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HPLC of anthocyanins in port wines: Determination of ageing rates

by

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HPLC-Analyse der Anthocyane in Portweinen: Die Bestimmung der Alterungsgeschwindigkeit

Zusammenfassung: Der Gehalt der gesamten sowie der einzelnen Anthocyane in Portweinen, die unterschiedlich schnell alterten, wurde während der ersten 46 Wochen ihrer Lagerung bei 15 °C mittels HPLC bestimmt. Die Anthocyanabnahme war eine logarithmische Funktion der Zeit. Zwischen den Hauptanthocyanen (Malvidin-3-Glucosid, Malvidin-3-Acetylglucosid und Malvidin-3-p-Cumarglucosid) bestanden geringe Unterschiede der Alterungsgeschwindigkeit. Die Abnahme der Gesamtanthocyane spiegelte die Alterungsgeschwindigkeit der Anthocyane in Portweinen am besten wider. Während der anfänglichen Alterung in Gegenwart von freiem Acetaldehyd war der molare Verlust an Anthocyan höher als derjenige des Acetaldehyds, woraus sowohl auf eine Kondensation durch Vermittlung von Acetaldehyd als auch auf eine direkte Kondensation ohne Beteiligung von Acetaldehyd in dieser Phase geschlossen werden kann. Erhöhung der Acetaldehydkonzentration beschleunigte die Abnahme der Anthocyane. Acetaldehyd war auch noch wirksam, wenn nur noch wenig Anthocyan übrig war; dies deutet auf die Bildung komplexer verzweigter Polymere hin. Der Anteil der Färbung, der auf polymeres Material zurückzuführen war, betrug in den jungen Portweinen zwischen 23 und 30 %; nach 46 Wochen lag er zwischen 78 und 98 %. Der Portwein mit dem höchsten Gehalt an freiem Acetaldehyd zeigte den schnellsten Zuwachs bei der polymer bedingten Färbung. Die durch Polymere verursachte Absorption und die Stabilität dieser Färbung hingen vom Gehalt an freiem Acetaldehyd ab.

Key words: dessert wine, ageing, anthocyanin, acetaldehyde.

Introduction

The colour of a young red wine is due mostly to anthocyanins extracted from the fruit. However, as the wine ages the colour is increasingly due to polymeric pigments formed from anthocyanins by condensation with other flavonoid compounds (SOMERS 1966 and 1971; RIBÉREAU-GAYON 1974; GLORIES 1978) and acetaldehyde (TIMBERLAKE and BRIDLE 1976). During ageing of ruby ports, the colour becomes darker over a period of about 6 months, after which it becomes lighter. The ageing mechanisms underlying these changes in ports have been described (BAKKER and TIMBERLAKE 1986).

To quantify the extent of polymeric pigment formation in young red wines SOMERS and EVANS (1977) developed a spectral method. Although quick and easy to use, this method has been shown by BAKKER et al. (1986) to underestimate the amount of polymeric material formed due to partial bleaching with bisulphite of oligomeric pigments. The latter used high performance liquid chromatography (HPLC) to separate individual anthocyanins into distinct peaks without interference from polymeric material. Integration of the total peak area quantified the total anthocyanin content. As both the total pigment measurement and the total anthocyanin determination by HPLC were

obtained in acid, polymeric pigment colour could be calculated as the difference between these two values. The polymeric pigment colour/total pigment colour × 100 represents the percentage colour due to polymeric pigments.

BARANOWSKI and NAGEL (1983) reported a logarithmic loss with time of malvidin 3-glucoside in the presence of catechin with or without acetaldehyde in model wine systems in the absence of air. BAKKER et al. (1986) found that in port wines the losses of total pigments and total anthocyanins determined by HPLC were logarithmic with time during ageing in the presence of air. They suggested that the rate constant of anthocyanin loss as determined by HPLC is a true measure of anthocyanin ageing in port wine.

The influence of acetaldehyde on colour changes during red wine ageing has been reported, but there is little information on the influence of acetaldehyde on the loss of anthocyanins. Thus it was of interest to study the losses of anthocyanins in ports maturing in different ways due to their different acetaldehyde contents, as now both the loss of acetaldehyde and the loss of anthocyanins can be determined accurately.

