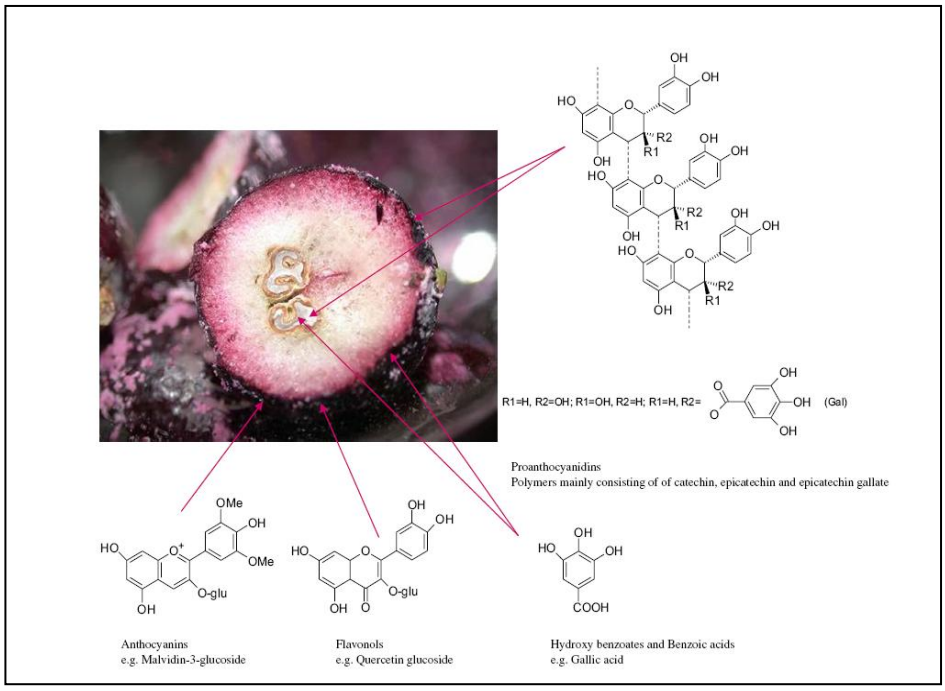
GRAPE AND WINE RESEARCH AND DEVELOPMENT CORPORATION

Australian Government
Grape and Wine Research and Development Corporation

GWRDC Tannin Review



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **GWR 0905**

Principal Investigator: **Dr Geoffrey R Scollary**

Research Organisation: **Private Consultant**

Date: **26 March 2010**

GWRDC Tannin Review

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Private Consultant

DATE **26 March 2010**

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ABSTRACT

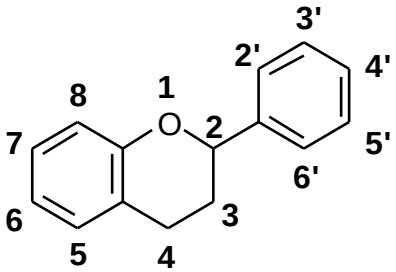
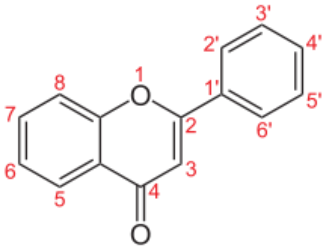
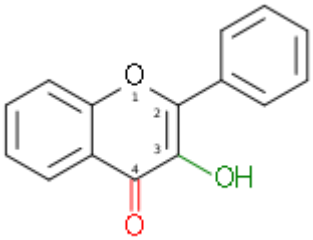
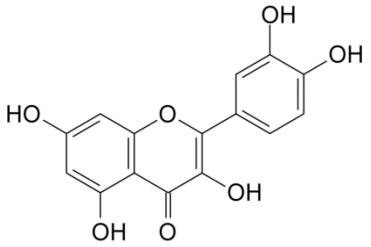
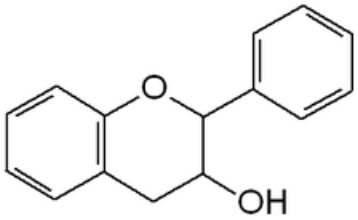
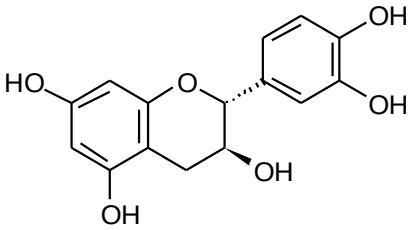
A scientific status report on grape and wine tannin research has been prepared. A review of the literature was performed to ascertain the present state of knowledge and to identify areas where further research is required. Responding to the brief provided by the GWRDC, this status report addresses the biosynthesis of tannins in grapes, the genetic and environmental factors that influence grape tannins, rapid methods for assessing tannin content in grapes and wine, the tannin content and composition in wine (especially the formation of polymeric pigments and other coloured compounds), the role of oxygen in tannin modification, the impact of exogenous tannins on colour stability and mouthfeel of wine and the links between tannin content and composition and sensory perception. Recommendations for further investment of research funds are provided. A greater focus on mechanistic studies is proposed as a key research direction in all areas that have been reviewed in this status report.

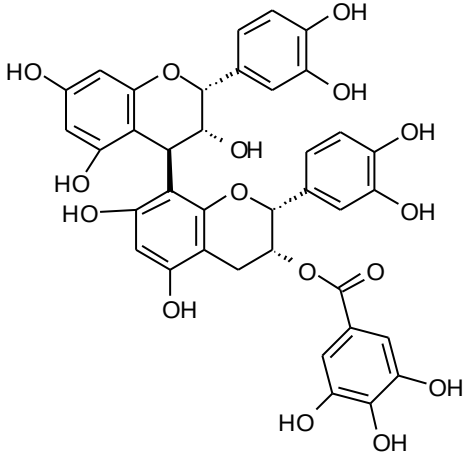
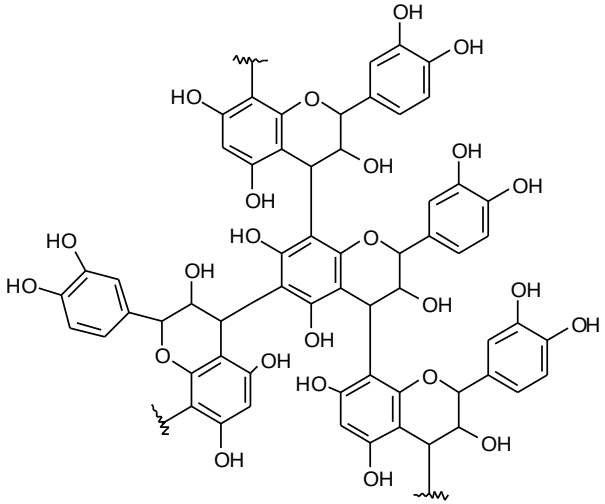
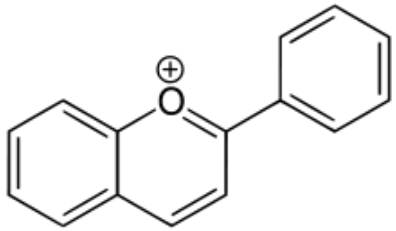
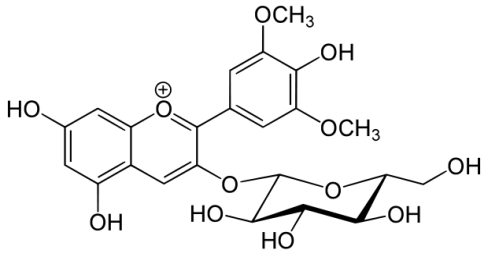
GLOSSARY OF ACRONYMS

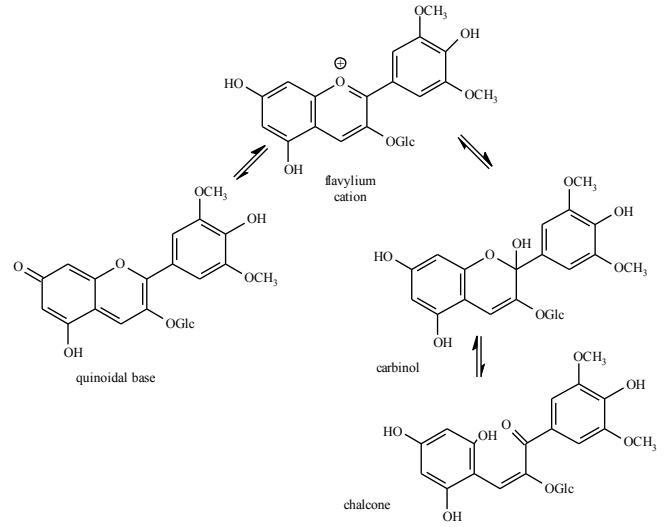
ANR	Anthocyanidin reductase
DAD	diode array detection
HPLC	high performance liquid chromatography
LAR	Leucoanthocyanidin reductase
LC-DAD-MS	liquid chromatography with diode array and mass spectrometry detection
LC-nmr	liquid chromatography with nuclear magnetic resonance detection
MCP	methyl cellulose precipitation (assay)
mDP	mean degree of polymerisation
MOX	micro-oxygenation
MS	mass spectrometry
MYB	Refers to a diverse class of genes that encode proteins of particular importance in transcriptional regulation in plants and elsewhere. The MYB symbol may well arise from initial research on the avian **my**ele**b**lastosis virus (underlined and bolded letters), but the scientific literature never refers to how MYB actually got its name.
nmr	nuclear magnetic resonance (spectroscopy)
RP-HPLC	reverse phase high performance liquid chromatography

GLOSSARY OF PHENOLIC COMPOUNDS

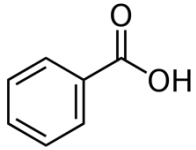
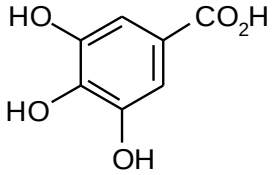
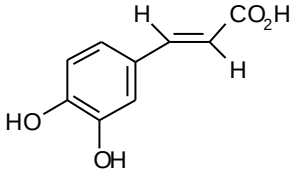
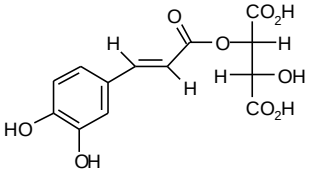
1. FLAVONOIDS

Compound	Description	Basic Structure	Example
Flavonoid	A class of compounds for which the common feature is the oxygen heterocyclic ring system. It is the building block of many phenolic compounds in wine		
Flavone	The basic structure for flavonols, characterised by a ketone oxygen at the position 4 as well as the heterocyclic oxygen atom at position 1.		
Flavonol	Also known as 3-hydroxy-flavone. The –on refers to the double bond oxygen link (position 4 in red) and the –ol refers to the hydroxyl (-OH link at position 3 in green)		Quercetin 
Flavan-3-ol	Characterised by a –OH group at the 3 position. Sometimes referred to as flavanols Proanthocyanidins are dimers, trimers or longer chain polymers of flavan-3-ols. They are also referred to as ‘tannins’ (see below)		(+)-catechin 

Procyanidins	Dimers consisting of 2 flavan-3-ol molecules		Procyanidin Dimer B2 3-gallate 
Condensed tannins	Consist of linked flavan-3-ols. Known by a variety of names including • non-hydrolysable tannins • ‘tannins’ • proanthocyanidins • grape tannins • wine tannins		
Anthocyanins	Based on a flavylium cation skeleton Contain a glucose molecule attached to the 3-position Known as ‘anthocyanidins’ if the glucose molecule is absent.		Malvidin-3-glucoside 

Anthocyanin equilibria	Colour depends on the structure: flavylium cation: red quinoidal base: coloured, with blue and red being ascribed to its colour expression carbinol: colourless chalcone: colourless		

2. NON-FLAVONOIDS

Phenolic acids-I	The basic structure is benzoic acid. This example is based on a C6-C1 structure		Gallic acid 
Phenolic acids-II	This example is based on a C6-C3 structure. Other names are • hydroxycinnamic acids • phenylpropanoids		Caffeic acid 
Phenolic acid esters	Characterised by an ester link between the –COOH unit of a hydroxycinnamic acid and a –OH group of tartaric acid		Caftaric acid 

EXECUTIVE SUMMARY

1. A scientific status report on grape and wine tannin research has been prepared.

2. The GWRDC Tannin Review project posed the following topic areas for review:
   (a) Biosynthesis of tannins in grapes – pathways in synthesis and degradation, and the cellular locations of these various reactions;
   (b) Analytical methods for assessing tannin content and composition in grapes and wine;
   (c) Tannin content and composition in grapes, as affected by genetic and environmental factors;
   (d) Tannin content and composition in wine, including the formation of stable polymeric pigments (polymers containing anthocyanins) and understanding issues associated with their extraction into wine (especially interactions with cell wall proteins, etc.);
   (e) Role of oxygen in tannin modification in grapes and wine, especially micro-oxygenation;
   (f) Role that the addition of exogenous tannins plays in modifying colour stability, mouthfeel and astringency of wine;
   (g) Links between tannin content and composition and sensory perception of astringency and mouthfeel in wines (including interactions with polysaccharides).

3. The methodologies adopted in preparing this status report were a review of the scientific literature (peer-reviewed) and selected technical articles, discussions with researchers in Australia and conversations with researchers in other countries, with a focus on their perceptions of gaps in knowledge regarding tannin research.

4. The scope proposed by the GWRDC was wide-ranging and this status report reflects the breadth of research that has been published. This status report is not a literature review in the traditional sense. Rather, the approach taken has been to identify and analyse critical scientific articles that best address the state of knowledge in tannin research. In many cases, scientific reviews on particular topics and book chapters were used to source information. From this review and analysis, a range of research issues have been identified that are presented for the GWRDC's and researchers' consideration.

5. Significant progress has been made towards our understanding of how grapes make tannins, although much needs to be done to complete the story. Much of the published work on tannins in wine, however, could perhaps be categorised as 'observational'. There is a clear need for more mechanistic studies to enhance our knowledge of the fundamental science that underpins the behaviour of condensed tannins in grapes and wine. The fundamental science studies need to be developed together with well-designed field and winemaking trials.

MAJOR FINDINGS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In the wine literature, the term 'tannin' is used to identify a class of compounds that are formed in the grape berry. These tannins are extracted from the berry during winemaking and may be chemically modified as part of the winemaking/wine development process. Terms that have been used to describe these tannins include condensed tannins, non-hydrolysable

tannins, procyanidins, proanthocyanidins, grape tannins and wine tannins. In this report, the term 'condensed tannins' will be used.

It is important to note that 'condensed tannins' originate in the grape. Tannins which are extracted from oak or which are added during winemaking are referred to as 'exogenous tannins'.

Biosynthesis of tannins in grapes
Significant progress in our understanding of 'when' and 'how' flavonoids are made has been achieved. This includes anthocyanin synthesis and condensed tannin formation. Tannin subunits, (+)-catechin and (-)-epicatechin, are synthesised by two enzymes, anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR). This is now well established, but the issue of how the subunits are converted into tannins is not understood.

The main questions that remain to be answered include:

- how are condensed tannin polymers formed?
- what mechanisms regulate their formation?
- when and how are the galloylated subunits formed?
- are the formation mechanisms enzymatically driven or chemically driven?
- is the apparent decline in condensed tannin extractability from véraison to harvest (the so-called 'tannin maturation') simply a consequence of tannin binding within the cells or are other degradation or oxidation mechanism in operation?

Knowledge of the mechanisms that determine condensed tannin production and composition will open up the possibility of improving the link between condensed tannin production in grapes and the impact of tannins on wine sensory properties. The recent identification of the MYB transcription factor that controls the expression of LAR and ANR is an important outcome for greater understanding of the synthesis and composition of condensed tannins. Continuing work in this field is essential.

Analytical methods for assessing tannin content and composition in grapes and wine
Significant advances have been made in the development of rapid assays for the assessment of the total tannin concentration. The methyl cellulose precipitation (MCP) and Adams-Harbertson methods have received the most attention. Further refinement and comparison of the methods would be beneficial in confirming the wide applicability of one or other of these two methods.

The work that is proposed to complete the refinement and validation includes:

- Impact of prolonged storage on integrity of condensed tannin material – does the tannin extracted from grape skins and grape seeds degrade in storage;
- Extracting solvents focusing on 70% acetone and 50% ethanol
    - why is acetone more effective?
    - comparative extraction of both solvents to grape homogenates as well as grape skins and grape seeds
    - is the application of either solvent meaningful in terms of fermentation extraction?
- Precision validation of the Adams-Harbertson assay – why is it so poor when used in winery laboratories compared with research laboratories?
- MCP assay precision validation – round-robin comparative analysis with winery laboratories

- Confirming links between assay values and astringency descriptors
    - Predictor modelling – can MCP and Adams-Harbertson assays actually predict astringency?
    - Comparative studies across several vintages, varieties and regions – do correlations hold?

There is a 2010 publication that presents a comparison of three extracting methods for the evaluation of phenolic ripening in several varieties of red grapes. The MCP procedure was one of the methods chosen. This is an important step forward in the international acceptance of the MCP method. This work also investigated a predictor model between the methods they employed.

Assessing tannin content in grape berry skins and seeds: composition and cellular location

The composition of the condensed tannins found in the skins and seeds in terms of the subunit composition following extraction of the tannins and cleavage into their component compounds has demonstrated the wide diversity of condensed tannins in berry parts. It is apparent that only limited knowledge regarding the cell wall and the association of tannins with cell wall components is available. Further research in this area is vital and needs to address:

- condensed tannin localisation and extraction in grape berry skins and seeds;
- improvement in methodology for visualisation and extraction of condensed tannins;
- development of methodologies for the enhanced release of condensed tannins from the cell wall.

One recent approach has examined the interactions between condensed tannins and cell wall material, while two publications on enzymatic cell wall degradation have opened up the potential for the enhanced release of phenolic compounds, including condensed tannins, from grapes. The areas of research that require further study are:

- the relationship between enzyme type and activity and the extent of cell wall degradation in skins and seeds;
- the influence of enzyme treatment on the amount and subunit composition of released condensed tannins and anthocyanins as well as the formation of tannin-anthocyanin adducts;
- microscopic and histochemical analysis of the changes in cell wall composition after enzyme treatment;
- changes in the polysaccharide composition of cell walls as a function of enzyme activity;

Changes in the polysaccharide composition need also to be considered in terms of its effect on the proposed colloidal structure in wine and possible influences on mouthfeel properties.

