoids) by bisulfite. Formation of polymeric pigments be-

gins at grape crushing and reaches considerable proportions during fermentation on the skins.

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