Materials and methods

Port wines

Port wines were made on a pilot scale at Long Ashton from Portuguese grapes (*Vitis vinifera,* cv. Touriga Nacional) and fortifying spirit flown to Bristol from the Douro valley in Portugal. To make ports with different acetaldehyde contents, grapes were weighed, destemmed, crushed with an addition of 150 mg l^{-1} SO_2, transferred into 20 l stainless steel fermentation vessels maintained at 28 °C and after approximately 6 h inoculated with Montrachet yeast (Uvaferm CM, Swiss Ferment Company Ltd.) (10^7 cells ml^{-1}; inoculate 1 ml kg^{-1} grapes). At specific gravitiy (SG) 1.045 the fermenting mash was pressed, the must was fermented to dryness, and divided into four parts. One part was fortified with special Portuguese fortifying spirit (77 % alcohol by volume) to give a calculated alcoholic strength of 19.5 % by volume (v/v) as a control with a normal content of aldehyde (port CA). The second part was fortified with spirit and 200 mg l^{-1} of acetaldehyde was added (port HA). The third part was fortified with spirit and 100 mg l^{-1} SO_2 was added (port S). The fourth part was used to make a port as low as possible in aldehydes (port LA), as follows. Ethanol (BP, aldehyde free) was used for fortification instead of spirit and small amounts of SO_2, calculated to bind all free aldehydes, were added at the beginning and at intervals during the first 6 months of storage. All particulate matter was removed by centrifuging and the ports were stored in sealed glass jars (10 l) with minimum headspace in a dark room at 15 °C.

A normal sweet port (port N) was prepared using the standard procedure, whereby the fermenting must was pressed at specific gravity 1.045, and the must fortified with spirit immediately after pressing.

Total aldehydes

Aldehydes were determined iodimetrically (BURROUGHS and SPARKS 1973).

Free aldehydes

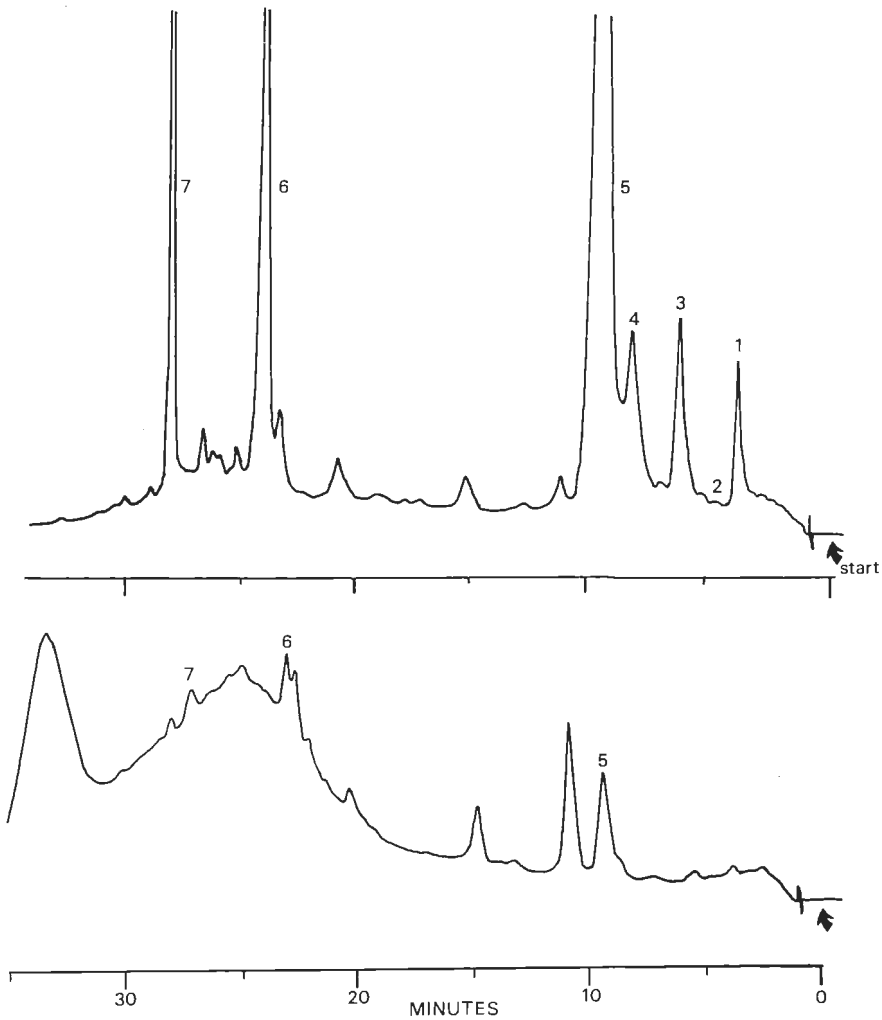Free aldehyde was calculated as described by BAKKER and TIMBERLAKE (1986).

