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IN VINO VERITAS: OLIGOMERIC PROCYANIDINS AND THE AGEING OF RED WINES

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Key Word Index —*Vitis vinifera*; Vitaceae; flavan-3-ols; dimeric procyanidins; polymeric procyanidins; ageing of red wine.

Abstract —The role of oligomeric procyanidins in the ageing of wine has been examined by the addition of these substances to red and white wines. Observations show that in wines the acid-catalysed C—C bond-breaking and bond-making process, characteristic of procyanidin chemistry, is set up and leads to the eventual precipitation of polymers. In red wines, procyanidin polymers are also additionally precipitated by reaction possibly with anthocyanins or products derived therefrom.

INTRODUCTION

"The making of red wine, which involves the skins and the pips as well as the juice of grapes, leaves extra substances dissolved, above all tannin. This gives the wine the special quality of hardness of drying up your mouth. These extras need time to resolve themselves to carry out slow and obscure chemical changes which make all the difference in the world to the eventual glass of red wine" [1].

The process of ageing of red wines has been of considerable fascination to man for centuries. There is no doubt that during this process the polyphenols present are important but progress towards defining their role has been slow and in an admirable review of the state of the art 10 years ago Singleton and Esau [2] commented ... "Unfortunately what we would really like to know—the specific amount of each individual substance and how it participates and changes in wine in each type of storage reaction—requires much more detailed knowledge than is now available". Despite further work in the past decade many uncertainties remain and in this communication evidence is presented to delineate as far as is now possible the part played by substances which are frequently referred to as the oligomeric and polymeric condensed tannins of grape [2], namely the flavan-3-ols (+)-catechin (**3**) and (−)-epicatechin (**4**) and the dimeric and oligomeric procyanidins.

The biosynthesis of plant procyanidins [3, 4] follows a pathway which is shown in Fig. 1. They are believed to be formed as by-products during the two step reduction of the flav-3-en-3-ol to the flavan-3-ol, (Fig. 1, **1** to **3** or **4**), when the supply of NADPH is rate limiting. The intermediate carbocation (**2**) is presumed to escape from the active-site of the reductase and to react with the end-product flavan-3-ol (predominantly at position 8 but also at position 6) to give dimeric procyanidins. Further analogous reactions of the dimers with **2** yields higher oligomers and finally polymeric forms whose structures are predominantly linear (C-4 to C-8 interflavan linkages) but simply on a statistical basis will contain some C-4 to C-6 linkages and hence will possess a degree of branching within them. In any plant tissue where this process occurs there is invariably found a range of procyanidin types from the monomeric flavan-3-ols (**3** and/or **4**) to the polymers (**9**) and the balance is probably influenced for each tissue by the corresponding balance between the metabolic flux through the pathway to the flav-3-en-3-ol (**1**) and the rate of supply of NADPH. Tissues in which the flux is high and NADPH supply is low contain high concentrations of the polymeric forms—in some plants, e.g. the ripened seed coat of sorghum [5], polymeric procyanidins are present almost exclusively, whilst in others where the supply of NADPH more nearly balances the metabolic flux a range of procyanidins is found [6]. With increasing polymerization the procyanidins become more difficult to dissolve in aqueous and alcoholic media [7–9] and it has been suggested [10] that those which can be solubilized may have molecular weights up to 7000—corresponding to the accumulation of up to 20 flavan-3-ol units (**9**, $n = 19$) in the polymer. Finally it is important to note that the essence of a great deal of the chemistry of the procyanidins revolves around the ready reversibility of the biosynthetic carbon—carbon bond forming reaction in acidic media [2–6].

RESULTS AND DISCUSSION

Weinges and Piretti [11] have isolated procyanidin B-1 (**5**) as its deca-acetate and (+)-catechin (**3**) and (−)-epicatechin (**4**) as their penta-acetates from wine grapes. They also noted the presence of the diastereoisomeric procyanidins B-2, B-3 and B-4 (**6–8**) in small amounts and stated that up to 25% of the polyphenol fraction consisted of polymeric procyanidins. We have examined the procyanidin profile [4, 6] of the leaves, stalks, grape skins and seeds of varieties of *Vitis vinifera* and our results corroborate the results of Weinges and Piretti [11] and earlier workers [12–15]. In the grape skins, in addition to various flavonoids and hydroxystilbenes, the balance of