Tannin content and composition in grapes, as affected by genetic and environmental factors

The areas reviewed are climate and temperature effects, viticultural influences including vine vigour and its link to shading, irrigation and water stress, varietal influences, rootstocks and environmental influences on gene expression.

Drawing general conclusions from the published studies is difficult, if not impossible, given the number of variables involved. The studies analysed here are spread over several varieties,

grown in different regions and countries. Most were field trials, although one pot trial has been included. Vintage variations were observed and noted by some authors and not others. The vintages ranged from 1992 to 2006. Some studies were single vintage only. Research designed to understand the mechanisms at work appears to be addressed in one publication only.

There is a clear need to examine, through properly designed experiments, the relationship between environmental influences and condensed tannin production. The study needs to include, as a minimum, varietal influences, rootstock influences, vintage variation, the timing and extent of environmental stress (light, temperature, water), berry ripening process and berry size development, the condensed tannin concentration and composition, the extractability of the condensed tannins, the transfer of the condensed tannins from the grape into wine and the sensory parameters of wines made from different treatments.

The focus on gene expression under various conditions of environmental stress is of critical importance in furthering our understanding.

Tannin content and composition in wine

Full characterisation of condensed tannins is faced with several significant analytical and structural challenges. The approach that has been generally adopted is to determine:

- the nature and number of monomeric flavan-3-ol units, sometimes referred to as constitutive units of terminal and extension units;
- the mean molecular mass of the polymer;
- the mean degree of polymerisation;
- the types and number of interflavanic links (that is, bonds between the flavan-3-ol monomers within the polymer.

Acid-catalysed cleavage of the carbon-carbon bond in the presence of a nucleophilic agent (toluene-α-thiol or phloroglucinol), followed by reverse phase HPLC (RP-HPLC) has become standard practice for identifying the terminal and extension units in condensed tannins. Both procedures have their supporters and detractors but neither is totally successful for full characterisation of the condensed tannin.

Limitations of existing analytical methods include the determination of mDP and the efficiency of the acid cleavage reaction. There does not seem any research directed towards a more efficient cleavage reaction.

The major limitation is the absence of standard reference materials. This must be rectified as a matter of urgency.

Molecular associations in the wine matrix are receiving greater attention. There is increasing evidence that colloidal interactions are important in influencing wine stability, clarification and taste. Colloidal particles may be of sufficient size to modify the rheological or flow properties. Small and finite particles might induce a friction-based taste sensation.

The implications of molecular associations for understanding sensory properties are significant and demand further work on wine rather than on model systems. Analytical methods such as Couette shear flow and ultracentrifugation, in combination with fluorescence spectroscopy offer the potential to gain further insight into the colloidal structures within the wine matrix.

Formation of stable polymeric pigments, especially polymers containing anthocyanins
The formation of stable pigments has been the subject of extensive research and a summary of the research would require a textbook to be written. More than one hundred derived pigments have been detected in red wine, although the extent to which most actually contribute to red wine colour is questionable.

A wide range of mechanisms have been proposed to explain the formation of stable pigments. These include anthocyanin-flavanol (A-F) direct condensation reactions, flavanol-anthocyanin (F-A) direct condensation reactions, acetaldehyde mediated anthocyanin-flavanol and anthocyanin-anthocyanin condensation reactions, pyranoanthocyanin formation and flavanol-flavanol condensation reactions. Many of the mechanisms have been developed in model studies, frequently using monomeric species as the starting material. Dimers and trimers of the type F-A^+ and F-A-A^+, as well as oligomers $(F)_n$-A^+, have been isolated from wine.

With respect to pigment production during wine ageing, some of the critical issues to be resolved are:

- are there actually polymeric pigments *per se* or are anthocyanins physically, rather than chemically, attached to molecular assemblies in the wine matrix?
- are pyranoanthocyanins major contributors to the pigment pool, especially as the wine ages;
- are the concentrations of ethyl-bridged anthocyanin-flavanol compounds and direct condensation anthocyanin-flavanol compounds high enough to impact on colour, especially if their concentrations decrease over time?

A more detailed understanding of the mechanisms of the reactions that are associated with pigment evolution is required. This would require the following to be studied:

- information of the kinetics of the various mechanisms for polymer formation to provide insight into the formation and/or re-arrangement of pigmented polymers as the wine ages;
- an examination of the impact of reduced oxygen availability from efficient bottling and packaging on the development of colour, especially pigment production that requires an oxidation step;
- a re-examination to the resistance towards SO_2 bleaching under wine conditions to determine the relative importance of pigment types to colour expression;
- a better understanding of the mechanisms and relevance of copigmentation as a contributor to wine colour expression;
- a clearer understanding of the structure/function relationships that determine the interactions between proteins and tannins;
- a more detailed assessment of changes in wine composition that occur as a consequence of fining with proteins: that is, are more astringent molecules removed from solution or encapsulated in some way that minimises their impact on sensory responses;
- clarification of the mechanism of polysaccharide inhibition of protein-tannin interaction.

The real challenge now is to understand how the various reactions occur in the wine matrix. Model studies have provided vital advances in our knowledge of these processes.

Extrapolation from model studies to the real context of wine chemistry needs to be done with care, especially when predictions regarding the impact of individual wine components on wine sensory descriptors, both mouthfeel and colour perception, are drawn.

Extraction of tannins and polymeric pigments into wine
This is generally well established with one obvious question being why the fermentation only extracts about 50% of condensed tannins. This is significant as it suggests that protocols that claim to simulate extraction during the maceration and fermentation are potentially limited as they are unable to reproduce the effect of increasing alcohol concentration, temperature fluctuations and duration of the maceration/fermentation.

The review of published work has opened up several possible lines for further enquiry:

- understanding the factors that determine the differential extraction of condensed tannins from skins and;
- validation of the rapid extraction and tannin assessment protocols against what happens in maceration/fermentation;
- examination of the relationship between berry ripeness and the release of polysaccharides;
- an examination of the tannin-anthocyanin ratio as a possible predictor of colour stability;
- tracking the fate of anthocyanins with the aim of maintaining higher concentrations, preferably stabilised, in the finished wine;

Role of oxygen in tannin modification in grapes and wine
The importance of oxygen in tannin modification is often discussed, but there are surprisingly few peer-reviewed publications on the topic.

This analysis of published information on oxygen and tannin modification suggests that further research in the following areas would be beneficial:

- polyphenolic structural and compositional changes that occur as a result of oxygen ingress that is representative of winemaking and wine ageing conditions;
- a better understanding of the formation and role played by A-type procyanidins;
- further development of wine oxidation processes/mechanisms including knowledge of the chemical speciation of metal ions;
- rigorous kinetic data for the competing reactions that could be involved in wine oxidation.

Structural changes to condensed tannins through oxidation will almost certainly influence sensory perception and a greater understanding of the chemical changes is required. Research in this area is important.

Micro-oxygenation
The introduction of MOX created a wave of enthusiasm amongst winemakers across the world. The winemaker enthusiasm seems to have moderated of late, although there are still a significant number of research papers appearing.

While research trials on MOX and red wine have been extensive, the concept of MOX presents a classic example of the difficulty of transferring information from research trials to commercial practice. That is, research trials in small volume (250 to 300 L) tanks do not

represent the spatial distribution processes that need to occur in commercial tanks of up to 200 kilolitre or more.

Most of the published trials have understandably focused on phenolic chemistry. However, there is a distinct lack of kinetic data relevant to the competing reactions that can occur within the wine matrix. The next phase in understanding the MOX process must be the production of kinetic data for the various competing reactions that can occur within the wine matrix following the addition of oxygen. Until this kinetic information is available, knowledge of the MOX process and the potential for more efficient and effective control will be limited.

Role that the addition of exogenous tannins plays in modifying colour stability, mouthfeel and astringency of wine

Exogenous tannins are compounds present in wine that were not present in the original grape material used to prepare the wine. The sources can be from oak used in winemaking or by selected addition. A classification of commercially available exogenous tannins is gallotannins, ellagitannins, grape derived tannins (red skin, white skin, seed, stalk) and mixtures of the above.

The main issues arising from this area of activity are:

- The use of exogenous tannins as a winemaking aid now falls more in the area of company specific trials to ascertain the more effective product for a given wine style. The same is essentially the case for the link between exogenous tannin addition and micro-oxidation.
- There is work being performed in France on tannin/protein interactions (not yet published) with the aim of using exogenous tannins in a sacrificial way to preserve the natural grape-derived tannins. This is an area that could develop into a new research field.
- Mechanistic studies to ascertain the molecular basis for colour stabilisation in the presence of exogenous tannins (presently underway in France) may open up new avenues for research in colour stabilisation.
- Understanding the mechanism of the sensory response to exogenous tannins is a potential new research area as it could provide a basis for matching exogenous tannin type to wine style.

Links between tannin content and composition and sensory perception of astringency and mouthfeel in wines

There has been extensive research on sensory aspects over the last 20 years or more and intriguingly the same questions keep arising:

- what are the main drivers of mouthfeel responses, including astringency, drying and so on?
- is astringency perception only a consequence of precipitation with proteins, especially salivary proteins?
- can the mDP of condensed tannins be correlated with sensory perception?
- what is the influence of tannin modification, including formation of acetaldehyde linked compounds, during wine ageing on sensory perception?

More recent questions link to a broader appreciation that components of the wine matrix other than condensed tannins can possibly influence the response of the taster:

- is there an influence on sensory response from colloidal particles that may form in wine from condensed tannin-protein interactions?
- to what extent do polysaccharides influence sensory response?
- does fining wine with proteins actually remove the compounds responsible for astringency from the wine?
- can rapid tannin assays be used to predict sensory response?
- can one or more analytical markers be found as indicators of sensory responses?
- what is the link between winemaker perception of astringency and the response of consumers?

Critical areas where additional research is required include:

- a comprehensive understanding of the mechanism of mouthfeel response;
- a better understanding of condensed tannin structure/function activity in relation to astringency – does astringency increase with polymer size and is the loss of astringency in ageing due to hydrolysis of flavan-3-ol polymers;
- a re-examination of the relative astringency of skin versus seed condensed tannins in a winemaking context;
- are there colloidal particles of sufficient size in the actual wine matrix to cause changes in flow properties with the possible induction of frictional responses affecting mouthfeel;
- a comprehensive understanding of the mechanisms of tannin/protein/polysaccharide interactions, both in model systems (to understand the effect) and in the wine matrix;
- a search for markers of mouthfeel (chemometrics, metabolomics) as potential methods of rapid assessment in place of lengthy and expensive trained sensory panel analysis;

BACKGROUND AND TERMINOLOGY

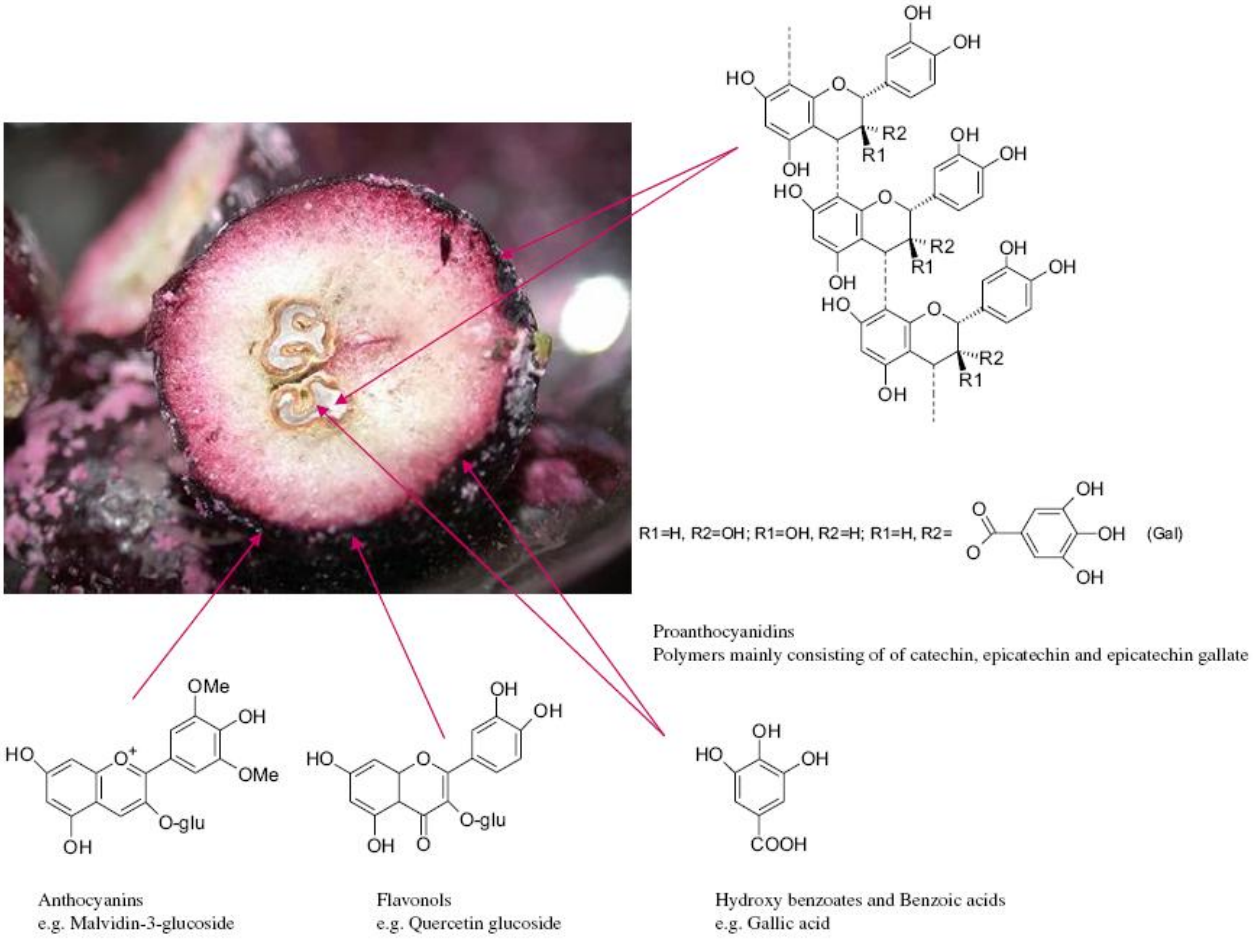
In this report, the term 'condensed tannin' will be used to describe tannins found in grapes as well as the tannins present in wine that originate from the grapes used in the winemaking process. Cross-referencing to other terms that are used in the scientific literature (proanthocyanins; procyanidins) will be given where appropriate.

Figure 1 presents an image of a cross-section of a red grape berry. This cross-section clearly shows the three major components of the berry: skin, pulp and seeds. The phenolic compounds in the skin and seeds are also shown in Figure 1. These can be categorised as:

- Skins: condensed tannins (labelled proanthocyanins in the Figure), anthocyanins, flavonols, hydroxy benzoates and benzoic acids
- Seeds: condensed tannins (proanthocyanins), hydroxy benzoates and benzoic acids.

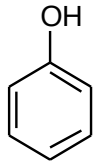
It is the condensed tannins in the skins and seeds that are of most relevance for the purposes of this tannin research status report. The anthocyanins, the red coloured molecules within the various classes of phenolic compounds found in the grape berry, are located in the skins. It is intriguing that, even though the berry used for the cross-section shown in Figure 1 is frozen, there has been noticeable leakage of anthocyanins from the skin to the pulp. The mobility of anthocyanins is an issue that will be discussed in detail later in this report.

Figure 1. Cross section of red grape berry showing the skin, pulp and seeds. Phenolic compounds in the skin and seeds are clearly identified. From Pinelo et al. (2006). *Reproduced with permission.*



Basic terminology
Polyphenolic compounds are based on phenol, a C6 aromatic ring compound with one hydroxyl group attached (Figure 2). Polyphenols are characterised by having more than one phenol type unit per molecule. For example, catechol (*ortho*-dihydroxyphenol) is the B-ring component of (+)-catechin (see Figure 5).

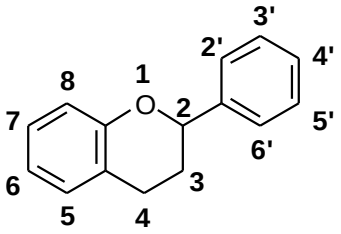
Figure 2. Structure of phenol.



Condensed tannins extracted from grapes are often referred to as grape tannins or proanthocyanins. In some cases, the term 'non-hydrolysable tannin' is also used. Grape tannins are extracted during winemaking into the fermenting system, where changes may occur over time. The term 'wine tannins' is sometimes used to denote tannins found in wine – modification of some grape tannins will occur during processing and ageing, giving new compounds. Other types of tannins may be added during winemaking or extracted from oak: these are termed 'exogenous tannins' in this report.

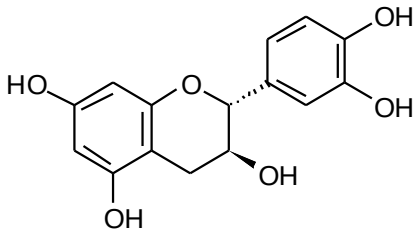
Condensed tannins are composed of flavonoid units. The basic structure of a flavonoid is shown in Figure 3.

Figure 3. The flavonoid ring system with the numbering scheme used to differentiate between the different atoms on each ring.

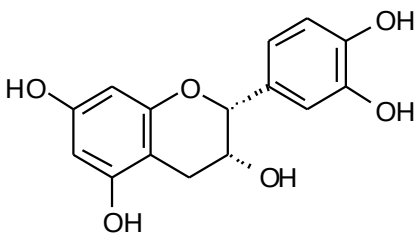


Building blocks
The two most important flavonoids with respect to tannin composition are the flavanols, (+)-catechin and (-)-epicatechin (Figure 4), described more appropriately in the literature as flavan-3-ols, to indicate that a –OH group is located at position 3 on the ring.

Figure 4. Structures of (+)-catechin and (-)-epicatechin



(+)-Catechin

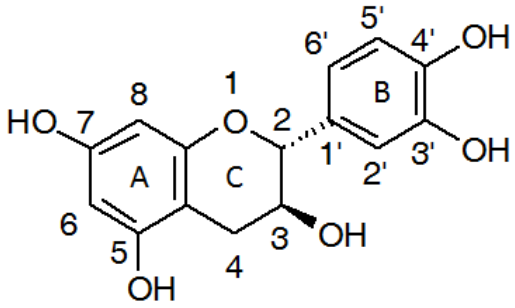


(-)-Epicatechin

These two compounds are diastereoisomers. That is, their structures are identical with the exception of the orientation of the hydroxy (-OH) group at position 3. This –OH group is above the plane of the molecule for (+)-catechin (solid line) and below the plane for (-)-epicatechin (dotted line). This structural arrangement can give rise to different properties, especially when the molecules are linked to form polymers.