Fig. 1: Top: HPLC trace of port wine CA at first analysis. — Bottom: HPLC trace of port wine CA after 46 weeks of storage. — 1: Delphinidin 3-glucoside; 2: cyanidin 3-glucoside; 3: petunidin 3-glucoside; 4: peonidin 3-glucoside; 5: malvidin 3-glucoside; 6: malvidin 3-acetylglucoside; 7: malvidin 3-p-coumarylglucoside.

Oben: HPLC-Diagramm des Portweines CA bei der ersten Analyse. — Unten: HPLC-Diagramm dieses Weines nach 46wöchiger Lagerung.

Total pigment

Total pigment (or wine colour in acid, WCA) was measured by diluting wine with N HCl as described previously (BAKKER et al. 1985).

High performance liquid chromatography

The equipment used for HPLC consisted of a Pye Unicam gradient programmer and visible detector, an Altex pump and a Spherisorb Hexyl reversed-phase column as
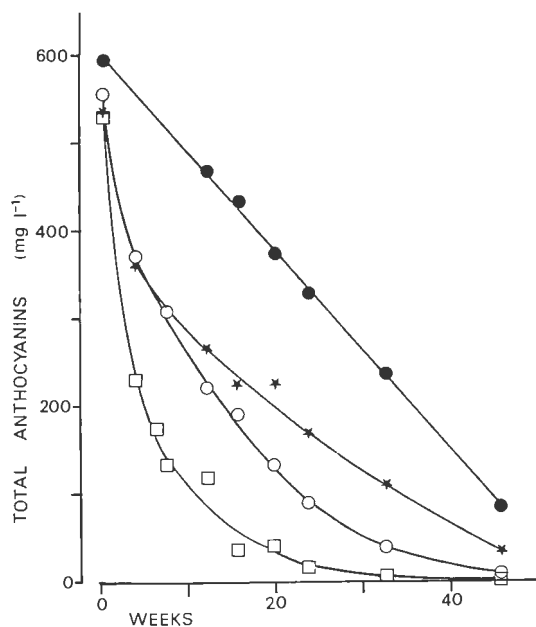
Fig. 2: Losses of total anthocyanins (mg l⁻¹) determined by HPLC during ageing of port wines. —
○: Port CA; ★: port LA; □: port HA; ●: port S.

Abnahme der Gesamtanthocyane (mg l⁻¹) während der Alterung von Portweinen; HPLC-Analysen.

described previously (BAKKER *et al.* 1986). The total anthocyanin concentration (ACA) was obtained by integration of all the individual anthocyanin peaks. The total k n o w n anthocyanin concentration was obtained by integration only of the seven identified anthocyanins (malvidin 3-glucoside, malvidin 3-acetylglucoside, malvidin 3-p-coumarylglucoside, petunidin 3-glucoside, peonidin 3-glucoside, delphinidin 3-glucoside and cyanindin 3-glucoside) (BAKKER and TIMBERLAKE 1986).

Standardisation of HPLC

The concentration of anthocyanins in ports was quantified by using an external standard of malvidin 3-glucoside chloride as described previously (BAKKER and TIMBERLAKE 1986; BAKKER *et al.* 1986), and expressed in terms of this anthocyanin.

Results and discussion

Losses of anthocyanins

HPLC analyses of the experimental ports during the first 46 weeks of storage showed a decrease in the concentration of monomers and an increase in the concentration of polymeric material present. A typical HPLC trace of the control port at the first analysis and after 46 weeks is shown in Fig. 1. Using HPLC it is possible to quantify only the concentration of monomeric anthocyanins. Thus the loss of anthocyanins can

be calculated accurately, but the polymeric material which is formed cannot be meas-
ured since it elutes in broad peaks or humps concurrent with late eluting acylated
anthocyanins. As the ports age, the size of the humps increases, while the anthocyanin
concentrations decrease. Even at the first analysis the baseline rise (after about
21 min) was in the form of a small, but discrete hump, indicating that some polmeric
material had formed already. This early formation of polymeric material has been
reported and discussed previoulsy by BAKKER *et al.* (1986).