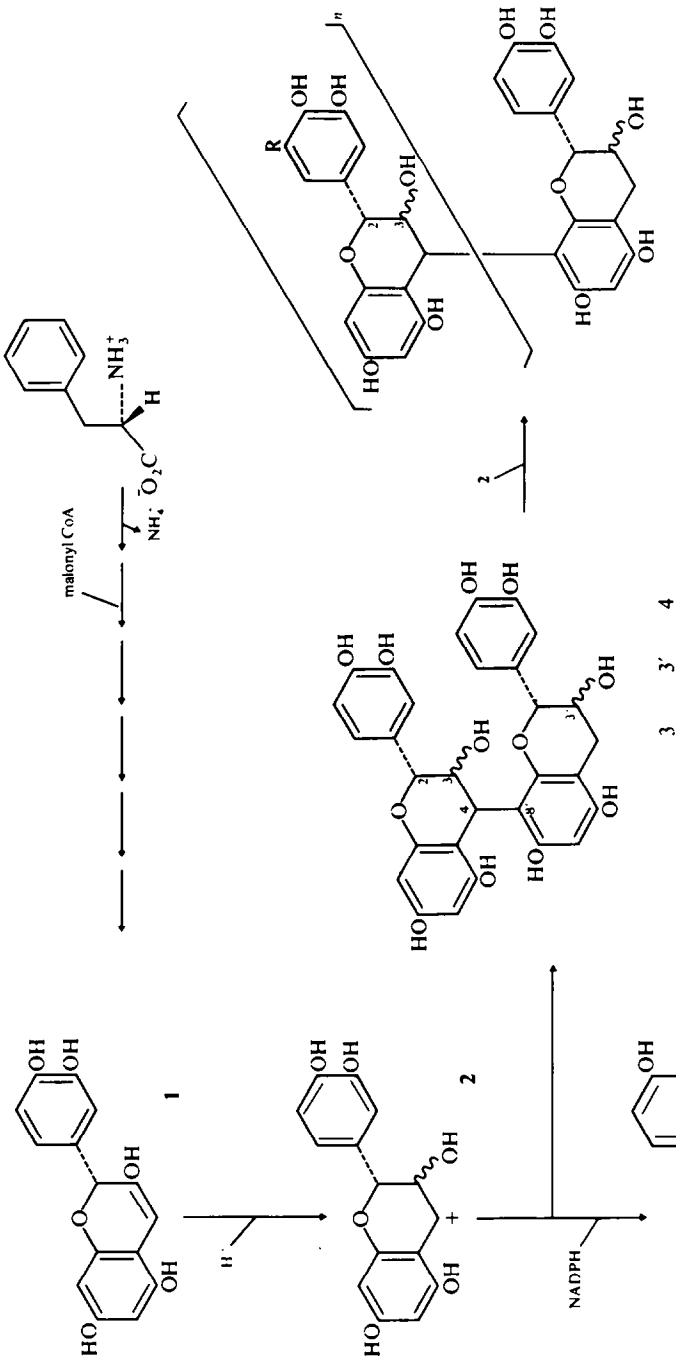

Fig. 1. Procyanidin metabolism in higher plants.

proanthocyanidins is in the polymeric forms, (+)-catechin (**3**) is the major flavan-3-ol present and (−)-epicatechin (**4**) occurs in smaller quantities. The concentration of dimeric procyanidins is relatively low and procyanidin B-1 (**5**) and B-2 (**6**) are the major diastereoisomers present. The polymeric proanthocyanidins may be partially solubilized and extracted with acetone–water (1:1 v/v) mixtures. A polymer fraction was obtained by chromatography on Sephadex LH-20 and the polymer on treatment with *n*-butanol hydrochloric acid (80°) gave both cyanidin and delphinidin (ratio approx. 4:1) thus suggesting that it contains both procyanidin and prodelphinidin units. The polymer when dissolved in dioxan water and treated with dilute acid [9] in the presence of (+)-catechin (**3**) gave procyanidin B-1 (**5**) and when similarly treated with acid in the presence of (−)-epicatechin (**4**) it yielded procyanidin B-2. Based on earlier rationalizations [5, 9] this leads to the formulation of the average structure of the polymeric procyanidin—prodelphinidin in grape skins as **9** with (+)-catechin (**3**) or (−)-epicatechin (**4**) chain terminal units, the polymer chain composed predominantly of flavan-3-ol units (R = H) with 2,3-*cis* stereochemistry and containing occasional prodelphinidin units (R = OH). Although it is not clear to what extent it represents the conditions during the manufacture of wine, if wine grapes (skins, seeds, pulp and stalks) are extracted flavan-3-ols, hydroxystilbenes and procyanidins are the principal polyphenols isolated. It is of interest to note that various dessert grapes when examined have similar polyphenol constituents, the seeds are a rich source of procyanidins; however the skins contain considerably diminished quantities of these substances, which are almost exclusively in the polymeric forms.