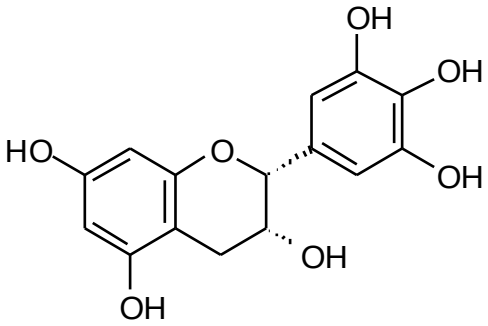
The labelling of the rings and atom number scheme for the flavan-3-ols is shown in Figure 5.

Figure 5. Ring labelling and atom numbering in catechin-type phenolic compounds.



In addition to (+)-catechin and (-)-epicatechin, there are several other flavan-3-ol compounds that participate as building units of condensed tannins. These include (-)-epigallocatechin (Figure 6), (-)-epicatechin-3-O-gallate (Figure 7), (+)-gallocatechin (Figure 8), (+)-gallocatechin-3-O-gallate (Figure 9) and (-)-epigallocatechin-3-O-gallate (Figure 10).

Figure 6. Structure of (-)-epigallocatechin



The difference between (-)-epicatechin (Figure 4) and (-)-epigallocatechin (Figure 6) is in the B-ring: there are three hydroxyl groups in the case of (-)-epicatechin gallate compared to two in (-)-epicatechin. The three hydroxyl groups give a ‘gallic acid’ component (see Figure 11 below) to the molecule that is reflected in its name. This is also the case for (+)-gallocatechin (Figure 8).

Gallic acid is attached to the hydroxyl position at C3 in (-)-epicatechin-3-O-gallate (Figure 7) and in (+)-gallocatechin-3-O-gallate (Figure 9) and (-)-epigallocatechin-3-O-gallate (Figure 10). The ‘-3-O-’ term in the name reflects this bond position. The B-ring in (+)-gallocatechin-3-O-gallate and (-)-epigallocatechin-3-O-gallate also contains 3 hydroxyl groups as in (-)-epigallocatechin (Figure 6).

Figure 7. Structure of (-)-epicatechin-3-O-gallate.

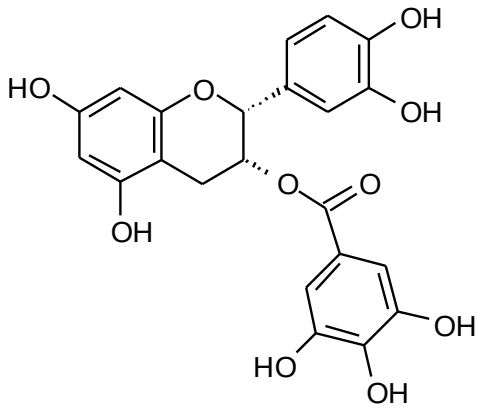


Figure 8. Structure of (+)-gallocatechin

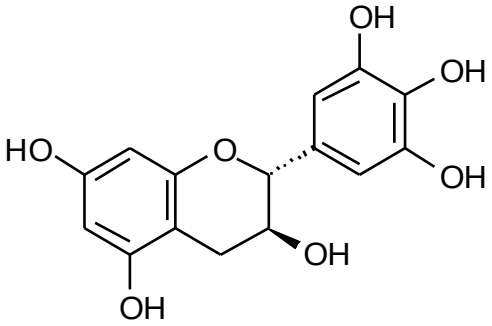


Figure 9. Structure of (+)-gallocatechin-3-O-gallate

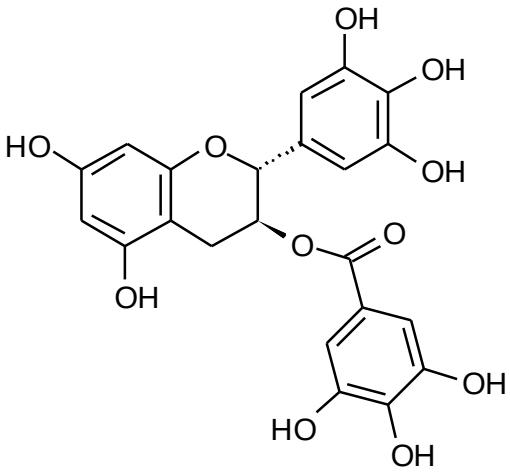
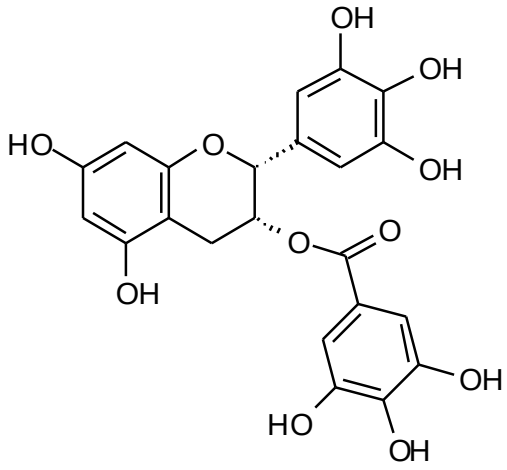


Figure 10. Structure of (-)-epigallocatechin-3-O-gallate



The structural complexities of these molecules, especially the 3-O-gallates, imply that there may be steric limitations to the polymerisation process.

Basic tannin structures

Figure 9 presents a schematic representation of a condensed tannin. It is a polymer formed through linkages between flavan-3-ol molecules. The molecule depicted in Figure 9 has four flavan-3-ol subunits. There are two catechin terminal units (labelled T) and two epicatechin extension units (labelled E). The linkage for the lower right T unit is C8 to C4 on the extension unit, whereas the lower left T unit linkage is C4 to C6 of the extension unit. The linkage between the two E units is C8 (lower) to C4 (upper). Note that only the A and C rings are involved in the linkage arrangements.

Figure 9. Schematic representation of a condensed tannin molecule.

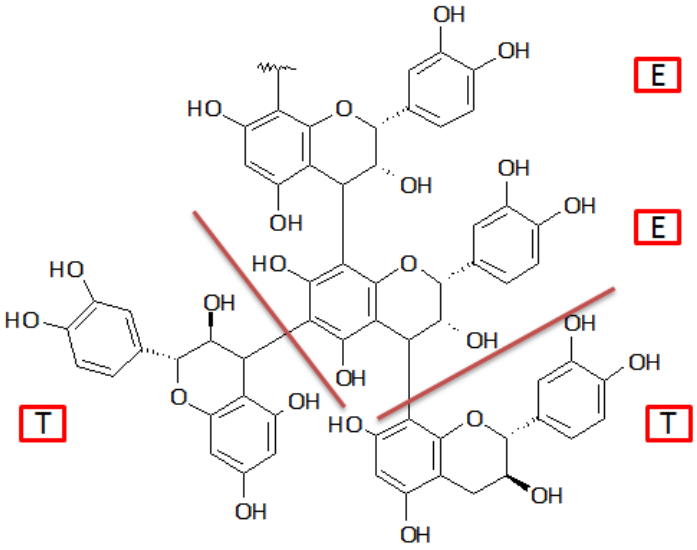
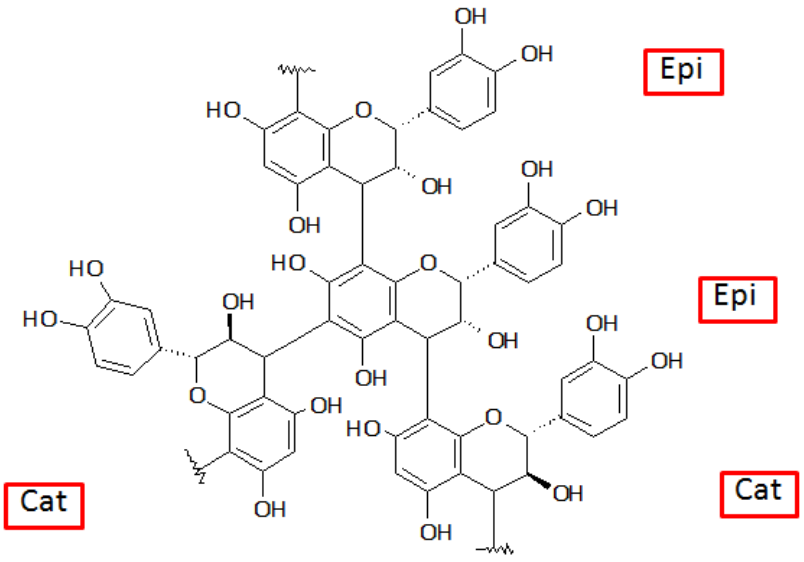


Figure 10 shows a series of extension units, consisting of catechin (Cat) and epicatechin (Epi) subunits with C6 to C4 and C8 to C4 links. The structural complexity of the condensed tannins is evident from these representations, especially given the different structural units that are available.

Figure 10.Catechin (Cat) and epicatechin (Epi) extension units



Degree of polymerisation
The size of the polymer is often described by the degree of polymerisation (DP). DP represents the number of flavan-3-ol molecules in the polymer. The component of the molecule shown in Figure 9 would be described as DP4 (there are four extension units). DP80 means that 80 monomer units are linked in the tannin.

Separating the different types of polymers remains an analytical challenge. Frequently, fractionation steps produce fractions that contain polymers of several different lengths. In this case, the average or mean degree of polymerisation (mDP) is used.

Hydrolysable tannins
The non-grape derived hydrolysable tannins are polymers which generally are based on two hydroxybenzoic acids (see below):

- Gallic acid, a component of gallotannins
- Ellagic acid, a component of ellagitannins

Figures 11 and 12 present the structures of these two compounds. Ellagic acid is actually a dilactone where the carboxyl group of one 'gallic acid' unit has condensed with the hydroxyl group on the other 'gallic acid' unit. Gallic acid is one the hydroxybenzoic acids of highest concentrations in wine. Both gallic acid and ellagic acid can be released into wine from oak following the hydrolysis of hydrolysable tannins.

Figure 11. Structure of gallic acid

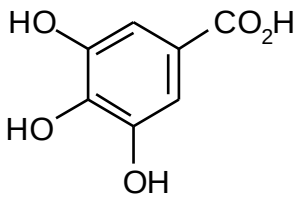


Figure 12. Structure of ellagic acid

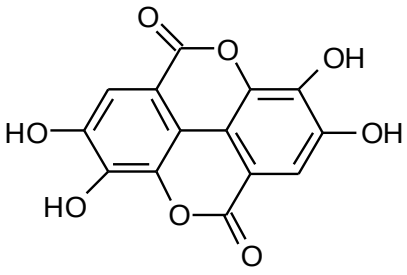


Figure 13. Structure of the ellagitannin, casuarictin.

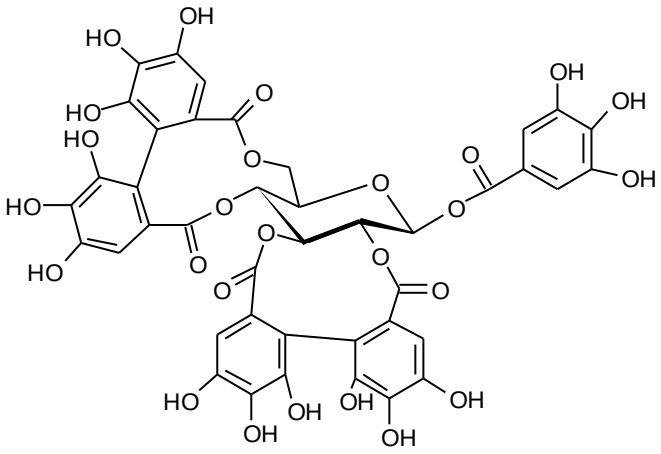


Figure 13 shows the structure of an ellagitannin. Hydrolysable tannins are characterised by having a carbohydrate molecule (usually D-glucose) at the centre. This clearly differentiates hydrolysable tannins from condensed tannins. The hydroxyl groups of the carbohydrate are

partly or totally esterified with the acid groups of gallic acid or ellagic acid. Hydrolysable tannins are hydrolysed in acidic or basic conditions to yield the free phenolic acids and the carbohydrate.

Phenolic acids

The phenolic acids in wine, originating predominately from the pulp of the grape, are based on the structure of phenol (Figure 2) and also contain a carboxyl (-COOH) group. They are often characterised as non-flavonoid phenolic compounds. Non-flavonoid phenolic compounds are generally non-coloured, but some exhibit a pale yellow colour due to 'tailing' of the absorbance into the visible region.

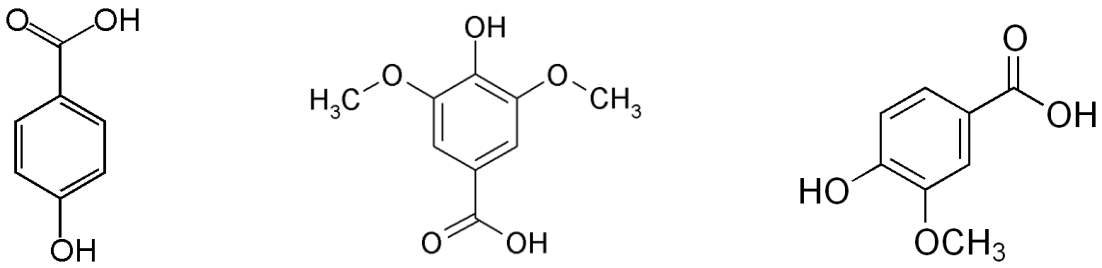
The non-flavonoid compounds can participate in several colour development and stabilisation reactions including:

- conversion of the carboxyl (-COOH) group to an aldehyde (-CHO) group that then participates in condensation reactions with catechin-type phenolic compounds;
- direct reaction with anthocyanins to generate pyranoanthocyanins;
- undergoing decarboxylation during fermentation and then reaction with anthocyanins
- oxidative cleavage to form reactive aldehyde that then reacts with a catechin-type phenolic compound.

There are two classes of phenolic acids found in wine:

- the C6-C1 type that is based from benzoic acid. Gallic acid (Figure 11) is one example. Further examples are shown in Figure 14;
- the C6-C3 type, commonly known as hydroxycinnamic acids.

Figure 14. Structures of *p*-hydroxybenzoic acid, syringic acid and vanillic acid



Hydroxycinnamic acids

Two examples of the C6-C3 phenolic acids are caffeic acid (Figure 15) and p-coumaric acid (Figure 16). This class of compounds is sometimes referred to as phenylpropanoids, referring to the C3 side chain (propyl) attached to the aromatic (phenyl) ring.

In juice and young wines, the free forms of these acids occur in low amounts only. Rather, they exist as esters with tartaric acid. Figure 17 presents one example: caffeic acid is linked through its carboxyl group to a hydroxyl group of tartaric acid. An increase in the free forms is observed as the wine ages. Glucosides (glucose molecule attached to a hydroxyl group on the aromatic ring) can also be found in wine. The reaction between caftaric acid and glutathione produces 2-S-glutathionylcaftaric acid, a major phenolic product formed during juice enzymatic oxidation.

Hydroxycinnamic acids can react with anthocyanins during winemaking and wine ageing and can have a major influence on red wine colour. This chemistry is outside the scope of this status report.

Figure 15. Structure of caffeic acid

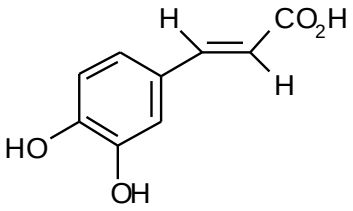


Figure 16. Structure of p-coumaric acid

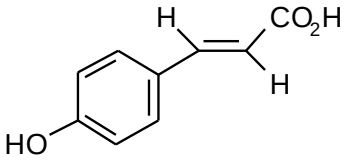
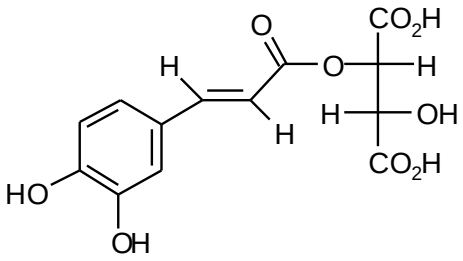
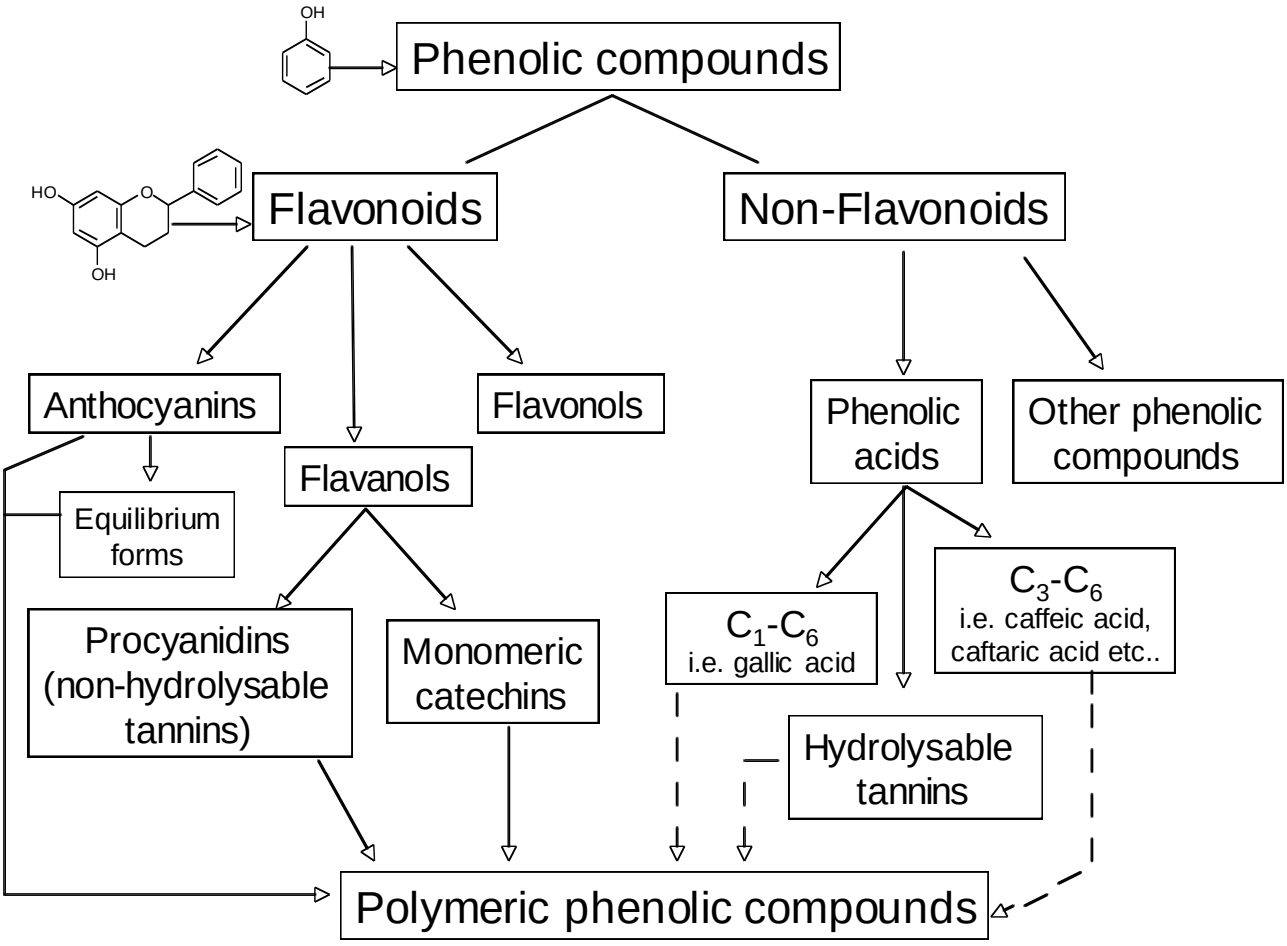


Figure 17. Structure of caftaric acid



Summary: phenolic diversity
There is considerable phenolic diversity in wine and Figure 18 presents a generalised overview of the main types of phenolic compounds. This status report focuses on the lower box in Figure 18; that is polymeric phenolic compounds.