Total anthocyanins in the ports were lost at different rates and after 46 weeks only
small quantities remained (Fig. 2). After 12 weeks the concentration of anthocyanins in
the control port (CA) was reduced from 557 to 221 mg l^{-1}, (i. e. more than half the con-
centration present at the first analysis had been lost). After 46 weeks only 11 mg l^{-1}
anthocyanins remained. Thus the major pigments contributing to the colour of the port
at these stages were the polymers. The port low in aldehydes (LA) lost anthocyanins at
a slower rate than the control. After 16 and 46 weeks, 224 mg l^{-1} and 33 mg l^{-1} respec-
tively of anthocyanins remained. The port high in aldehydes (HA) lost anthocyanins
much faster than the control. After 12 weeks only 120 mg l^{-1} anthocyanins remained,
but thereafter the rate of loss slowed down. When the port with excess SO_2 (S) was ana-
lysed, the anthocyanins with short retention times eluted as doublets, presumably due
to differences in retention times between the bleached anthocyanin-SO_2 adduct and
the non-bleached anthocyanins. Hence it was necessary to make an addition of acetal-
dehyde to such samples approximately 10 min before their analysis by HPLC to bind
SO_2 and so liberate free anthocanins. Port S exhibited the slowest rate of anthocyanin
loss. After 24 weeks 328 mg l^{-1} of the initial 597 mg l^{-1} anthocyanins remained, and
after 46 weeks there were still 83 mg l^{-1} anthocyanins left. The rate of loss of anthocy-
anins appeared to be linear over the period of analyses.

The ageing of a sweet port (port N) made using the standard procedure was also
monitored. Due to the different fermentation procedure port N is analytically different
from dry port CA described above, and is not a true control. At its first analysis it con-
tained 627 mg l^{-1} anthocyanins, while after 12 and 46 weeks 280 and 24 mg l^{-1} respec-
tively remained.

Losses of anthocyanins in food products kept over a period of time at a constant
temperature are reported to be of a logarithmic nature (MARKAKIS 1982). BAKKER *et al.*
(1986) reported that the loss of anthocyanins in an ageing ruby port was also logar-
ithmic with time at a constant temperature (15 °C). They suggested that the rate of
polymerisation of anthyocyanins in port can be depicted by the rate constant (k), which
is given by the slope of the straight line obtained by plotting $\log_{10}$ concentration of
anthocyanins remaining (c; mg l^{-1}) against time according to the equation k = 2.303
($\Delta\log_{10}$ c/Δtime). Fig. 3 shows that the losses of anthocyanins at constant storage tem-
perature (15 °C) in all four port wines were logarithmic with time. Calculated rate con-
stants (k, week^{-1}) were as follows: 0.108 for port HA, 0.083 for port CA and 0.053 for
port LA. Port S lost anthocyanins very slowly over the first 32 weeks, reflected in a k of
0.028 week^{-1}. After this time the logarithmic loss was no longer linear; the anthocy-
anins were lost more rapidly as free acetaldehyde now became available (due to the
continuous loss of SO_2 by oxidation), which reacted quickly with the remaining avail-
able anthocyanins. The k value for the normal sweet port (N) was 0.074 week^{-1} (BAK-
KER *et al.* 1986).

Losses of acetaldehyde and anthocyanins

It has been established (BAKKER and TIMBERLAKE 1986) that free acetaldehyde
plays an important role in the formation of aldehyde-linked polymers of anthocyanins
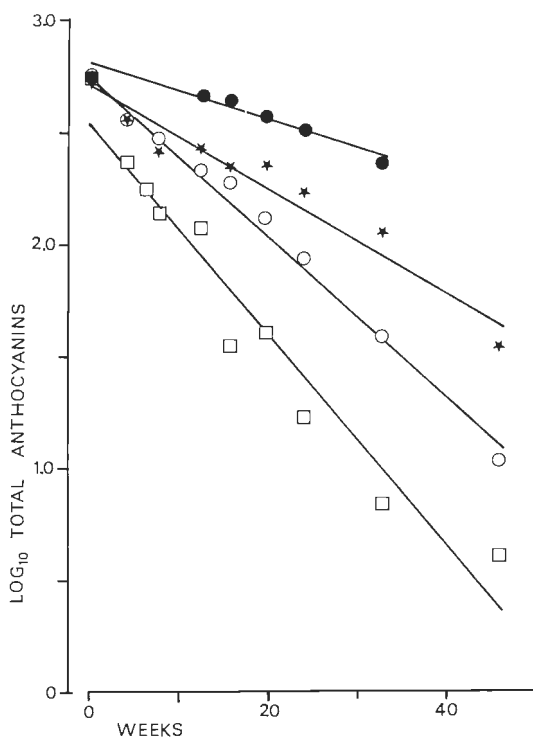
Fig. 3: Logarithmic changes of total anthocyanins determined by HPLC during ageing of port wines. — ○: Port CA; ★: port LA; □: port HA; ●: port S.