This evidence clearly implies that in the vinification of red wines the expressed juices in the fermentation will contain the flavan-3-ols (**3**, **4**), some procyanidin dimers (**5**–**8**) and dependent on their solubility proanthocyanidin polymers of varying molecular weight and composition. Weinges and Piretti [11] suggested from their observations that the polymers in grapes which are of most interest in wine preparation are built up of not more than eight flavan-3-ol units (e.g. **9**, $n = 7$) and accordingly have at most a MW of 2300–2500.

We have examined the transformation in white and red wines of the flavan-3-ols (**3** and **4**), procyanidin B-2 (**6**) and B-3 (**7**) and because of its ready availability a polymeric procyanidin [5] from *Sorghum bicolor* (NK 300) which has an average structure and stereochemistry (**10**, $n = 5$ or 6) and average MW approximately the same as that suggested by Weinges and Piretti [11] for the natural polymers derived from grapes. Polyphenols were added to a white wine, a white wine adulterated with anthocyanin pigments, an immature red wine (1 yr) and a mature red wine (4 yr) and the solutions kept at 20° under nitrogen. In the case of red wines changes in the anthocyanin content were followed approximately by measurement of the change in absorption at λ_{max} 520–540 nm before and after the addition of one drop of concentrated hydrochloric acid. Solutions were also analysed [6] by extraction with ethyl acetate, two-dimensional paper chromatography, chromatography on Sephadex LH-20 and HPLC.

(a) *White wine (4 years old)*

The sorghum procyanidin polymer initially gave a clear solution but developed a turbidity (15–25 days – dependent on the initial concentration of polymer) and finally gave a precipitate. The recovered precipitate (48 days) represented 50–70% of the original polymer, it gave a positive procyanidin test and on a quantitative comparison yielded 75–80% of the amount of cyanidin derived from an equivalent weight of the original sorghum polymer. This precipitate was *not* observed in experiments conducted with the sorghum procyanidin polymer in the presence of (+)-catechin (**3**) or (−)-epicatechin (**4**). These solutions remained clear and translucent (48 days). Extraction with ethyl acetate gave phenolic extracts which when analysed [4, 6] showed that when (+)-catechin was the additive procyanidin B-1 (**5**) was produced in solution and that when (−)-epicatechin was added procyanidins B-2 (**6**), B-1 (**5**) and (+)-catechin were formed. Similarly solutions of procyanidin B-2 (**6**) and (+)-catechin (**3**) in white wine steadily formed procyanidin B-1 (**5**). These observations provide firm evidence that under the mildly acidic conditions of the white wine solution (pH 3–4) the familiar and characteristic C–C bond breaking and making equilibrium (see above) of procyanidin chemistry [4, 8] has been set up. The process is illustrated (Fig. 2) for the procyanidin polymer from sorghum (**10**). Although fission of the interflavan bonds will be essentially random and will lead to carbocations and procyanidin oligomers of varying size the appearance of particular products in the experiments can be satisfactorily explained by the breaking of specific bonds between flavan-3-ol units. Thus fission of the terminal interflavan bond (a) will give (+)-catechin (**3**) as one of the products and fission of the adjacent interflavan bond (b) gives procyanidin B-1 (**5**). Rupture of the alternative terminal flavan unit bond (c) gives the carbocation (**11**) whose capture by (−)-epicatechin (**4**) or (+)-catechin (**3**) leads to the formation of procyanidin B-2 (**6**) and procyanidin B-1 (**5**) respectively. In the absence of an excess of one of the added flavan-3-ols (**3** or **4**) the various carbocations produced by random fission of interflavan bonds in **10** may then react further with other polymer species to produce molecules of increased size and complexity or degenerate by alternative pathways to produce heterogeneous polymeric species which have been loosely termed phlobaphens [16]. Both of these phenomena are probably responsible for the eventual precipitation of the oligomeric procyanidin from solution when it is dissolved alone in white wine. This precipitation does not occur when an excess of one of the flavan-3-ols (**3** or **4**) is present. In these circumstances presumably the various intermediate carbocations are captured by **3** and **4** to produce on average molecules of smaller size and increased solubility in the medium.