Figure 18. Phenolic diversity in wine.
Dashed lines indicate that the conversion pathways are less likely than those marked with solid lines. Image extracted from the wine chemistry teaching notes at CSU (M Allen and A Clark).



STATUS OF RESEARCH INTO TANNINS IN WINE

An analysis of the status of research into condensed tannins in wine has been performed at the request of the GWRDC. Responses to the scope of the review set by the GWRDC are presented in eleven sections:

- Biosynthesis of tannins in grapes - pathways in synthesis and degradation, and the cellular locations of these various reactions;
- Analytical methods for assessing tannin content and composition in grapes and wine;
- Assessing tannin content in grape berry skins and seeds: composition and cellular location;
- Tannin content and composition in grapes, as affected by genetic and environmental factors;
- Tannin content and composition in wine;
- Formation of stable polymeric pigments, especially polymers containing anthocyanins;
- Extraction of tannins and polymeric pigments into wine;
- Role of oxygen in tannin modification in grapes and wine;
- Micro-oxygenation;
- Role that the addition of exogenous tannins plays in modifying colour stability, mouthfeel and astringency of wine;
- Links between tannin content and composition and sensory perception of astringency and mouthfeel in wines.

The methodologies adopted in preparing this status report include:

- Review of the scientific literature (peer-reviewed) and selected technical articles;
- Discussions with researchers in Australia;
- Discussions with researchers in other countries, with a focus on their perceptions of gaps in knowledge regarding tannin research.

The scope proposed by the GWRDC was wide-ranging and this status report reflects the breadth of research that has been published. This status report is not a literature review in the traditional sense. Rather, the approach taken has been to identify and analyse critical scientific articles that best address the state of knowledge in tannin research. In many cases, scientific reviews on particular topics and book chapters were used to source information. From this review and analysis, a range of research issues have been identified that are presented for the GWRDC's and researchers' consideration.

There is a vast literature on condensed tannins in wine. Significant progress has been made towards our understanding of how grapes make tannins, although much needs to be done to complete the story. Much of the published work on tannins in wine, however, could perhaps be categorised as 'observational': that is, *this is what was found under the conditions of our experiments*. While this may be seen as an oversimplification, there is a clear need for more mechanistic studies to enhance our knowledge of the fundamental science that underpins the behaviour of condensed tannins in grapes and wine. The fundamental science studies need to be developed together with well-designed field and winemaking trials.

SECTION 1
Biosynthesis of tannins in grapes – pathways in synthesis and degradation, and the cellular locations of these various reactions

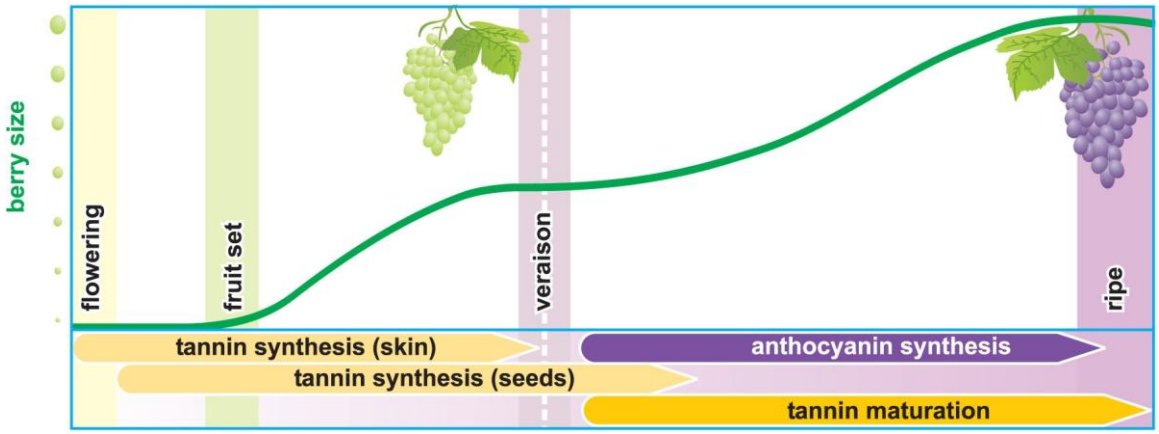
CSIRO Plant Industry has made significant progress in our understanding of 'when' and 'how' flavonoids are made. This includes anthocyanin synthesis and condensed tannin formation. The work is among the best in the world and has greatly assisted the wine industry in understanding colour and factors that influence colour development as well as condensed tannin development.

CRCV project 3.3.1 (Flavonoid pathway genes in grapes) produced critical outputs regarding the time at which condensed tannins are produced in grape berries. Figure 19, extracted from CRCV project report 3.3.1, demonstrates that tannin synthesis occurs early in the berry ripening process. The main points can be summarised as:

- tannin synthesis is essentially complete before véraison;
- synthesis of tannins occurs in the skins and in the seeds, but not in the pulp of the berry;
- tannin synthesis might be considered as a branch reaction to the main flavonoids synthetic pathway that leads to anthocyanins (see below for detail);
- following the accumulation of tannins up to véraison, a period of 'tannin maturation' occurs up to physiological ripeness;
- this 'maturation' period is associated with a loss of extractability using chemical extracting solutions;
- the tannin synthesis genes are not expressed in the véraison to maturity period: the pathways are more directed towards anthocyanin synthesis.

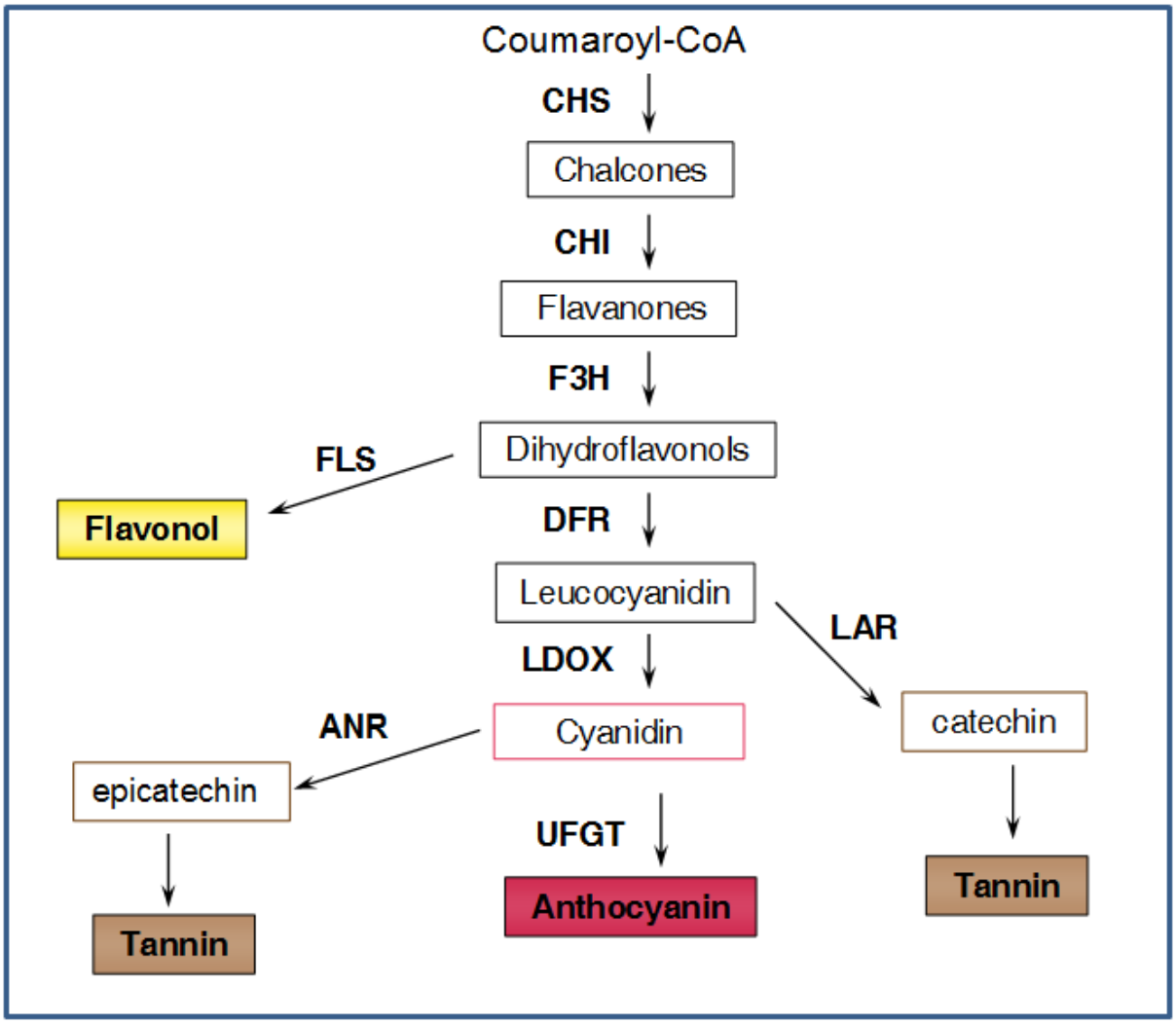
The tannin content and composition (subunit distribution) in the skins and seeds will depend on the gene activity during the synthesis phase. Expression of the genes involved in the synthesis can be influenced by climatic and viticultural management practices as well as by variety (Section 4). The location and possible binding of the tannins with skin and seed cell walls may be critical in determining the effectiveness in extracting the tannins from the berry parts into wine (Section 3).

Figure 19. Schematic representation of the synthesis of tannins in skins and seeds, anthocyanin synthesis and tannin maturation processes during grape berry development. *Extracted from CRCV project report 3.3.1. Berry size is represented by the green curve.*



CSIRO Plant Industry, in CRCV project 3.3.1, characterised the main steps in the flavonoid pathway that operates in grapes. A schematic representation of the pathway is presented in Figure 20. Full details of the pathway can be found in the CRCV project 3.3.1 report (GWRDC report 99/16A). There are also two excellent technical publications that provide an overview of the critical aspects of this research (Robinson and Walker, 2006; Kennedy et al., 2007).

Figure 20. Schematic representation of the flavonoid pathway leading to the production of anthocyanins, flavonols and tannins in grapes. *Extracted from CRCV report 3.3.1.*
The enzymes are: CHS = Chalcone synthase; CHI = Chalcone isomerase; F3H = Flavanone-3-hydroxylase; FLS = Flavonol synthase; DFR = Dihydroflavonol reductase; LDOX = Leucoanthocyanidin dioxygenase; UFGT = UDP-glucose : flavonoid glycosyltransferase; LAR = Leucoanthocyanidin reductase; ANR = Anthocyanidin reductase.



The critical aspects of this pathway are:

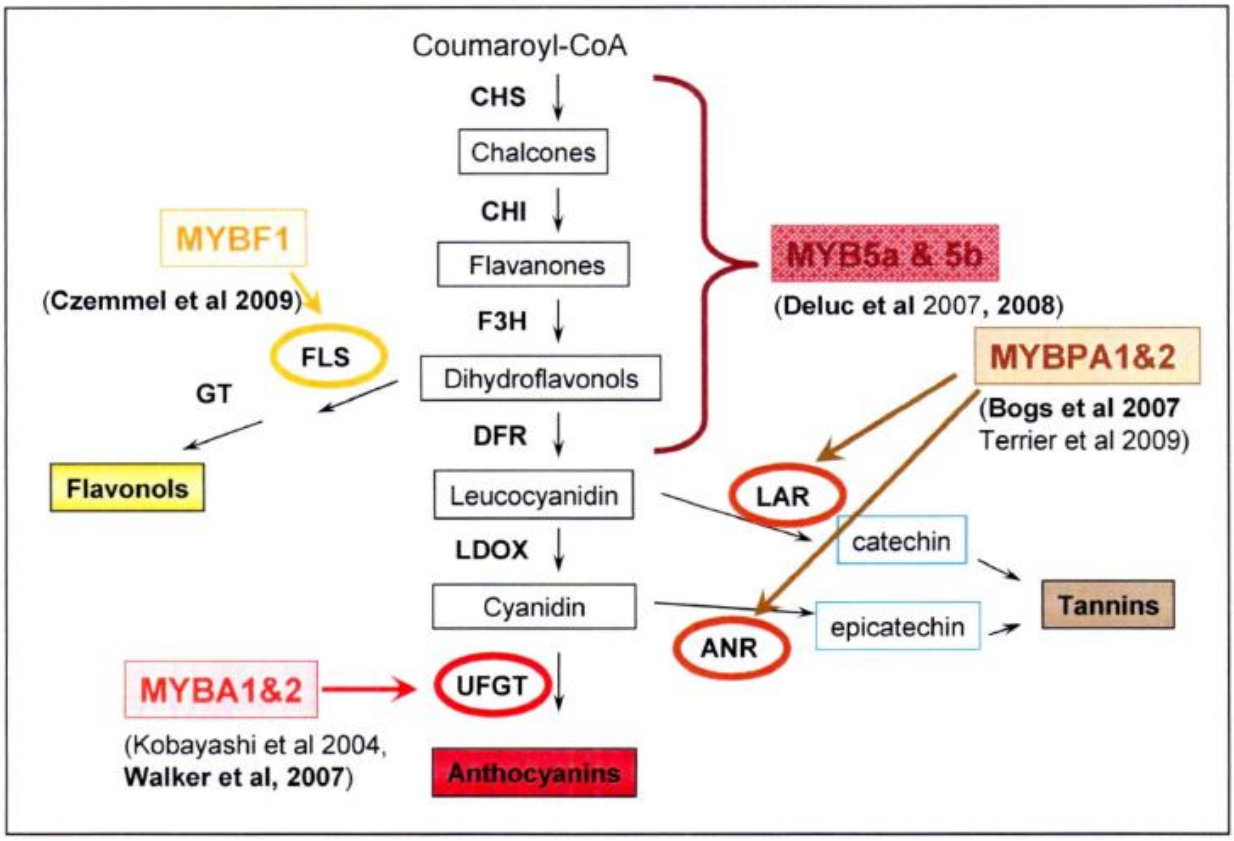
- the pathway leads to the synthesis of anthocyanins, flavonols and condensed tannins, with some 'shared enzymes' required for the general pathway;
- each step in the pathway is carried out by a specific enzyme;
- the enzymes (proteins) are encoded by structural genes;
- the genes can be used as indicators of the synthetic pathway.

Two enzymes, ANR and LAR (see Figure 20) are important for the production of the flavan-3-ol monomers, (+)-catechin and (-)-epicatechin needed for the formation of condensed tannins (Bogs et al., 2005). Genes encoding these enzymes have been isolated from Shiraz and fully characterised (Bogs et al., 2005). The respective contribution of these enzymes to condensed tannin synthesis remains to be clarified. It is however a critical issue.

Knowledge of the genes involved in the flavonoid pathway is the critical first step in developing a detailed mechanism for the synthesis of condensed tannins. The second step is knowledge of the regulation of the pathway as this will provide a mechanistic basis to manipulate the steps involved in tannin synthesis. This requires identification of the transcription factors that regulate the structural genes of the pathway shown in Figure 20.

Work at CSIRO Plant Industry and at INRA in Montpellier, France, has identified many of the MYB transcription factors responsible for the regulation of the flavonoids pathway. This work is summarised in Figure 21.

Figure 21. Schematic representation of the flavonoid pathway showing the parts of the pathway controlled by MYB regulator genes. References to published papers are included below the MYB acronyms. Figure courtesy of Simon Robinson and Mandy Walker, CSIRO Plant Industry.



Significant progress has been made in identifying regulators of condensed tannin synthesis (Bogs et al., 2007; Terrier et al., 2009). These regulators, symbolised by VvMYBPA1 and VvMYBPA2, both appear to have the capacity to activate the condensed tannin specific genes that encode LAR and ANR (see Figure 21). VvMYBPA1 would appear to have a wider role in regulating some of the main pathway genes as well (DeLuc et al., 2006; DeLuc et al., 2008). Induction of changes in condensed tannin profiles were observed following ectopic

expression of either VvMYBPA1 orVvMYBPA2 in grapevine hairy roots (Terrier et al., 2009).