Logarithmische Veränderungen der Gesamtanthocyane während der Alterung von Portweinen; HPLC-Analysen.

and other phenols in ports. The initial polymerisation reaction is the bridging by an acetaldehyde molecule of a catechin molecule and an anthocyanin molecule, resulting in equimolar losses of anthocyanins and acetaldehyde. This process is in competition with polymerisation of anthocyanins and other phenols by their direct condensation, a process not involving acetaldehyde, and expected to be more prominent in red wines because of their generally lower acetaldehyde contents. Fig. 4 shows the molar losses of acetaldehyde plotted against the molar losses of anthocyanins in ports CA and N. If equimolar concentrations of total acetaldehyde and anthocyanins had been lost, a straight line through the origin at an angle of 45 ° would be expected. However, the graph shows that during the initial ageing reactions port CA lost anthocyanins faster than total acetaldehyde. Only after about 12 weeks were equimolar concentrations of total acetaldehyde and anthocyanins lost. During the 46 weeks of ageing the total acetaldehyde concentrations decreased from 2.2 mMol to 1.5 mMol, but the free acetaldehyde concentration increased from 0.6 mMol to 1.6 mMol (due to its liberation from acetaldehyde-bisulphite by oxidation). After 4 weeks of storage the concentration of free acetaldehyde exceeded the concentration of anthocyanins, due to losses of anthocyanins and increases in free acetaldehyde. Port N also lost more anthocyanins than acetaldehyde on a molar basis during the inital storage period, but the molar ratio
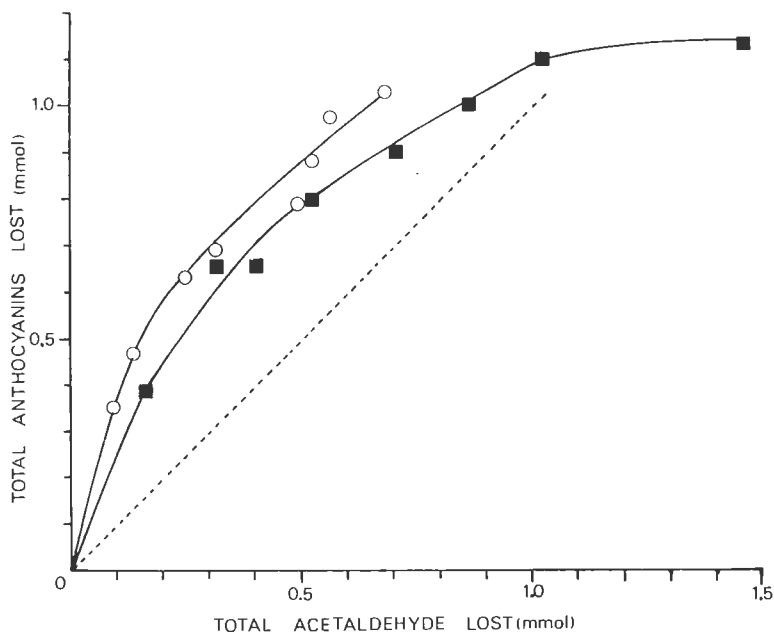
Fig. 4: Molar loss of total anthocyanins lost versus molar loss of total acetaldehyde lost. Dotted line represents equimolar losses. — O: Port CA; ■: port N.

Abnahme der Gesamtanthocyane (molar) in Beziehung zur Abnahme der Gesamtaldehyde (molar). Die gestrichelte Linie gilt für äquimolare Abnahme.