(b) *Red wine (1 and 4 years old)*

Red wines to which the sorghum procyanidin polymer has been added show the formation of a precipitate after 4–5 days. This precipitate is formed in the presence of the added flavan-3-ols (**3** or **4**). Removal of the precipitate (60 days) showed it to represent approximately 60–70% (in weight) of the original sorghum polymer. It was deep red-brown in appearance and, compared to the original polymer, insoluble in most typical hydrophilic solvents such as ethanol, methanol, acetone and water. In its ability however to release cyanidin on treatment with acid it gave values of 65–75% when compared directly to the original procyanidin polymer and this supported the view that substantial regions of the original structure remain intact in the precipitate. Its chromatographic behaviour

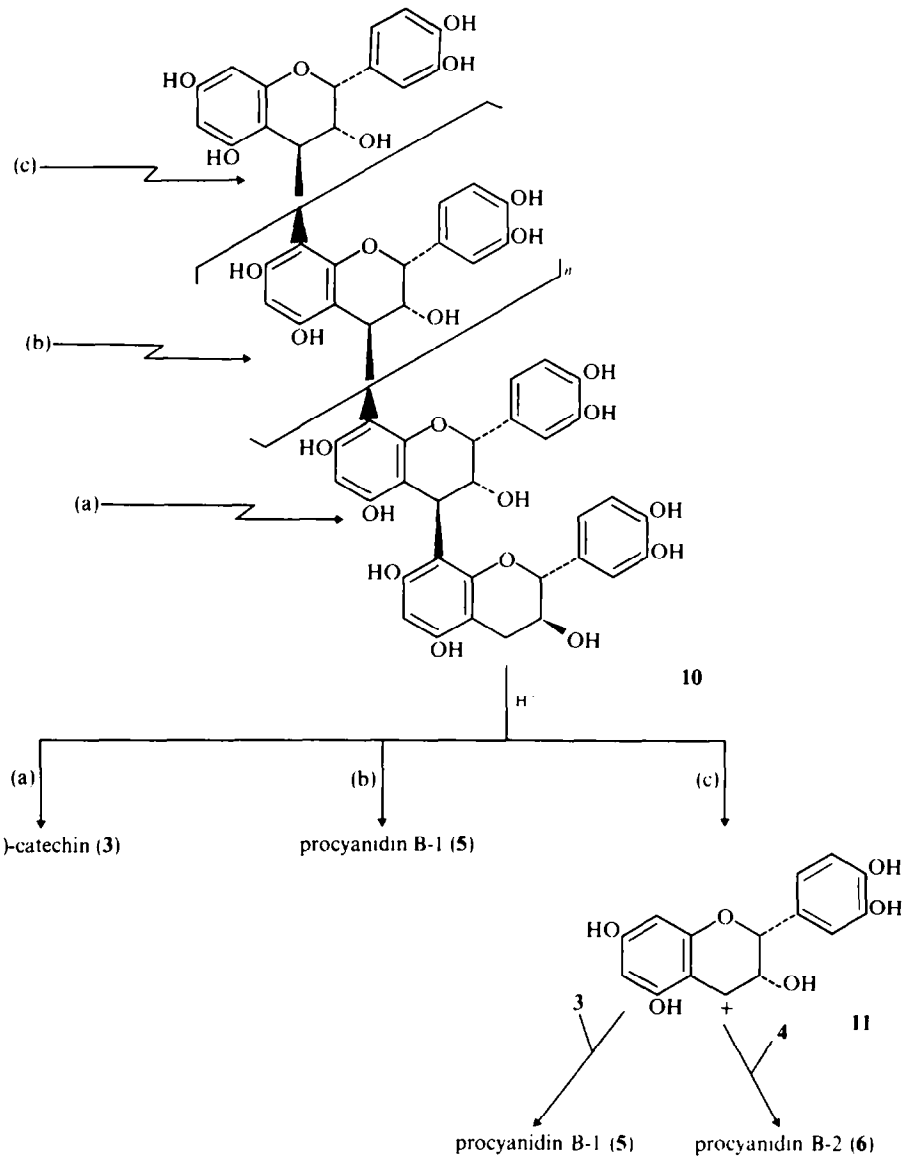

Fig. 2. Acid-catalysed degradation of *Sorghum* procyanidin polymer in wine.

resembled in some respects that of the polymeric pigments isolated from red wine by Somers [17, 18]. Red wine treated similarly with tannic acid gave no immediate precipitate and it therefore seems unlikely that the precipitation phenomenon is associated with the formation of a protein–polyphenol complex. In addition it was noted that red wine which had been aged at room temperature for periods of up to 90 days and which were then treated with the sorghum polymer showed a decreased rate of precipitation of the polymer from solution.