Terrier et al. (2009) have examined what they term the 'spatiotemporal' expression of VvMYBPA1 and VvMYBPA2 and linked their observations to those of Bogs et al. (2007). Terrier et al. (2009) report:

- expression of VvMYBPA2 is restricted to skin and leaves;
- the expression pattern of VvMYBPA2 suggests a preferential role in condensed tannin synthesis in grape skins;
- VvMYBPA1 expression is expressed pre-véraison and correlates with condensed tannin synthesis in seeds (Bogs et al., 2007);
- both VvMYBPA1 and VvMYBPA2 activate ANR and LAR1, but not LAR2 – these observations about LAR are in agreement with the earlier report of Bogs et al., (2007);
- overexpression of VvMYBPA2 results in the accumulation of VvMYBPA1 transcripts. This observation was interpreted as suggesting that the VvMYBPA2 signal acts upstream to VvMYBPA1. However, overexpression studies can be misleading when assigning functionality to a gene.

The studies by the research teams at CSIRO Plant Industry and INRA Montpellier, (France) have made significant progress in our understanding of the flavonoids pathway and the genes that regulate the process.

The major question that remains is how the flavan-3-ol monomers are converted into polymers.

Tannin polymer synthesis

The pathway for the production of anthocyanins is now reasonably well established, but the detailed steps involved in condensed tannin synthesis are less well known. From the studies on the flavonoid pathway, it is known that (Figure 20):

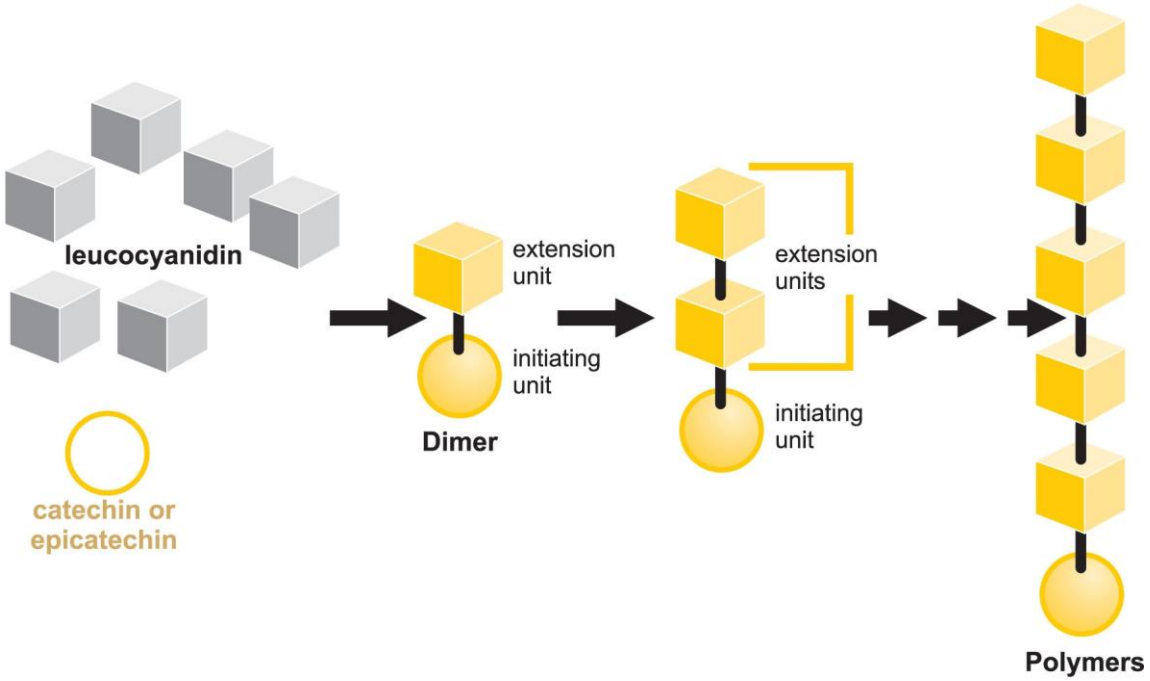
- (+)-catechin is derived from leucocyanidin via LAR and
- (-)-epicatechin is derived from cyanidin via ANR

A schematic representation for the production of condensed tannins is shown in Figure 22. Leucocyanidin is presently thought to be the source of the extension units as well as being involved, via LAR, in the production of the catechin initiating unit.

Our present state of knowledge only allows determination of terminal units and not the initiating unit *per se*. Terminal subunits in skins, for example, tend to be mostly catechin, with measurable amounts of epicatechin gallate and traces of epicatechin. Extension subunits have a high proportion of epicatechin followed by epigallocatechin, epicatechin gallate and traces of catechin. There is also a site influence on the extension subunit composition (see Downey, GWRDC Tannin workshop, July 2008). More data on the distribution of terminal and extension subunits is presented in Section 3.

The major challenge that remains is an understanding of the mechanistic process that controls the production of the condensed tannin polymers. The focus of research has been on the biochemistry of anthocyanin synthesis because of the industry's request to understand colour. Fortunately, one outcome of the studies on anthocyanin production has been information on

Figure 22. Schematic representation of the formation of tannin polymers. *Image extracted from CRCV Report 3.3.1.*



the molecular processes that lead to the generation of flavan-3-ol monomers (catechin and epicatechin), the building blocks for condensed tannins. The source of the galloylated flavan-3-ols remains an open question.

A detailed understanding the mechanistic process represented schematically in Figure 22 is vital to progress in our knowledge of tannin chemistry and biochemistry. As noted above, we now have a good understanding of anthocyanin production. It is essential that we achieve at least the same level of understanding with respect to condensed tannin production so that operations for regulating or manipulating production can be developed.

The difficulty is that the polymerisation process has so far proved resistant to the scientific process. This is not just the case for grapevines but generally for plants that contain condensed tannins. This scientific challenge is one that must be addressed.

Related questions to the mechanism of condensed tannin production include:

- what are the limiting factors in determining the size of the condensed tannin polymer? In some cases, there might be 4 or 6 subunits maximum, in others 80 subunits. Is there a chemical or biochemical basis for this?
- when and how are galloylated subunits formed?
- do the same processes operate in skins and seeds?
- in which cell components do the accumulation processes occur?

There are climate and viticultural practices that influence condensed tannin production. These are discussed in Section 4.

Tannin maturation

Following véraison, there is an apparent decline in condensed tannin concentration. While this is in part due the increasing volume of the berry, with consequent dilution of tannins

synthesised pre-véraison, there is also a marked decline in the total amount of tannin that can be extracted chemically from skins and seeds. The period of berry development from véraison to harvest is sometimes referred to as 'tannin maturation' (Figure 19).

There is now increasing evidence to suggest that this maturation period may be a reflection of interactions between the cell wall and condensed tannins, rather than degradation of the tannins themselves.

Sections 2 and 3 discuss these points in more detail. It is sufficient to note here that knowledge of the mechanism of condensed tannin synthesis and the cell components in which tannin accumulation occurs will add considerable value to explaining the processes that occur during the so-called maturation phase.

Pigmented polymers

On some occasions, extraction of condensed tannins from grape skins or seeds gives rise to a group of compounds termed 'pigmented polymers'. These are tannin polymers containing, it is assumed, one or more anthocyanin molecules to generate the colour.

Somers (1971) first proposed the existence of pigmented polymers from dialysis experiments and the argument about the composition and structure of these pigments still continues. This debate is summarised in Section 6 and is not discussed further here.

The occurrence of pigmented polymers in tannin extracts from grapes may be the result of reactions that take place after the cell wall has degraded. Alternatively, it may be an artefact of the physical and mechanical processes involved in the extraction process itself. Figure 1 shows the high mobility of anthocyanins from the skin of a cut grape even when the grape is frozen. This mobility suggests that condensed tannins and anthocyanins may come into contact during processing for extraction and not prior to it.

The question therefore remains unresolved. If the actual formation of tannin-anthocyanin polymers in the berry can be demonstrated, it may provide insight into the mechanism of pigmented polymer formation.

Research methodologies

Molecular approaches to altering condensed tannin synthesis can be achieved by reducing the activity of a critical enzyme, for example ANR or LAR. Reducing or silencing the enzyme activity can be achieved by:

- a stable transformation or
- hairy root transformation

Grapevine hairy roots are the outcome of transformation of grapevine organs (eg: petioles) with Agrobacterium rhizogenes. These hairy root systems are in use in confidential work at CSIRO Plant Industry and were also used in the recently published INRA (Montpellier) study (Terrier et al., 2009). The advantage of this methodology is that it is relatively fast, requiring about 6 months compared with 4 or more years for transgenic plants. The disadvantages are that the amount of metabolite material produced is small, making subsequent tannin subunit analysis a major analytical challenge, and root growth is not always uniform. Irrespective, silencing experiments are providing information on the role played by ANR and LAR on the formation of extension subunits. Similarity of the results of silencing experiments in hairy

root systems and transgenic vines provides confirmation of the methodology and gives confidence to the data interpretation.

Grapevine transformation is a complex process, but often essential for studies on specific gene activity when other systems cannot be used as 'models'. For example, Arabidopsis, so successful in many gene activity studies, is not useful for grape tannin work as tannin synthesis is confined to a single cell layer. The methodology for grapevine transformation was originally described by Franks et al. (1998) and Torregrosa et al. (2002). CSIRO Plant Industry has recently made several improvements to the transformation methods that have provided a major enhancement to the production of transgenic vines.

Once the transformations are stable, the vines with altered gene expression will provide sufficient fruit for analysis of tannin composition. In addition, microfermentation experiments can be carried out to produce wines for analysis. The benefits of this work extend past tannin production: information on anthocyanin production and flavonoid synthesis generally can be obtained. For example, the observation of a light inducible MYB transcription factor controlling flavonol synthesis (Czemmel et al., 2009) could potentially be extended to explaining light exposure effects in field trials (Ristic et al., 2007).

Summary

Tannin subunits, (+)-catechin and (-)-epicatechin, are synthesised by two enzymes, ANR and LAR. This is now well established, but the issue of how the subunits are converted into condensed tannins is not understood. This lack of knowledge applies to plant material in general.

The questions that remain to be answered include:

- how are tannin polymers formed?
- what mechanisms regulate their formation?
- when and how are the galloylated subunits formed?
- are the formation mechanisms enzymatically driven or chemical driven?
- is the apparent decline in tannin extractability from véraison to harvest (the so-called 'tannin maturation') simply a consequence of tannin binding within the cells (the cell wall – see Section 3) or are other degradation or oxidation mechanism in operation?

Knowledge of the mechanisms that determine condensed tannin production and composition will open up the possibility of improving the link between tannin production in grapes and the impact of tannins on wine sensory properties.

The molecular and transgenic techniques developed by CSIRO Plant Industry are vital to the success of research in this field. The recent identification of the MYB transcription factors that control the expression of LAR and ANR by CSIRO Plant Industry is an important outcome for greater understanding of the synthesis and composition of condensed tannins. There is also significant competition in this research area from other groups outside Australia. Continuing work in this field is essential.

SECTION 2:
Analytical methods for assessing tannin content and composition in grapes and wine

This section reviews the literature relevant to the determination of condensed tannins in grape extracts as well as the assessment of the total tannin content in wine. The focus is more on the methodology that could be applied in commercial winery laboratories, rather than research laboratories. The determination of structural complexity and composition of individual tannins is described in Section 5.

A wide variety of analytical procedures have been used in what is essentially a four-step process for grape analysis:

- Preparation of the grape material
- Extraction of the tannin material
- Measurement of the amount of tannin extracted
- Relationship between the tannin measured by the assay and wine properties, especially sensory descriptors.

Only the final two steps are relevant to assays applied to wine.

The field has been reviewed by De Beer et al. (2004), Herderich and Smith (2005), Cheynier (2006) and Seddon and Downey (2008). The methodologies are diverse and there are only a limited and restricted number of comparative studies. As a consequence, the results tend to be 'operationally defined'; that is the value for the tannin concentration is dependent on the analytical procedure itself. This makes interpretation of the literature complicated and minimises progress towards understanding condensed tannin content in grapes and grape parts.

Preparation of grape material

Cheynier (2006) refers to the extraction of flavonoids from fresh grapes, frozen grapes or freeze-dried material. The purpose of freezing or freeze-drying the material is to allow the material to be stored for subsequent analysis. There does not appear to be any study that compares the stability of condensed tannins during protracted storage after extraction.

Homogenisation of the grapes is common practice prior to extraction of tannin material. Methods include blending and grinding or milling the grape material. The method of homogenisation can influence the results, especially with the break-up of seeds (Herderich and Smith, 2005). A standard homogenisation procedure is required before any meaningful cross-research group data can be interpreted.

Extraction of tannin material

A wide variety of solvents has been used for extracting condensed tannins. In some cases, attempts have been made to use the same solvent for the simultaneous extraction of several classes of phenolic compounds, including flavonols, anthocyanins and condensed tannins. The application of acidified solvents (for example 1% hydrochloric acid in methanol) has its limitations as, while the extent of extraction may be greater, partial hydrolysis of some phenolic compounds may occur.

The following lists the majority of solvent mixtures that have been examined:

- Aqueous acetone (commonly 60 to 70 % acetone by volume) for grape homogenates (Cheynier, 2006), seeds (Prieur etal., 1994), skins (Souquet et al., 1996) and stems (Souquet et al., 2000);
- Ethyl acetate:water in the ratio of 90:10 (Pekić et al., 1998);
- Methanol:water (60:40) (Sun et al., 1996);
- Ethanol:water (50:50) (Sarneckis et al., 2006).

The logic behind the choice of solvent for a particular study is not always apparent and in many cases, it seems to be based more on ease of transfer of extract solution to subsequent steps in the analysis rather than solvent efficiency.

There have been only limited studies comparing solvent efficiency and the results of each study may be confounded by the effectiveness of the measurement step (see below). Generally, 70% aqueous acetone is regarded as a more effective condensed tannin extraction solvent (Kallithraka et al, 1995). Work published by Harbertson and Downey (2009) and unpublished work by Downey and Hanlin (submitted) tends to confirm the higher extraction efficiency of 70% acetone.

The question that arises from this survey of extraction solvents is the relevance of each to a fermenting system. If the unpublished data of Downey and Hanlin can be confirmed, 50% ethanol extracts at least twice as much condensed tannin material from grape skins compared to 10% ethanol. That is, 70% acetone and 50% ethanol may provide insight into the total potential extractable condensed tannins in grape homogenates and grape skins, but the concentrations will generally overestimate the amount that can be extracted in a fermentation.

The other variables in the extraction process are the ratio of solid material to the volume of the extracting solvent and the contact time between the solvent and the grape solid. These variables are generally optimised in published procedures, but must be re-examined and optimised for new types of analyses. For example, if an extraction process is optimised for grape homogenates, it needs to be re-optimised when applied to grape seeds or grape skins. This is slow and tedious, but vital, work to ensure integrity of the extraction process.

Measurement of the amount of tannin extracted

There are a wide range of measurement techniques applied to the determination of condensed tannins in grape homogenates, grape skins and seeds and as well as wines. The measurement capacity ranges from the simple UV spectral measurement at a single wavelength to high cost sophisticated instrumentation such as nuclear magnetic resonance (nmr), LC-mass spectrometry (LC-MS) and LC-MS-MS (tandem mass spectrometers).

The status of the measurement tools available has been reviewed by Herderich and Smith (2005). This review examines some advances that have taken place since the 2005 review. The focus in this section is on methods that could be readily applied in wineries that have a small laboratory. The application of more sophisticated instrumentation is discussed in Section 5.

The following comments assume that the tools to be discussed are being applied to extracts of grapes. A discussion of the measurement of tannin concentration in wine will be presented later in this section.

Some of the more commonly available measurement tools are:

- Single wavelength (280 nm) measurement – the so-called Somers method
- Multiple wavelength measurement – a new, as yet unpublished, method that is going through industry trials
- Colorimetric methods that utilise a colour development reaction
- Tannin precipitation by proteins
- Tannin precipitation by non-protein material

An intriguing extension of the simple single wavelength approach is under study at the AWRI[1]. By selecting four wavelengths, rather than just one as in the Somers approach, a correlation between the measured A_{280} tannin and the predicted A_{280} tannin was found to be remarkably strong ($r^2 = 0.92$). The proposed method is being offered to industry via the web: all that will be required is to enter the four absorbance values and the web program will calculate the tannin concentration. The simplicity of this method and its validation to date suggests that it has the potential to become an effective tool for wineries.

Colorimetric methods abound in the literature. The Folin-Ciocalteu method is perhaps one of the better known and widely used until recently. The colorimetric methods are generally time consuming, use chemicals that require special handling conditions and, importantly, lack specificity. Herderich and Smith (2005) have reviewed the general literature and there has not been any significant advance since then. Simply put, there are several wet chemical methods now available that provide more accurate and precise information than can be obtained through colorimetric methods.

Precipitation methods also abound in the literature and the Herderich and Smith review (2005) is again a major source of information. Of the wide range of methods published, only two will be addressed here:

- the protein-based precipitation method that uses bovine serum albumin (BSA), re-suspension of the precipitate and a subsequent colour development reaction using iron(III) chloride; this is sometimes referred to as the Adams-Harbertson assay (see Harbertson et al., 2003)
- precipitation using the polysaccharide, methyl cellulose; this MCP method was developed at the AWRI (Sarneckis et al., 2006) and revised by Mercurio et al. (2007).

Both methods are widely used for rapid condensed tannin assays and both have their supporters and detractors. There are only a small number of published works that compare the two methods, but even then, there are differences in methodologies that make comparisons difficult.

A detailed description of the two methodologies is not given here. Rather, the focus is on the comparison of the methods in terms of robustness and potential suitability for routine winery analysis.

Comparison of the Adams-Harbertson and MCP methods
Mercurio and Smith (2008) have published a detailed comparison between the MCP and Adams-Harbertson assays for grape homogenates and red wine. Comparative studies for condensed tannins in grape skins have been reported by Seddon and Downey (2008) and

[1] From information provided during discussions at the AWRI as part of this review. See also Mercurio et al. (2007.

Harbertson and Downey (2009). De Beer et al. (2004) have also presented a comparison of several methods, but the comparison applies only to wine and is discussed in the next section.

Mercurio and Smith (2008) used 50% aqueous ethanol for extracting tannins from grape homogenates and applied the MCP and Adams-Harbertson assay to the same extract. They present data to justify the following points:

- the MCP analysis is faster, provided a 96-well plate reader is available: 48 duplicate samples can be analysed in 45 minutes (< 1 minute per sample) compared to 10 to 15 samples in 90 minutes by the Adams-Harbertson assay.
- the correlation between the two methods when applied to grape homogenates is strong with an r^2 value of 0.9636. However, the gradient of the linear regression between the two data sets is 0.358, indicating that the difference between the values obtained by the two methods differ by almost a factor of three.