(anthocyanin lost/acetaldehyde lost) was less than in port CA. After about 12 weeks this ratio became smaller than unity. At the first analsis port N contained 1.2 mMol anthocyanins and 3.0 mMol total acetaldehyde with a calculated free acetaldehyde content of 0.9 mMol. The free acetaldehyde increased to 1.8 mMol after 33 weeks, and then decreased to 1.4 mMol after 46 weeks. Both the total and the free acetaldehyde concentration in port N were higher than in port CA throughout the 46 week period.

Free acetaldehyde would be expected to react mainly with anthocyanins and other phenols in ports, and to a much lesser extent with other wine components. Some aldehyde may also be formed in ports presumably by oxidation of ethanol (BAKKER and TIMBERLAKE 1986), so that the true amounts of aldehyde reacted may be greater than the net losses described here. While it has not been possible to quantify the amounts formed, they are estimated to be small compared with the large concentrations usually present in ports, and values of ratios anthocyanins lost/acetaldehyde lost are only slightly overestimated. Therefore values of this ratio greater than unity observed in ports CA and N during the first 12 weeks of storage suggest that anthocyanins were lost by reactions not involving acetaldehyde, and indicating direct condensation between anthocyanins and other phenols. The lower molar ratio in port N than in port CA is attributed to the higher free acetaldehyde concentration in port N. The influence of acetaldehyde concentration was particularly evident in port HA, which contained a large molar excess of acetaldehyde (6.6 mMol total and 4.9 mMol free acetaldehyde) and had a ratio anthocyanins lost/acetaldehyde lost much greater than unity. Thus, during

Table 1

Percentages of colour due to polymeric pigments — [(WCA – ACA) : WCA] × 100 — in ports of ageing experiment

Prozentualer Anteil der polymeren Pigmente an der Portweinfärbung im Verlauf des Alterungsversuches

Age (weeks)	[(WCA – ACA) : WCA] × 100 (%)				
	CA	LA	HA	S	N
0	28	30	28	23	22
4	48	49	64	—	46
8	51	39	74	—	60
12	61	56	74	38	57
16	63	59	91	38	66
20	70	55	88	42	61
24	79	64	94	48	80
33	89	74	97	56	90
46	95	88	98	78	93

WCA: Total pigment colour.
ACA: Total anthocyanin colour.
CA: Control port.
LA: Port low in total and free aldehyde.
HA: Port with 200 mg l^{-1} acetaldehyde added.
S: Port with 200 mg l^{-1} SO_2 added.
N: Normal sweet port.

the initial ageing direct condensation forms an important part of the ageing process, more so when the initial free acetaldehyde concentration is low. The continuing loss of aldehydes after most anthocyanins have polymerised can be attributed to reactions of acetaldehyde with small polymers, resulting in more complex branched structures.

Formation of polymers

It has been established that polymeric pigments are formed already during the fermentation of ports (BAKKER et al. 1986), but with the available analytical techniques it is not possible to quantify the amounts formed. However, the percentage of colour due to polymeric material can be estimated from [(WCA − ACA) : WCA] × 100 %, where WCA is the total pigment colour and ACA is the total anthocyanin colour calculated from the measured HPLC content (BAKKER et al. 1986). At the first analysis of port CA polymeric material contributed 28 % of the colour (Table 1). This percentage continued to increase in all ports; and after 46 weeks it was 95 % in port CA. Port HA contained 94 % after already 24 weeks, compared with 79 % in port CA and only 48 % in port S. The percentage was lowest throughout in port S and after 46 weeks it was still only 78 %.