Model red wines were prepared as described by Somers [17, 18] and by the addition of a black grape pigment extract to the white wine used in earlier experiments. In both cases precipitation of the sorghum procyanidin polymer was observed with these model wines but the rate of precipitation was much slower than with the authentic red wines and very similar to that observed in the white wine alone.

The monomeric flavan-3-ols (**3** and **4**) and the dimeric procyanidins (**6** and **7**) gave no precipitate with the red wine after 60 days and only traces of insoluble matter after 90 days. The solutions changed however to an amber tawny red colour. Ethyl acetate extraction of the phenols present (100 days) and subsequent analysis showed that the flavan-3-ols (**3** and **4**) were still present, although in reduced quantity, but that the dimers (**6**, **7**) were no longer detectable. When red wine was aged with mixtures of (+)-catechin (**3**) and procyanidin B-2 (**6**) or (−)-epicatechin (**4**) and procyanidin B-3 (**7**) further evidence was obtained to support the conclusion, obtained earlier with white wine, that at wine pH (pH 3–4) the familiar acid-catalysed equilibration of procyanidins was occurring. Thus mixtures of **3** and **6** showed after 40 days the presence of procyanidin B-1 (**5**) and mixtures of **4** and **7** showed the formation of the diastereoisomeric procyanidin B-4 (**8**).

The changes in anthocyanin content in all the red wine solutions followed a very similar qualitative trend to that in the unadulterated red wine itself although differences in absolute values were noted. In the unadulterated red wine the anthocyanin content after 120 days (based on the

criteria noted above) was ~20% of that at the commencement of the experiment. That of the various solutions to which the sorghum procyanidin polymer had been added was some 10–15% of the original value and broadly dependent on the concentration of the amount of polyphenol initially added. The changes in visible absorption in the region λ 450–550 nm of red wine varied markedly with the different polyphenol additives. In all cases, and in agreement with earlier observations, [2] the λ_{max} shifted to lower wavelengths ($\Delta\lambda$ −10 to −20 nm) and the absorbance rose initially and then decreased. Significantly the point of maximum absorbance was attained after substantially different time intervals. Immature red wine (1 yr), without additives, gave a maximum absorption after 85 days, but red wines to which the sorghum polymer had been added gave a maximum absorption after 20–30 days.

The weight of evidence thus points to the conclusion that during the ageing of red wines two principal processes are responsible for the loss of 'tannin' and hence for the decrease in astringency. The first of these is the familiar acid-catalysed bond breaking and bond making process which characterises procyanidin chemistry and the consequences of which have been outlined (see above). In addition it seems probable that procyanidins and flavan-3-ols react with other substances present in red wines and in the case of procyanidin polymers this also leads to precipitation from solution. The evidence pointing to the nature of these substances is however equivocal. Jurd [19] has suggested [18] that anthocyanins may react in red wines with flavan-3-ols and procyanidins and although this is, in part, supported by the data it also appears probable that additional compounds—possibly hydrolytic degradation products of the anthocyanins —are important in the precipitation of the procyanidin oligomers from solution. There is little evidence to suggest that oxidative reactions lead to the precipitation of procyanidin oligomers from red wines. Thus gallic acid was present as a major constituent of the phenols extracted from the red wines utilised in this study. Its concentration did not change very markedly over the time course of the model reactions which were examined as might have been anticipated if phenol oxidative changes were taking part in solution.

EXPERIMENTAL

Procyanidins, (+)-catechin and (−)-epicatechin were isolated by previously described methods [5, 6]. PC was performed at 20 ± 2° on Whatman No. 2 paper (27.5 cm^2) in the solvent systems (A) 6% HOAc and (B) butan-2-ol HOAc–H_2O (14:1:5). Procyanidins and flavan-3-ols were detected by spraying with (i) $FeCl_3$–$K_3FeC_6N_6$, (ii) 2,6-dibromobenzoquinone-*N*-chlorimide–$KHCO_3$ solution, (iii) vanillin–*p*-TosOH in MeOH. Anthocyanidins were determined by measurement of their absorption at λ 535–545 nm and by chromatography on MN-300 cellulose precoated plastic sheets with Forestal solvent. HPLC analysis was performed using a Du-Pont 860 HPLC system with a Zorbax NH_2 adsorbent and isochratic systems of varying composition acetonitrile–0.05 M NaH_2PO_4. Quantitative measurements of procyanidins and flavan-3-ols were made by adding an aliquot of methyl gallate (10 mg) to the wine solutions before extraction with EtOAc. Changes were measured by comparison with the standard methyl gallate signal.