In searching for an explanation for the differences between the two methods, the study by Mercurio and Smith (2008) led them to conclude that the difference is related to methods of detection used by the MCP and Adams-Harbertson assays. They also concluded that tannin quantification will be dependent on the method employed and that more work is needed to elucidate the basis for the difference between the two methods. The lack of condensed tannin standards minimises the opportunity for a full understanding of the methodological variation.

Seddon and Downey (2008) have also compared the application of the MCP and Adams-Harbertson methods to the determination of the condensed tannin concentration in grape skins. The correlation between the values obtained for the two methods was $r^2 = 0.4107$, considerably lower than that found by Mercurio and Smith (2008). There are however significant differences between the two studies which confound any comparison:

- Seddon and Downey (2008) used grapes skins while Mercurio and Smith (2008) used grape homogenate
- Seddon and Downey (2008) used 70% acetone as the extracting solvent for the Adams-Harbertson assay and 50% ethanol for the MCP assay; that is, they followed the reported protocols for each assay. Mercurio and Smith (2008), on the other hand, used 50% ethanol as the extracting solvent for both assays.

In essence, any comparison between the studies is severely limited by the methodological differences.

Harbertson and Downey (2009) attempted to address these methodological differences by using both 70% acetone and 50% ethanol. The extractions were applied to grape skins and not grape homogenate. As expected (see above), 70% acetone gave higher concentrations by both methods. The MCP assay generated lower values than the Adams-Harbertson assay, the opposite result to that found by Mercurio and Smith (2008). As mentioned above, solvent differences between the studies may be important. The possibility of the tannin distribution being different in grape skins to grape homogenate, leading to different extractabilities, and thereby confounding the results has not been explored.

While Mercurio and Smith (2008) reported acceptable precision (measured as repeatability) for the Adams-Harbertson assay as well as the MCP assay, Brooks et al. (2008) have published a damning report of the Adams-Harbertson method's precision: the paper's title is

'Adams-Harbertson protein-based precipitation wine tannin method found invalid'[2]. Both Mercurio and Smith (2008) and Brooks et al. (2008) examined the precision for wine analysis and not grape analysis – imprecision is likely to be higher for grape analysis, given the number of additional steps in the sample work-up.

Recently, Jensen et al. (2008) have argued that the ratio of tannin (in wine in their study) to added protein as the precipitating agent can influence the result. Through a dilution study, they found that there is a critical 'tannin to added protein' operating range where valid results can be obtained. This study clearly points to the need to ensure optimisation of all operating parameters in method development and application and a summary addressing proposed future work is presented at the end of this section.

Wine analysis

Condensed tannins in red wine are frequently determined using HPLC, as the main emphasis has been attempts to determine tannin structure, rather than quantification of the total amount of condensed tannin material present in wine. De Beer et al. (2004) have compared a range of methods for phenolic compounds in red (and white) wine, although the emphasis was not on condensed tannins *per se*. HPLC is more of a research tool and further discussion of its application is left over until Section 5.

Several wet chemical procedures for total tannin determination are also available. These methods are similar to those described above for grape analysis and include:

- absorbance at 280 nm
- Folin-Ciocalteu method
- precipitation with proteins including the Adams-Harbertson method and methods using proteins other than BSA
- MCP assay

The Adams-Harbertson and MCP assays have received more attention in the last 5 years and only these two will be considered further. Determination of condensed tannin in wine is likely to be more reliable by these methods, as considerably fewer steps are involved in the analytical process. Mercurio and Smith (2008) found that the coefficients of variation (%CV) for triplicate analyses of six wines were less than 7% for both the MCP and Adams-Harbertson assays. However, as noted above, Brooks et al. (2008) have presented data that challenges the precision of the Adams-Harbertson assay. In their study, four wineries and one commercial laboratory applied the Adams-Harbertson assay to several wine samples. %CV values up to 27% were found. Method validity, as determined by comparison values with a HPLC procedure, could only be assumed to be 50% (Brooks et al., 2008). That is, Brooks et al. (2008) argue that significant underestimation of the tannin content of wine would occur if wineries only used the Adams-Harbertson assay for monitoring the winemaking process.

It is difficult to reconcile the differences for reliability obtained by Mercurio and Smith (2008) with that of Brook et al. (2008) for the Adams-Harbertson assay. It may simply be the case that assays of this type work well for those who have extensive experience with the manipulations involved. Perhaps it may lack robustness for general use or there is a need for more effective analyst training.

[2] The publication by Brooks et al. (2008) stimulated considerable debate regarding the approach taken. See, for example, the letters by E. Boselli and J Kennedy in J AOAC International, (2009), **92**, 37A.

The MCP method is widely used in some teaching programs and so by default there is 'analyst training'. However, it appears that there has not been a 'round-robin' comparative study of the MCP assay, similar to that described by Brooks et al. (2008) for the Adams-Harbertson assay; that is an interwinery comparative study. The industry would benefit from the wider use of the MCP method as a routine analytical tool.

As always in these assay type analyses for condensed tannin, the accuracy of the method cannot be formally assessed. Accuracy is defined as the difference between the measured result and the true value for the sample under examination. There are no certified standard wines with a known condensed tannin concentration available and there are no condensed tannin standards of known composition available. This limitation in our knowledge is discussed in detail in Section 5. The present approach is to use another method, assumed to be more accurate, as indicating the 'true value' of condensed tannin in wine. Generally HPLC is used, simply as there is no alternative. More efforts are needed to generate independently validated data for the MCP and Adams-Harbertson assays.

Relationship between the tannin measured by the assay and wine properties

It is well established that sensory properties of a red wine can be varied by modulating the condensed tannin concentration. This Section examines only the relationship between various condensed tannin assays and the relationship with astringency and related sensory descriptors.

Several investigators have used protein based assays, on the basis that astringency is linked to tannin-protein interactions in the mouth. For example, Monteleone et al. (2004) have developed a successful predictive model for astringency based on the turbidity induced through polyphenol-mucin precipitation. This work was further developed in a subsequent report (Condelli et al., 2006). Correlations (r^2) between the astringency of grape seed extract and induced turbidity values were as high as 0.98.

Kennedy et al. (2006) used several assay and instrumental measures of condensed tannin in their quest to find meaningful correlations between the measured value and sensory assessment of astringency. Methods used include absorbance at 280 nm, the Adams-Harbertson assay and HPLC. A strong correlation between sensory assessment of astringency and the values from the Adams-Harbertson assay was found ($r^2 = 0.82$). Forty wines (Cabernet Sauvignon, Merlot and Syrah; two vintages) were used. The strength of the correlation indicates the possibility of a relatively simple approach to sensory astringency prediction. The potential limitations of the Adams-Harbertson assay described above must be borne in mind here.

Mercurio and Smith (2008) have applied both the Adams-Harbertson and MCP assays to 20 wines (10 Cabernet Sauvignon and 10 Shiraz; two vintages). Correlations (r^2) between the sensory parameter of drying and the tannin assay parameters were 0.90 for the Adams-Harbertson assay and 0.83 for the MCP assay. Similar correlations were obtained for other sensory descriptors such as surface texture and adhesiveness. These data again suggest potential for a rapid assessment of astringency related sensory descriptors.

Further comparative studies across several vintages, varieties and regions may well confirm the effectiveness of either or both condensed tannin assays as predictors of a wine's astringency. This may well be more important than using the assay to obtain an accurate value for the tannin concentration itself. Surprisingly, it appears that predictor modelling for

either Adams-Harbertson or MCP assays with astringency (similar to that described by Monteleone et al. (2004) and Condelli et al. (2006)) has not been investigated.

There has not been any work on comparing the assay values (MCP or Adams-Harbertson) for condensed tannin in grape homogenates or grape skins with sensory assessment of astringency of the finished wine. The variations of winemaking approaches that determine how much tannin is or can be extracted make such a study of limited or no value. On the other hand, measurement of condensed tannins in grapes may provide an indicator of the maximum amount of tannin that could be extracted. Cell wall considerations and tannin compartmentalisation will also be important here (see Section 3).

Summary
Significant advances have been made in the development of rapid assays for the assessment of the total tannin concentration. The MCP and Adams-Harbertson methods have received the most attention. Further refinement and comparison of the methods would be beneficial in confirming the wide applicability of one or other of these two methods.

The work that is proposed to complete the refinement and validation includes:

- Impact of prolonged storage on integrity of condensed tannin material – does the tannin extracted from grape skins and grape seeds degrade in storage?
- Extracting solvents focusing on 70% acetone and 50% ethanol
    - why is acetone more effective?
    - comparative extraction of both solvents to grape homogenates as well as grape skins and grape seeds
    - is the application of either solvent meaningful in terms of fermentation extraction?
- Precision validation of the Adams-Harbertson assay – why is it so poor when used in winery laboratories compared with research laboratories?
- MCP assay precision validation – round-robin comparative analysis with winery laboratories

- Confirming links between assay values and astringency descriptors
    - Predictor modelling – can MCP and A-H actually predict astringency?
    - Comparative studies across several vintages, varieties and regions – do correlations hold?

Fragoso et al. (2010) have recently published a comparison of three assay methods for the evaluation of phenolic ripening in several varieties of red grapes. The MCP procedure was one of the methods chosen. This is an important step forward in the international acceptance of the MCP method. The work by Fragoso et al. (2010) also investigated a predictor model between the methods they employed.

SECTION 3
Assessing tannin content in grape berry skins and seeds: composition and cellular location

Condensed tannins exist in grape skins (Souquet et al., 1996), grape seeds (Prieur et al., 1994) and grape stems (Souquet et al., 2000, Jordão et al, 2001). This section of the review will address only grape skins and seeds, as these are the more relevant berry components in the Australian winemaking situation.

In Section 2, the application of rapid assay techniques to total condensed tannin determination in grape skins (as well as berry homogenates) was described. The focus of this section is more specific: it addresses the composition of the condensed tannins found in the skins and seeds in terms of the subunit composition following extraction of the tannins and cleavage into their component compounds.

The general steps in the process are:

- Separation of the seeds or skins from the whole berries
- Extraction of tannins from the skins or seeds
- Chemical cleavage of the tannins into subunits
- Determination of the mean degree of polymerisation
- Identification by HPLC of the subunits (terminal and extension units)

Table 1 summarises the results from a range of studies that include variety, region/country, extraction method, stages of berry development and water stress. The papers have been selected to represent the type of experiments that have been reported. HPLC methodologies are reviewed in Section 5 and the acid cleavage methods of thiolysis and phloroglucinolysis are also discussed in Section 5. Determination of mDP is related to the analytical method used, as described in Section 5.

The most efficient extraction solvent for condensed tannin compositional studies is aqueous acetone, about 60 to 70%. This is in agreement with the results of the solvent comparison study of Sun et al. (1996). Even though aqueous acetone is effective in removing tannins from skins and seeds, the report by Downey et al. (2003) clearly demonstrates that there is residual condensed tannin material after the extraction process. This observation is discussed in more detail below.

Mattivi et al. (2009) used a totally different extraction approach for condensed tannin composition studies. A 'wine-like solution' consisting of 12% aqueous ethanol, SO_2 and tartaric acid at pH 3.2 was used. Whole skins or seeds were soaked in this extracting solvent for 5 days at 30^{o}C, presumably to replicate fermentation conditions. Only shorter chain tannins (oligomers) were extracted, as the mDP was generally < 8. Flavan-3-ol monomers were also abundant in the extract solutions. It is not possible to draw any meaningful conclusions from the results of the study of Mattivi et al. (2009) in terms of their relevance to fermentation condition (especially the changing sugar concentration and variable temperature), but it is clearly an area worthy of further study. The other important point that comes out of the results of Mattivi et al. (2009) is the large effect of variety on mDP and subunit composition for both grape skins and seeds. This adds an extra layer of complexity in any attempt to determine a generalised model for the extraction behaviour of condensed tannins from berry parts.

Table 1. Tannin composition in grape skins and grape skins

Berry part	Variety	Region	Extraction	Chemical cleavage	Analysis methods	Results	Other study areas	Reference
Seeds	Not stated	Portugal	Solvent comparison		Fractionation of extract Vanillin colorimetric assay for catechins and oligomers HPLC for polymers	In terms of amount extracted: 80% methanol and 75% acetone most efficient for catechins and oligomers Methanol (50%, 80%) and acetone (75%) better for polymers		Sun et al., 1996
Seeds	Not stated	France	60% aqueous acetone	Thiolysis	Fractionation of extract HPLC for subunit determination	Five fractions isolated, all containing C, EC and ECG Extension subunits: EC major Terminal subunits: C more common Galloyated units: increased with mDP mDP: 2.3 – 15.1 by thiolysis and 2.4 – 16.7 by GPC		Prieur et al., 1994
Seeds	Cabernet Sauvignon	Napa Valley USA	66% aqueous acetone	Thiolysis	Intact tannins by NP-HPLC Subunits after thiolysis by RP-HPLC	Extractive yields decreased with maturity Incompatibility between mDP when measured for intact tannins versus thiolysis mDP at véraison: 8.3 by thiolysis mDP at maturity: 5.63 by thiolysis Results suggest oxidative process generating cross-linking to polyphenols, carbohydrates or proteins	Maturity study Vine water status	Kennedy et al., 2000a
Seeds	Shiraz	Barossa Valley, Australia	2:1 acetone : water	Phloroglucinolysis	Intact tannins by NP-HPLC Subunits after phloroglucinolysis by RP-HPLC	Amount of tannin declined from véraison to maturity and composition changed mDP: decreased from véraison (9.2) to maturity (5.7) Extension subunits: C, EC, ECG with constant ratio C:EC:ECG of 8:75:17 Terminal subunits: C, EC, ECG with relative amounts changing during ripening Epr study showed presence of free radicals that supports existence of oxidative event during ripening	Maturity study Epr study	Kennedy et al., 2000b
Skins	Merlot	France	60% aqueous acetone	Thiolysis	Fractionation of extract by NP-HPLC Subunit analysis after thiolysis by RP-HPLC	Six fractions obtained C, EC, ECG, EGC, CG and EGCG found Extension subunits: 60% EC Terminal subunits: 67% C mDP ranged from 3 (fraction I) to 80 (fraction VI). All fractions contained prodelfinidins		Souquet et al., 1996
Skins	Shiraz	Adelaide, Australia	2:1 acetone : water	Phloroglucinolysis	Subunit analysis by RP-HPLC ^{13}C nmr, ESI-MS and elemental analysis	Berry development correlated with mDP Increase during ripening of amount of anthocyanin associated with tannin fraction Subunit analysis found EGC, EC, C, ECG Extension subunits: increase in proportion of EGC during ripening Terminal subunits: C (EC and ECG absent or insignificant) MS data suggests binding of tannins to pectins; elemental analysis indicates protein not involved.	Maturity study	Kennedy et al, 2001

Seeds and skins	Shiraz	Willunga, Australia	70% aqueous acetone	Phloroglucinolysis	Subunit analysis by RP-HPLC Residue from acetone extraction subjected to phloroglucinolysis	Weekly sampling from fruit set until commercial harvest Skin tannins accumulate until 1 – 2 weeks after véraison Subunit composition different between seeds and skins and composition changed during berry development Seed terminal units: C+EG+E in changing proportions Seed extension subunits: EC (65%) and relatively consistent Skin terminal units: mostly C Skin extension subunits: EC+C+ECG+EGC in changing proportions mDP: seed tannins shorter than skin tannins (5 compared to 40) Subunit analysis of residue remaining after acetone extraction showed that, for seeds, the additional (unextractable) subunits could account for most of post-véraison loss, but not so for skins. Most (75%) of extractable tannins were in seeds at harvest Accumulation mechanism for seeds and skins appear to be different	Maturity study	Downey et al., 2003
Seeds and skins	Cabernet Sauvignon, Carmenere, Mazemino, Merlot Pinot Noir, Syrah Teroldego	Trentino, Italy	'wine-like' solution at 12% ethanol	Thiolysis	HPLC with DAD and MS detection	Significant varietal influence on free monomers and tannin extraction. Shorter, oligomeric, tannins extracted Seed mDP = 2.0 – 6.5; Skin mDP – 2.1 – 10.8 Seeds contained procyanidin; Skins contained procyanidins and prodelphinidins Varietal dependence of the distribution of both extension and terminal subunits	Varietal comparison Focus on extraction of flavanol monomers	Mattivi et al., 2009
Seeds and skins	Monastrell, Syrah, Monastrell X Syrah cross	Murcia, Spain	2:1 acetone : water	Phloroglucinolysis	Subunit analysis by RP-HPLC	Tannin composition of crosses similar qualitatively to parents, but some quantitative differences Potential for breeding to develop tannin profile suitable to produce high quality wines relevant to a region		Hernández-Jiménez et al., 2009

C: catechin; EC: epicatechin; ECG: epicatechin gallate; EGC: epigallocatechin; GC: gallocatechin; EGCG: epigallocatechin gallate

NP-HPLC: normal phase HPLC
RP-HPLC: reverse phase HPLC
DAD: diode array detector
MS: mass spectrometry

The debate between the relative efficiencies of ethanol (50%) versus acetone (60 to 70%) was discussed in Section 2. There does not appear to be any published work where grape seeds or skins have been extracted with 50% ethanol, followed by acid cleavage to determine subunit composition. This work is essential before a recommendation regarding the preferred extracting solvent can be made. This comparison between 50% ethanol and 60 – 70% acetone needs to be set against fermentation-type extracting solution as used by Mattivi et al. (2009).

Grape maturity studies (Table 1) generally suggest a decrease in the amount of extractable condensed tannin material as well as a reduction in the mDP. Whether this is due to an actual loss of tannin compounds through catabolic processes or whether the tannin compounds become bound and no longer extractable was raised as an issue in several studies listed in Table 1. For example, Downey et al. (2003) found that acid cleavage of the residue remaining after acetone extraction increased the subunit amount, accounting for the post-véraison loss for seeds, but not for skins.