The total pigment measurement (WCA) in the ports decreased due to the loss of anthocyanins and the formation of polymers, less coloured at pH < 1 (N HCl) than anthocyanins (BAKKER et al. 1986), and these losses were also logarithmic with time. The absorbance due to polymeric pigments, calculated by subtracting the anthocyanin colour (ACA) from WCA, is plotted in Fig. 5. In port CA it increased during the first 4 weeks, reached a plateau, and decreased slowly after 20 weeks. The absorbance of
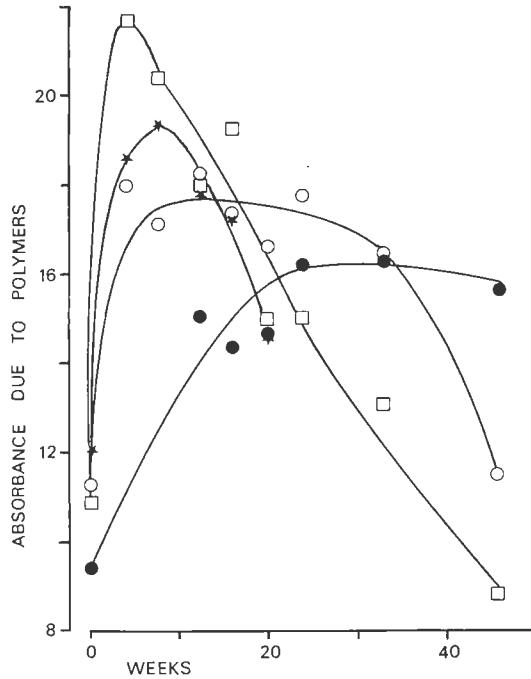
Fig. 5: Absorbance due to polymers (WCA−ACA) during ageing of port wines. — ○: Port CA; ★: port LA; □: port HA; ●: port S.

Durch die polymeren Pigmente (WCA − ACA) bedingte Absorption während der Alterung von Port-weinen.

polymers in port HA went up rapidly, but once the highest value was reached it decreased rapidly. Port S showed a slow increase in polymer absorbance over 24 weeks, which fell off slightly after 33 weeks. The polymer absorbance of port LA increased, fol-lowed by an immediate decrease. The rapid decrease in polymeric pigment colour in port HA could be due to precipitation of large polymeric molecules. Another explana-tion could be that large polymeric molecules containing anthocyanins are less coloured than oligomers. In port S the high concentration of SO_2 bound to acetaldehyde pre-vented the formation of any free acetaldehyyde during the first 24 weeks of storage, and during this period total acetaldehyde loss was negligible, indicating that the poly-mer formation must have been due only to direct condensation. However, the high SO_2 concentration slowed down the direct condensation. Polymeric colour formed was least in amount, as the amount of anthocyanins remaining was greater than in any of the other ports (see above). During the 20 weeks port LA was stored with a calculated free acetaldehyde concentration of 0, the absorbance colour increased and decreased, indi-cating that some free acetaldehyde was present. The polymerisation would have been much slower if the concentration of free acetaldehyde had been truely 0.

Port N also reached a plateau absorbance value after about 4 weeks; the amount of colour due to polymers decreased after 33 weeks. The larger amount of colour due to polymeric pigment in comparison with port CA may be due to its higher free acetalde-hyde content and possible solubilising effect of the sugar on the polymeric material.
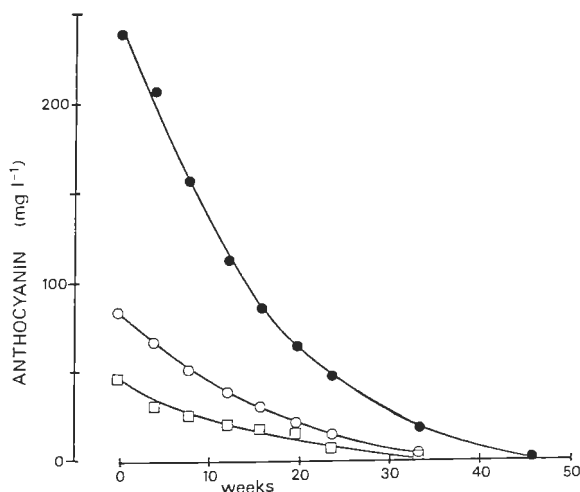
Fig. 6: Losses of malvidin 3-glucoside (●), malvidin 3-acetylglucoside (○) and malvidin 3-p-cou-
marylglucoside (□) during ageing of port CA.

Abnahme des Malvidin-3-Glucosids (●), des Malvidin-3-Acetylglucosids (○) und des Malvidin-3-
p-Cumarylglucosids (□) während der Alterung des Portweines CA.

Loss of anthocyanins depending on structure

The losses of malvidin 3-glucoside, malvidin 3-acetylglucoside and malvidin 3-p-coumarylglucoside in port CA are shown in Fig. 6. Malvidin 3-glucoside was lost rapidly during the first 12 weeks, but thereafter more slowly. The initial concentrations of the acylated anthocyanins malvidin 3-acetylglucoside and malvidin 3-p-coumarylglucoside were much lower than the concentration of malvidin 3-glucoside. The concentrations of malvidin 3-p-coumarylglucoside were determined least accurately, because this anthocyanin eluted superimposed onto a polymeric hump which increased with time (ref JSFA). After 46 weeks only 3 mg l^{-1} malvidin 3-glucoside remained from the initial 239 mg l^{-}, whilst only trace quantities of malvidin 3-acetylglucoside and malvidin 3-p-coumarylglucoside could be detected.