Wines were stored at 0° but allowed to equilibrate at 20 ± 2° before polyphenols were added. Reactions were followed at 20 ± 2°.

Isolation of polyphenols from wine grapes. The skins, stalks and seeds of wine grapes (2 kg *Vitis vinifera* var. kindly supplied after harvesting by Major Rooke, Stragglethorpe Hall, Nr. Lincoln) were extracted with MeOH (4 × 2000 ml) and the EtOAc-soluble phenols (18 g) isolated as a light-buff powder as previously described. The extract was fractionated (350 × 10 ml fractions) on Sephadex LH-20 (7.5 × 50 cm column) in EtOH. (+)-Catechin, (−)-epicatechin, procyanidin B-1 and procyanidin B-2 were isolated and separated as their acetate derivatives by HPLC on silica.

A polymeric proanthocyanidin fraction (0.85 g) was obtained by extraction of the residual plant debris (from the original extraction with methanol) with Me_2CO–H_2O (1:1, 3000 ml) for 7 days at 20°. Concentration of the extract gave a ppt. of the polymeric proanthocyanidin which was collected and dried at 100° for 48 hr. Its ability to release anthocyanidins was determined after treatment with *n*-BuOH–HCl (12N), 30% v/v at 100° for 2 hr.

Quantitative determination of cyanidin from procyanidin polymers. Separate aliquots of polymer (0.75, 1.5, 2.25, 2.75, 3.25 mg) were treated with *n*-BuOH–HCl (12N), 30% v/v (10 ml) at 115° for 2.5 hr then diluted to 25 ml with *n*-BuOH. The absorptivity was measured at 545 nm and the results plotted graphically. The polymer derived from sorghum was employed as standard and measurements performed on the polymers precipitated from wines and from grape skins compared to the standard.

Wine experiments. (a) *White wine.* The wine employed was a white Mosel wine (Bernkasteler–Kurfürstlay) 4-yr-old. Polyphenols [(+)-catechin, (−)-epicatechin, procyanidins B-2 and B-3 and the sorghum procyanidin polymer] were dissolved in a minimum of EtOH–H_2O and added separately and in various combinations to white wine (25 and 50 ml) to give concentrations of the phenols of *ca* 2.5–3.5 mg/ml. The solutions were kept at 20° for 24 hr, filtered to remove traces of insoluble material, 1 drop of $CHCl_3$ was added and the solutions stored under N_2 at 20°. Solutions were analysed by EtOAc extraction after 30, 60 and 90 days (5 × equal vol.), separation of the phenols by chromatography on Sephadex LH-20 in EtOH, and identification by paper chromatography, HPLC, and TLC of acetate derivatives on silica. The ppt. formed in experiments with the sorghum procyanidin polymer was separated and dried at 100° for 48 hr at 0.05 mm Hg. (Found: C, 61.0; H, 5.0. $(C_{15}H_4O_4)_n$ requires C, 62.0; H, 4.8%.) The ppt. was analysed by quantitative determination of its cyanidin production in acid, (see above). Values of 78, 85 and 82% compared to the original polymer, were obtained. (b) *Red wine.* Analogous experiments with polyphenol additives were conducted with two samples of a red Bordeaux Wine (Chateau Latour), 1 yr and 4 yr old, (the immature wine was kindly donated by Mr. Harry Waugh and Monsieur J. P. Gardère, manager at Chateau Latour) and with the white wine (see above) to which an extract of the anthocyanin pigments from black wine grapes (*Vitis vinifera*, var.) from Chateau Latour had been added [18]. Solutions were analysed as above and the ppt. obtained from solutions containing the sorghum procyanidin polymer was collected and analysed by determination of the cyanidin formed in acidic media. Changes in anthocyanin content were determined by measurement of the optical density change at λ (520–540 nm) before and after the addition of a drop of HCl (12 N) to a 1 ml aliquot. Solutions were also monitored every 7 days by measurement of the UV spectrum (λ 380–560 nm).

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