Kennedy et al. (2000a), in a study on condensed tannins in seeds of Cabernet Sauvignon argued that the results suggested an oxidative process generating cross-linking to polyphenols, carbohydrates or proteins. An electron paramagnetic resonance (epr) experiment for free radical detection was applied to tannins extracted from Shiraz seeds (Kennedy et al., 2000b). The observation of free radicals was argued to support the occurrence of an oxidative process occurring during berry ripening. A mass spectrometric (MS) study on tannins extracted from Shiraz skins (Kennedy et al., 2001) suggested that binding of tannins to pectins was occurring during ripening. Elemental analysis of the tannin extract was low in nitrogen, discounting binding to proteins.

Cellular location of tannins

The general distribution of phenolic compounds in grape berries is well known from anatomical and histological studies (see Hardie et al., 1996 and references cited therein). In intact grape berry cells the phenolic compounds are mostly confined to vacuoles from where they are released upon degeneration or rupture of the vacuolar membrane (tonoplast). However there has been surprisingly little work until recently to discriminate condensed tannins from other classes of phenolic compounds in regard to cellular contents in grape berry skins and seeds. This is despite the importance of tannins in red winemaking and the known inability of the fermentation process to extract all condensed tannin from the grapes.

Cadot et al. (2006a) used a histochemical approach (combining specific staining and quantitative chemical analysis) to understanding the localisation of tannins and other components in seeds taken from Cabernet Franc berries grown in the Loire Valley. At five different stages from berry set to harvest, seeds were removed from berries, cut, fixed, thin-sectioned and stained for microscopy. The staining solutions used were for specific for lignin, polysaccharides, proteins and tannins. This approach allows the general spatialization of classes of compounds. However the fixation step is a well known source of membrane damage and sub-cellular disruption so care must be taken in interpretation at that level. The traditional chemical analysis approach involves crushing of the seeds (usually) and solvent extraction before acid cleavage and subunit analysis. This is likely to lead to extensive sub-cellular disruption.

The main observations of this study by Cadot et al. (2006a) are

- The seed coat comprises a tri-part integument (outer, middle and inner);
- Seed lignification took place in the middle integument and was achieved by véraison;

- Condensed tannins were localised in the epidermis and inner cells of the outer integument and the inner cell layer of the inner integument;
- Monomeric flavan-3-ol content was linked to changes in cell walls of the outer integument.

Monomeric flavon-3-ols, being concentrated in the outer integument, would be readily extracted during winemaking. Cadot et al. (2006a) also observed intensive lignifications of the middle integument resulting in increasing hardness and waterproofing. With the condensed tannins being located in the inner integument, the extraction of the compounds may well therefore be limited. This is an important consequence for wine making and also for chemical analysis studies involving solvent extraction. In essence, the hard and water proof middle integument will minimise access to the condensed tannin compounds in the inner integument.

Cadot et al. (2006a) suggested several mechanisms for the location of phenolic compounds in the inner integument, one of which relates to oxidative processes involving the phenolic compounds and subsequent cross-linking with carbohydrates and proteins. This is similar to that proposed by Kennedy et al. (2000b, 2001). No conclusive evidence was presented for any of the proposed mechanisms however.

Further evidence for the 'non-extractability of condensed tannins' was noted by Cadot et al. (2006a). Increasing intensity of the stain used to identify phenolic compounds during ripening is at odds with previous studies that showed a decrease during berry maturation (see Table 1). However, as found by Cadot et al. (2006a) and as noted above, solvent extraction access to condensed tannin material will be limited by the hard middle integument, unless this layer is first broken down prior to the extraction process.

In a complementary study, Cadot et al. (2006b) examined spatial distribution and chemical composition of flavonols, including condensed tannins, in berry skins of Cabernet Franc. The study is somewhat limited as it addresses changes only up to véraison and focuses on site effects. Histochemical analysis (qualitative) by staining with 4-dimethylaminocinnamaldehyde (DMACA) and quantitative analysis after extraction (60% aqueous acetone), thiolysis and RP-HPLC were compared. This comparative methodological study provided tentative evidence for the genesis of condensed tannin aggregation and the link between this aggregation and the average composition determined by HPLC. Extension of this methodology over the full period of grape skin maturation would be advantageous.

It is apparent from these two French studies that a greater understanding of the cell wall and condensed tannin association with cell wall components is vital to our understanding of condensed tannin localisation and extraction in grape berry skins and seeds.

Tannin Binding

Amrani-Joutei et al. (1994) proposed that condensed tannins bind to proteins of the internal surface of the tonoplast (vacuolar membrane) and also to cell wall polysaccharides, but the former is likely to be an artefact of the fixation process used to prepare the tissue for examination (see Hardie et al 1996). The extent to which the latter occurs in intact berries or is reliant on cellular degeneration or mechanical disruption (eg: associated with extraction for analysis or wine production) is not clear. This binding of tannins was claimed to occur during the maturation process (Amrani-Joutei et al., 1994). The observations of Kennedy et al. (2001) described above also support polysaccharide binding, but discount protein binding.

Cellular degeneration within the berry (Hardie et al., 1996) and plasmolysis and dehydration of the outer cell layers of the seeds (Cadot 2006a) occur from véraison and any associated leakage of tannins would allow binding to cell walls and other cellular contents. However, based on histological studies (Hardie et al., 1996; Diakou and Carde, 2001) and vital staining (Tilbrook and Tyerman, 2008, Krasnow et al., 2008, Clarke et al., submitted) degeneration of the most tannin rich cells in the skin does not appear to occur (if at all) until late in ripening, that is, about 30 days after véraison.

In summary, evidence for condensed tannin binding in intact berries is compromised by current methods of visualisation and extraction.

Tannin binding in mechanically disrupted grape tissues.
Gény et al. (2003) isolated cell walls from the seeds of Cabernet Sauvignon and determined the condensed tannin composition (subunit analysis) after cleavage by thiolysis. Modifying an established cell wall isolation procedure, Gény et al. (2003) obtained a cell wall fraction and an internal cell fraction. Extraction of the condensed tannins was achieved using a methanol/12 M hydrochloric acid (99.9:0.1) solution (compare Table 1). After thiolysis and RP-HPLC analysis, the subunit composition of the condensed tannins in both fractions was determined. The main results of the study were:

- The extraction process was very fast from the internal cell fraction, but took about 14 hours from the cell wall fraction;
- Condensed tannins extracted from the cell wall fraction had a higher mDP compared with those from the internal cell fraction (between 2 to 3 versus 4 to 5.5);
- The mDP increased in both fractions during ripening;
- The subunit composition was similar in both fractions consisting of (in order) epicatechin, catechin and epicatechin gallate;
- There was no attempt to determine terminal and extension subunit distribution;
- There was no significant effect on the condensed tannin distribution resulting from water stress.

A similar approach was adopted by Gagné et al (2006) in a study of the condensed tannin location in grape skins of Cabernet Sauvignon. The same extraction and fractionation process, as described above by Gény et al. (2003) for grape seeds was applied, although it was noted that the fractionation step did not prevent unspecific adsorption of the tannin material on cell walls. The main results were:

- The condensed tannin concentrations in both internal cell and cell wall fractions decreased from the end of véraison to maturity;
- There was a higher amount of condensed tannins in the internal cell fraction, although by maturity, the difference was much less than at véraison;
- mDP was higher for the cell wall fraction with maximum values around 8 – 10 compared with 1 – 5 for internal cell fraction;
- Subunit composition consisted of varying proportions of catechin, epicatechin, epigallocatechin and epigallocatechin gallate.

Interpretation of the data is confounded by the significant differences in growing conditions over the two vintages studies (Bordeaux, 2004 and 2005). However, it is clear from these two studies from the same research group in Bordeaux that the cell wall contains or retains a significant amount of condensed tannin after disruptive extraction.

Cell wall composition studies and release of condensed tannins

In what seems to be a largely ignored review entitled *Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release*, Pinelo et al. (2006) have presented an excellent overview of the state of knowledge related to cell wall location of condensed tannins as well as other phenolic compounds in grape skins. They identify two classes of phenolic compounds as:

- Cell wall phenolic compounds bound to polysaccharides by hydrophobic interactions and hydrogen bonds;
- Non cell wall phenolic compounds which include those confined in the vacuoles and those associated with the cell nucleus.

Pinelo et al. (2006) comment that

> *Degradation of cell wall polysaccharides is a fundamental step to improve the release of phenols from grape skins.... Cellulases, hemicellulases, pectinases...can be employed to decompose the cell wall structure.*

With respect to cell wall linked phenolic compounds, Pinelo et al. (2006) note that many studies have used model systems to understand the hydrogen bonding and hydrophobic interactions that may occur. However, they point out that there has been extensive work on condensed tannin interactions with apple cell wall material. Summarising work by Renard et al. (2001) and Le Bourvellec et al. (2004, 2005a,b), Pinelo et al. (2006) note that:

- The highest affinity between condensed tannins and cell wall polysaccharides is obtained with pectin;
- The amount of binding increases with increasing degree of polymerisation of the condensed tannin and with the proportion of (+)-catechin is important;
- Drying the cell wall decreases porosity which decreases the apparent affinity between cell wall polysaccharides and condensed tannins.

Similar conclusions to those of Pinelo et al. (2006) have been drawn by Hanlin et al. (2010). From their examination of the literature, Hanlin et al. (2010) report that

- There is good evidence for condensed tannin-cell wall interactions by the two processes of hydrogen bonding and hydrophobic interactions;
- Structural characteristics and composition of both the condensed tannins and cell wall material influence the interactions;
- The developing nature of the cell wall during maturation of the berry may influence its capacity to bind condensed tannins

Understanding the structure of the cell wall and the ways in which condensed tannins are bound, as well as the means by which the tannins can be released, is of fundamental importance for successful extraction of tannins that are within the grape. The capacity to extract larger amounts of condensed tannins from the grape may well obviate the need to add exogenous tannins as part of the winemaking process.

Enzymatic degradation studies

The use of enzymes to degrade pectins (polysaccharides) has been a wine making practice for many years. The focus on enzymes is now towards more targeted applications beyond the former uses of improving the pressing process and enhancing clarification. Multi-component pectolytic enzyme preparations are now under study as a means of improving extraction of phenolic compounds from grapes, as well as other fruits.

Bautista-Ortín et al. (2005) examined the effects of two different pectinase preparations on colour extraction from Monastrell grapes. They report that, while there were differences in colour between the control and enzyme-treated wines at the beginning of the winemaking process, these differences diminished as the wine developed. The impact of the enzyme treatments on levels of condensed tannins was not assessed.

Ortega-Regules et al. (2006) claim to have developed an anthocyanin 'extractability index' based on cell wall composition. After examining the cell wall composition for Cabernet Sauvignon, Merlot, Syrah and Monastrell, a model that accounted for 78% of the anthocyanin extraction variability was established. Again, the focus was on anthocyanins and not condensed tannins.

The same researchers (Ortega-Regules et al., 2008a, 2008b) extended the above study, evaluating the changes in skin cell wall composition during maturation of the same four varieties. Evolution of the protein content and total phenolic compound content was studied. No specific information on condensed tannins was presented. In what appears to be an overlapping study, the researchers (Ortega-Regules et al., 2008b) examined what they called 'technological implications' of the results of their previous work. In this technological study, the presence and activity of several naturally occurring enzymes was examined. Only pectinmethylesterase (PME) and α- and β-galactosidase were identified in the skin and pulp extracts of the four varieties. Based on the observations of their several studies, Ortega-Regules et al. (2008b) proposed that high anthocyanin extractability would be found with low concentrations of galactose, cellulose, rhamnose and xylose and a low degree of pectin methylation. This conclusion suggests a possible pathway for increasing condensed tannin extraction from the cell wall.

Romero-Cascales et al. (2008) characterised the main enzymatic activities in six commercially available macerating enzymes and linked these activities to the extraction of colour during winemaking with Monastrell grapes. The authors note that the main effect of the enzymes was an increase in the total phenolic content measured at 280 nm (Control: 680 mg L^{-1}; enzyme treated samples: 988 – 1110 mg L^{-1}). This increase they associated with the enzymes facilitating phenolic release. No examination of the cell structure with and without enzyme treated was carried out, however.

Arnous and Meyer (2010) examined the release of phenolic compounds in general during the enzymatic degradation of cell wall polysaccharides in Merlot and Cabernet Sauvignon skins. The enzymes showed pectinolytic and cellulolytic activity. The main results of this study are:

- Anthocyanin release occurred early during enzyme treatment but the anthocyanins were degraded during further enzyme treatment;
- Rutin (glycosylated flavonol) was converted to quercetin (deglycosylated) during enzyme treatment;
- There was an apparent link between the release of phenolic acids as a function of monosaccharide liberation;
- Some phenolic acids may be released from the degradation of lignin;
- There was some suggestion that p-coumaric acid may be released by enzyme degradation of acylated anthocyanins.

Arnous and Meyer (2010) claim that their data may provide a pathway for the use of multi-functional enzymes to promote:

- targeted release of phenolic compounds from grape skins;
- molecular changes in the structure of the phenolic compounds.

However, they did not address the release of condensed tannins as a specific target group.

Recently, Ducasse et al (2010) have reported the outcome of using macerating enzymes on the polyphenol and polysaccharide composition of Merlot wines over three vintages. This extensive and detailed study examined the effects of pectinase rich enzymes on the degradation of grape berry cell walls. The enzymes were added to the crushed grapes for 12 h of maceration at 12^{o}C prior to fermentation yeast inoculation. Wines were stored and analysed 20 months after the end of alcoholic fermentation. The authors examined the effect of enzyme treatment on

- wine polysaccharide treatment and composition;
- wine colour;
- polyphenol composition;
- vintage versus enzyme effect.

Importantly for this review, Ducasse et al. (2010) examined the quantitative and qualitative composition of the wines. The main observations were:

- the condensed tannin concentrations in the enzyme treated wines were higher than in the control for each vintage;
- the mDP for the enzyme treated wines was slightly higher than that for the control wines;
- the released condensed tannins were mainly skins tannins: the percentage of epigallocatechin subunits confirms the importance of skins;
- there was a slight increase in the proportion of epicatechin gallate subunits in two of the three vintages;
- inconsistent results were obtained for the effect of enzymes on the amount of anthocyanins and anthocyanin-tannin adducts.

Summary

It is apparent from the published work reviewed in this Section that we have only limited knowledge regarding the cell wall and the association of condensed tannins with cell wall components. Further research in this area is vital and needs to address:

- condensed tannin localisation and extraction in grape berry skins and seeds – the work of Cadot et al. (2006a,b) provides a framework for this research;
- improvement in methodology for visualisation and extraction of condensed tannins – current methods are compromising tannin binding in intact berries;
- development of methodologies for the enhanced release of condensed tannins from the cell wall – it is apparent that the cell wall contains or retains a significant amount of condensed tannin after disruptive extraction.

One recently published approach (Bindon et al., 2010) has examined the interactions between condensed tannins and cell wall material and this is supported by the work by Vicens et al. (2009) on polysaccharide and protein composition changes during ripening and overripening of Shiraz. The work of Arnous and Meyer (2010) and Ducasse et al. (2010) on enzymatic cell wall degradation has opened up the potential for the enhanced release of phenolic compounds, including condensed tannins, from grapes. The areas of research that require further study are:

- The relationship between enzyme type and activity (polygalacturonase, pectin methylesterase, cellulase, β-galactosidase) and the extent of cell wall degradation in skins and seeds;
- Microscopic and histochemical analysis of the changes in cell wall composition after enzyme treatment;
- Changes in the polysaccharide composition of cell walls as a function of enzyme activity;
- The influence of enzyme treatment of the amount and subunit composition of released condensed tannins;
- The influence of enzyme treatment on anthocyanin release and the formation of tannin-anthocyanin adducts, recognising that winemaking practices will also influence these parameters;
- The vintage, regional and varietal effects on each of the above points.

Changes in the polysaccharide composition need also to be considered in terms of its effect on the proposed colloidal structure, or molecular assembly, in wine. Alteration of the molecular assembly may influence mouth-feel properties, as is discussed in Section 11.

SECTION 4
Tannin content and composition in grapes, as affected by genetic and environmental factors

The relationship between environmental factors and condensed tannin content has been reviewed recently by Cohen and Kennedy (2010). Environmental factors that can be controlled such as viticultural practices and those over which there is no or less control (climate, biotic and abiotic stress) are described. This present scientific status report gives a summary of the critical environmental factors with an assessment of recommended further research. The reader of this report should consider the review by Cohen and Kennedy (2010)[3] in parallel with this science status report.

The biosynthesis of condensed tannins was discussed in Section 1. There has been some work on the relationship between environment and gene function. However, there does not appear to be a review of this field at this stage, perhaps confirming the limited research outputs.

Climate and temperature effects

One of the complexities associated with interpreting data from field studies is separating light exposure effects from variations in temperature. The use of controlled growth chambers, glass houses and films/shadecloth have all been tried with some success (see review by Cohen and Kennedy (2010) for details).

Cohen et al. (2008) have described a field experiment on Merlot in Washington, USA, in which vines had controlled exposure to sunlight and temperature manipulation was applied to individual clusters *in situ*. Six temperature regimes were applied: ambient, convective (blower) control, daytime heating, nighttime cooling, reduced as well as twice-reduced diurnal temperature range (damping and double damping, respectively) achieved by daytime cooling and nighttime heating. Some of the critical observations of this study with respect to condensed tannin development are:

- Seed condensed tannins (per berry) at véraison were higher in night-heated and damped fruit with respect to ambient fruit – on a per seed basis, the differences were not significant;
- The seed tannin sub-unit composition at véraison did not show a treatment effect;
- At harvest, there was little treatment variation in the amount of condensed tannins as well as in the tannin sub-unit composition;
- Skin condensed tannins at véraison were highest in the night-heated treatment
- Generally, the sub-unit composition of the condensed tannins was consistent, with some measurable increase in the proportion of EGC in the extension sub-units;
- Damping significantly reduced the mDP values in skin tannins at véraison, but there was no apparent trend between mDP values and treatments at harvest.

From the results of this study, Cohen et al. (2008) suggested that is a need to study gene expression as a function of these environmental effects. Related work has been proposed by some research groups in Australia, but an intensive study appears to have been put on hold.

Viticultural practices

The viticultural practices that are addressed here are vine vigour and its link to shading, irrigation and water stress, varietal influences, rootstocks and other management practices.

[3] The review by Cohen and Kennedy (2010) is accepted, but not yet published. Only the authors' submitted article was available at the time of writing this report.