The losses of these three anthocyanins were logarithmic with time in all ports; only the more typical ports CA, LA and N will be discussed. The correlation coefficients of the logarithmic losses of anthocyanins with time were very high ($P < 0.001$). Both malvidin 3-acetylglucoside and malvidin 3-p-coumaryl glucoside have higher k-values than malvidin 3-glucoside (Table 2). This could be due to their greater reactivity on ageing, but it would also occur if the acylated anthocyanins slowly hydrolysed to malvidin 3-glucoside, thus depressing the reaction rate of the latter (Table 2). Also, the k-value for malvidin 3-p-coumarylglucoside is underestimated to some extent because of increasing interference in its measurement by the polymers formed. It is thus difficult to reach any firm conclusions regarding the relative reactivity of the individual anthocyanins during ageing.

It is appropriate to consider the most accurate measurement reflecting the ageing rate of anthocyanins in a port. The k-value for the total anthocyanins is the lowest. This is due to the presence of small concentrations of unidentified anthocyanins which are less reactive, and which become proportionally more important as the port ages.

Table 2

Rates of loss of anthocyanins in ports expressed as k-values — k = 2.303 ($\Delta\log_{10}$ c/Δtime), where c = concentration of anthocyanins — during the first 46 weeks of storage

Verlauf der Anthocyanabnahme in den ersten 46 Wochen der Portweinlagerung, dargestellt durch die k-Werte · k = 2,303 ($\Delta\log_{10}$ c/ΔZeit), c = Anthocyankonzentration

Anthocyanin	k-Values		
	CA	LA	N
Malvidin 3-glucoside	0.094	0.055	0.076
Malvidin 3-acetylglucoside	0.110	0.074	0.110
Malvidin 3-p-coumarylglucoside	0.104	0.069	0.090
Total known anthocyanins	0.101	0.062	0.085
Total anthocyanins	0.083	0.053	0.074

CA: Control port.
LA: Port low in total and free aldehyde.
N: Normal sweet port.

The measurement of total anthocyanins also included small amounts of anthocyanins which are formed on ageing. The k-value of the total k n o w n anthocyanins is higher because it excludes these unidentified anthocyanins, and it is therefore the most meaningful measurement.

The k-values for the total known anthocyanins show that port CA ages faster than port LA, which must be due to the higher free acetaldehyde concentration. Port N ages slower than port CA, eventhough the concentration of free acetaldehyde is greater than in port CA. One possible reason may be the reduced solubility of oxygen in port N due to the high sugar concentration slowing down the rate of ageing. Ports HA and S, both atypical ports, confirm the influence of acetaldehyde on k-values: port HA has a high k-value (0.154), while port S has a low one (0.025).

Summary

The total and individual anthocyanin contents in several port wines ageing at different rates were determined by HPLC at regular intervals during the first 46 weeks of storage at 15 °C. The losses of anthocyanins were logarithmic with time. There were small differences in the rate of ageing of the major anthocyanins (malvidin 3-glucoside, malvidin 3-acetylglucoside and malvidin 3-p-coumarylglucoside), but no firm conclusions could be drawn after consideration of all factors involved. The rate of loss of total known anthocyanins best reflected the rate of ageing of anthocyanins in port wines. During the initial ageing in the presence of free acetaldehyde, the molar loss of anthocyanins was higher than the molar loss of acetaldehyde, indicating that both acetaldehyde condensation and direct condensation not involving acetaldehyde occurred at this stage. Increasing acetaldehyde contents increased the rate of loss of anthocyanins. Acetaldehyde was still reacting when little anthocyanins remained, indicating the formation of complex branched polymers. The percentage of colour due to polymeric material was between 23 and 30 % in the young ports, while after 46 weeks it was between 78 and 98 %; the port with the highest free acetaldehyde content showed the

fastest increase in the percentage of polmeric pigment colour. The absorbance due to polymers and the stability of its colour was dependent on the free acetaldehyde concentration.

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