Vigour

Jessica Cortell from Dr Jim Kennedy's former group in Oregon has published extensively on the influence of vigour on grape and wine phenolic composition. Pinot Noir was the variety used in the study. With regard to condensed tannins, Cortell et al. (2005) reported that:

- there was no significant effect of vigour on the amount of condensed tannins per seed and the sub-unit composition showed only minimal changes with response to vigour;
- skin condensed tannins showed a greater response to a reduction of vigour:
    - an increase in condensed tannins measured as mg/berry
    - the proportion of EGC increased and the proportion of EC decreased
    - mDP showed a variable response

Analysis of the wines made from grapes taken from the different vigour zones (Cortell et al., 2008) reported that there was an increase in extraction of skin condensed tannins from low vigour zones. While a response between vigour and condensed tannin composition, especially for skin tannins, was observed, the authors present a note of caution in interpreting the results: other environmental parameters such as light exposure, heat and temperature (see above) may also have had some influence on the values.

The same authors have also examined the influence of vigour on anthocyanin concentration and composition and pigmented polymers (Cortell et al., 2007a,b). Sensory evaluation of the wines (Cortell et al., 2008) was also performed.

Shading

While vigour and shading may be linked to some extent, several researchers have used boxes to eliminate or reduce the amount of light reaching grape bunches. The boxes are designed to maximise airflow to overcome the effects of temperature and humidity on berry development. Downey et al. (2004) have examined the influence of shading on Shiraz berry development and noted that

- there was no effect of shading on seed condensed tannin concentration or composition;
- skin condensed tannins were more influenced by shading with significant differences in the extension sub-unit composition. These differences were less pronounced as the fruit approached harvest.

The observed decrease in condensed tannin concentration as berries ripen is usually interpreted as a consequence of reduced extractability (see Section 3). However, Downey et al. (2004) note that while there was a decrease in the extraction of extension sub-units with maturity for the exposed fruit, this was not the case for shaded fruit. This observation again indicates the lack of knowledge with respect to the cell wall and, in this case, the influence of light/shading on the capacity of the cell wall to bind condensed tannins. The reflections of Downey et al. (2004) of the implications of the results in this study on gene expression are discussed below.

The same light reduction boxes were used by Cortell and Kennedy (2006) in a study of shading influences on Pinot Noir grown in Oregon, USA. Anthocyanins as well as condensed tannins were examined at véraison and harvest and extractions into a model wine system was also used. The main outcomes of this work with respect to condensed tannins are:

- cluster shading reduced the accumulation of skin condensed tannins (as mg/berry);

- for seed condensed tannins, the shaded bunches at harvest had higher extension and terminal subjects (as nmol/berry) compared with exposed bunches while there was little difference at véraison
- for skin condensed tannins, the shaded bunches were lower in all subunits at both véraison and harvest
- shading caused several changes in the sub-unit distribution;
- the percent extraction of skin condensed tannins was higher (17%) in the model wine extraction of the exposed fruit compared with the shaded fruit.

In essence, there are similarities between this work of Cortell and Kennedy (2006) and Downey et al. (2004). There are also similarities between the results of the shading and vigour studies of Cortell and Kennedy (2006) and Cortell et al. (2005). The study of Cortell Kennedy (2006), similar to that of Downey et al (2004), does not address the link between influence of shading on condensed tannin extraction and cell wall composition.

Irrigation/water stress

Cohen and Kennedy (2010) have reviewed this area extensively, drawing parallels between grapevine behaviour with other fruit crops. The following presents a summary of some of the main ideas published over the last 10 years with more detail available in the review of Cohen and Kennedy (2010).

Esteban et al. (2001) examined the response of Tempranillo (grown in an experimental vineyard in Madrid, Spain) to water deficits. Total skin tannins increased during ripening and the amounts were generally higher for the non-irrigated treatments. However, on a per berry basis, the concentration of tannins was always higher in the irrigated vines indicating that there was more tannin in the skins of the larger berries. Esteban et al. (2001) claim that their data implies that the higher crop yields resulting from irrigation do not adversely affect must composition.

Geny et al. (2003), working on the composition and cellular location of grape seed condensed tannin in Cabernet Sauvignon grown in Bordeaux, used three different irrigation treatments. Their results show no significant effects of water stress on the composition and polymerisation of seed tannins. Unfortunately, they did not utilise the irrigation treatments in their parallel study on grape skins (Gagné et al., 2006).

Roby et al. (2004a), working on Cabernet Sauvignon in California, report that induced water deficits increased the amounts and concentrations of skin condensed tannins, but had little effect on seed condensed tannins. Drawing on data in a parallel publication Roby and Matthews (2004b) suggest that differential growth responses of the skin and inner mesocarp tissue to water deficits may well be the primary mechanism for the increase in concentration of skin condensed tannins. The authors also reflect on the possibility that water deficit has an influence on phenolic biosynthesis, but do not pursue this point in detail.

A combination of water deficits and pruning regimes were used by Petrie et al. (2004) to examine the growth behaviour of Shiraz grown in the Murray Valley region. Phenolic development was not one of the major aspects of this study, although the authors note that water deficit treatments increased the phenolic content of berries and pruning treatments had no influence in the first year. In the season following the application of the water treatments, the accumulation of phenolics showed a consistent increase only for the minimally pruned

vines. Unfortunately, this study did not differentiate between total phenolics and condensed tannins.

Sivilotti et al. (2005) examined the impact of water stress on berry development in Merlot grapes grown in Udine, Italy. The treatments were applied from véraison to maturity. Total polyphenolic compounds only, and not condensed tannins, were assessed in this study. The total skin and seed polyphenolics increased in the severely stressed vines (~15% AW). Phenolic extraction increased during ripening, an observation at odds with the majority of research (Section 3). The authors used different solvents (ethanol, acidified methanol and a tartrate based wine-type buffer) to determine the link between water stress and polyphenolic compound extractability. While their results and interpretations are not always logical, the data they present suggests an approach that could be followed to examine the link between water stress and condensed tannin extractability for optimum wine production.

Container-grown Shiraz was used as the source of material for a study on the impact of pre- and post-véraison water deficits on the synthesis and concentration of skin phenolic compounds in Montpellier, France (Ojeda et al., 2002). The timing of water stress was found to be critical, with strong early season stress producing the lowest phenolic content, but stress applied post-véraison enhanced condensed tannin production. The authors found that water stress increased the degree of polymerisation (albeit a small increase) and argued that it might have important consequences for sensory perception (no data presented). Ojeda et al. (2002) also discuss their results in terms of biosynthesis, rather than in terms of extractability of condensed tannins. They do not present any data on gene expression as a function of water deficit.

Castellarin et al. (2007) applied early and late season water stress to field grown Cabernet Sauvignon vines in California. The focus of the study was on gene expression. The authors argue that their results demonstrate that early or late water stress has little effect on condensed tannins and on the expression of genes involved in their synthesis.

Drawing general conclusions from these studies is difficult, if not impossible, given the number of variables involved. The studies analysed here are spread over several varieties, grown in different regions and countries. Most were field trials, although one pot trial has been included in this summary. Vintage variations were observed and noted by some authors and not others. The vintages ranged from 1992 to 2006. Some studies were single vintage only.

This analysis of published studies indicates a need to examine, through properly designed experiments, the relationship between water management and condensed tannin production. The study needs to include, as a minimum:

- varietal influences;
- vintage variation;
- regional effects;
- the timing and extent of water stress;
- the expression of genes involved in the biosynthesis processes relevant to condensed tannin production (see below);
- berry ripening process and berry size development;
- the condensed tannin concentration and composition (sub-unit analysis and mDP values);
- the extractability of the condensed tannins;

- the transfer of the condensed tannins from the grape into wine;
- the sensory parameters of wines made from different treatments.

Rootstocks

There does not appear to be any published work in refereed journals on the influence of rootstocks on condensed tannin production. This is surprising, given the well-established role of rootstocks in modifying vine productivity.

Cohen and Kennedy (2010) cite work attributed to Sampaio et al. (2006) on rootstock effects for which only a brief conference abstract is available. Working on Pinot Noir, it is claimed that there is a rootstock influence on condensed tannin and anthocyanin content of fruit and wine. Further, Cohen and Kennedy (2010) suggest the data of Sampaio et al. (2006) imply compositional differences (whether in condensed tannins or anthocyanins is not clear) induced by rootstocks. Unfortunately the original data are not available for a more detailed assessment.

Dr Rob Walker (personal communication) has provided access to unpublished data on one rootstock trial. The initial results suggest that while there may be a rootstock influence of condensed tannin production within a vintage, the between-vintage variation overrides any within-vintage effect. This study would appear to have opened up more questions than it has answered.

The role of rootstocks in influencing condensed tannin development essentially remains an unresolved issue. There may well be considerable value in exploring rootstock influences in greater detail, given the changing climatic regimes under which grapes will be grown in the future.

Other factors

Cohen and Kennedy (2010) have reviewed what they call ‘exogenous factors’ on phenolic development. The research tends to be scattered and, in many cases, observational rather than mechanistic. The exogenous factors include nitrogen fertilisers, plant hormones, potassium and calcium ions, boron and copper.

It is difficult to sort out from the published data whether the effects of one or more of these variables is a direct effect or a secondary effect due to a change in the overall vine development. For example, excess nitrogen can enhance vine vigour and this in turn can affect tannin development.

At this stage, it is difficult to recommend any research activity involving these exogenous factors until the mechanism of condensed tannin production, their location within the cell, their extraction and transfer to wine are clarified.

Post-harvest treatments

Post-harvest drying or dehydration has been practised in many parts of Europe since ancient times. The practice is most probably the forerunner of today’s Amarone and Reciotto styles of the Verona region. Air-drying is the most common way of achieving the dehydration, whether on traditional drying racks or by using air blowers.

While the practice is not all that common outside Verona, some interesting data have been generated in a study by Moreno et al. (2008). Working with Pinot Noir in Oregon, USA, the authors studied changes in condensed tannin concentrations aswell as other phenolic

compounds (anthocyanins, for example) and aroma compounds. From their analyses, Moreno et al. (2008) found:

- drying reduced the amount of skin condensed tannin when measured per berry;
- mDP for skin condensed tannins decreased with drying and also with protracted time on the vine (that is, riper grapes). A similar observation was made for seed tannins;
- there was little impact of drying on extension subunit composition for skin tannins;
- catechin dominated the terminal sub-unit composition of the seed tannins while epi-catechin dominated the extension sub-unit composition. Extended ripening and drying had little impact on the compositional analysis;
- analysis of wines made from grapes of the different treatments showed a marked increase in condensed tannin concentration for the drying and extended ripening treatments;
- mDP values for the wine condensed tannins were slightly less than the seed mDPs, but markedly less than skins mDPs.

There are two important outputs from this study. First, the use of dehydration may find application in regions where achieving maturity is a challenge. The second output relates to the observation of a marked increase in the condensed tannin concentration in wines made from grapes that were either dehydrated or left for an extended period on the vine. Moreno et al, (2008) comment suggest that the drying process, be it either induced or through extended period on the vine, may well affect the diffusional barrier between the tannin storage compartments in the berry skins and seeds allowing greater transfer during the fermentation process.

This latter observation is in accord with the conclusion in Section 3 and re-affirms the need for a greater understanding of the cell wall and the binding of condensed tannins within the cell wall.

Gene function

Section 1 described the state of knowledge with respect to the biosynthesis of condensed tannins, the pathways involved and the expression of the relevant genes. This summary addresses environmental impacts on gene expression and the link between this expression and the biosynthesis of condensed tannins.

In essence, there appears to be only one direct study described in the literature. While some researchers have suggested a link between environmental influences and the production of condensed tannins, they do not actually address gene expression *per se*.

Castellarin et al. (2007) examined the expression of *BAN* and *LAR2* in berry skins as a function of early and late water deficit. Treatment effects were essentially not significant. That is, there were only limited effects on condensed tannin production and on the expression of the genes involved in their synthesis. The authors do suggest that the inherent biological heterogeneity requires a re-examination of the factors with more, rather than fewer, replicates.

Summary

The areas reviewed are climate and temperature effects, viticultural influences including vine vigour and its link to shading, irrigation and water stress, varietal influences, rootstocks and environmental influences on gene expression.

Drawing general conclusions from the published studies is difficult, if not impossible, given the number of variables involved. The studies analysed here are spread over several varieties, grown in different regions and countries. Most were field trials, although one pot trial has been included. Vintage variations were observed and noted by some authors and not others. The vintages ranged from 1992 to 2006. Some studies were single vintage only. Research designed to understand the mechanisms at work appears to be addressed in one publication only.

There is a clear need to examine, through properly designed experiments, the relationship between environmental influences and condensed tannin production. The study needs to include, as a minimum varietal influences, rootstock influences, vintage variation, the timing and extent of environmental stress (light, temperature, water), berry ripening process and berry size development, the condensed tannin concentration and composition, the extractability of the condensed tannins, the transfer of the condensed tannins from the grape into wine and the sensory parameters of wines made from different treatments.

The focus on gene expression under various conditions of environmental stress is of critical importance in furthering our understanding.

SECTION 5
Tannin content and composition in wine

There is a vast literature describing the formation and analysis of condensed tannins in wine. ASVO seminars in 1997 and 2005 presented state of knowledge reviews from both science and winemaking perspectives. Book chapters by Cheynier and Fulcrand (2003) and Cheynier (2006) present detailed scientific summaries on the analysis and chemistry of condensed tannins and tannin-like compounds.

The winemaking process extracts a wide range of phenolic compounds from grapes, including condensed tannins, anthocyanins and monomeric and oligomeric flavanols. These extracted compounds can remain in the chemical form in which they are extracted or react further to give new polymers and pigments. Sometimes there are attempts to differentiate between grape tannins (condensed tannins remaining in the form in which they extracted) and wine tannins (condensed tannins formed from reactions in wine).

Following the terminology initially proposed by Santos-Buelga and Scalbert (2000) and used by Cheynier and Fulcrand (2003), this section of the review will address two types of compounds:

- Condensed tannins, as polymers of catechin-type (flavan-3-ol) phenolic compounds;
- Tannin-like compounds, often arising from acetaldehyde mediated reactions; compound types include ethyl-bridged flavan-3-ols, ethyl bridged anthocyanin-flavanol adducts and pyranoanthocyanin-flavanol adducts.

This section will address:

- methods of analysis;
- limitations of these methods
- the possibility of molecular association within the wine matrix.

Mechanistic studies on the formation of tannins and tannin-like compounds are described in Section 6 while Section 7 describes studies on the binding of tannins to other components in the wine matrix.

Methods of analysis

The focus of this discussion is on instrumental methods for molecular size and compositional studies. Rapid methods of analysis for total tannin concentration were discussed in Section 2. The following comments are based on concepts presented in Cheynier and Fulcrand (2003), De Beer et al, (2004), Herderich and Smith (2005) and Cheynier (2006).

a) Fractionation by molecular size (molecular mass[4])

Traditionally, separation was based by adsorption chromatography using a column with solvent flowing down the column under gravity (sometimes referred to as low pressure chromatography). Fractionation between condensed tannins of different masses is not always successful.

Gel permeation chromatography is a form of size exclusion chromatography where the solvent is pumped through the column under pressure. Resolution (separation) of condensed tannins tends to be poor beyond a tetramer (Cheynier and Fulcrand, 2003).

[4] The term molecular mass, rather than the older 'molecular weight' is used here to align the text with more recent terminology practice.

Normal phase HPLC uses a polar column material (stationary phase) so that non-polar compounds are eluted early followed by polar compounds. There has been some success with this technique, but is requires specific workup for each application to ensure the successful separation of target analytes (condensed tannins) from other UV absorbing compounds such as anthocyanins.

Reverse phase chromatography uses a non-polar phase and has been highly successful in separating the products obtained after depolymerisation in the presence of so-called nucleophilic agents, as discussed below.

Counter current chromatography has found some success in condensed tannin separation, perhaps more so with tea than wine (Cheynier and Fulcrand, 2003). Vidal et al. (2004) have used counter current chromatography successfully for anthocyanin separation and analysis, but not for condensed tannins.

b) Quantitation and characterisation of condensed tannins
Cheynier and Fulcrand (2003) suggest that:

- heterogeneity of condensed tannins increases with the degree of polymerisation; that is there are more possible structural isomers for the same mDP, as the mDP increases;
- the increased structural complexity results in a loss of resolution/separation;
- it is virtually impossible with existing methodology to obtain condensed tannins in the pure state (that is, with full isomer separation) for mDPs greater than 5.

Full characterisation of condensed tannins is therefore faced with several significant analytical and structural challenges. The approach that has been generally adopted is to determine (Cheynier and Fulcrand, 2003):

- the nature and number of monomeric flavan-3-ol units, sometimes referred to as constitutive units of terminal and extension units;
- the mean molecular mass of the polymer;
- the mean degree of polymerisation;
- the types and number of interflavanic links (that is, bonds between the flavan-3-ol monomers) within the polymer.

Several colorimetric methods have been used with varying degrees of success. The Bate-Smith method involves oxidative cleavage of the interflavanic links using butanol-HCl (Porter's reagent) followed by a colorimetric reaction. Direct colour development for both monomers and polymers can be achieved by reaction with vanillin (Dai et al., 1995) or dimethylaminocinnamaldehyde (DMACA) (Gutmann and Feucht, 1991). These latter two reagents were used successfully as staining agents for cell wall studies by Cadot et al. (2006a) (see Section 3). Finding an appropriate calibration standard is a limitation of these colorimetric methods.

Acid-catalysed cleavage of the carbon-carbon in the presence of a nucleophilic agent, followed by reverse phase HPLC (RP-HPLC) has become standard practice for identifying and quantifying the terminal and extension units in condensed tannins. The acid cleavage step separates the terminal unit as the free flavan-3-ol while the extension units form carbocations (positively charged species). The carbocations then react with a nucleophilic (positive seeking) species, forming stable compounds that can be readily detected and identified.

The two most common nucleophilic agents used are:

- phenylmethanethiol, also known as toluene-α-thiol; the process is known as thiolysis (see Thompson et al. 1972).
- 1,3,5-trihydroxybenzene, also known as phloroglucinol: the process is known as phloroglucinolysis (see Foo and Porter, 1978).

Both procedures have their supporters and detractors. Phloroglucinol is sometimes preferred as it is essentially odourless while phenylmethanethiol has a highly pungent odour. The limitations of these reagents are discussed in more detail below.

There is a vast literature on the application of these acid cleavage/nucleophile reactions for condensed tannin analysis. Detection and partial identification by RP-HPLC with diode array detection (DAD) is common practice. There are slight methodological variations, but these are generally in-house adaptations of the general method. The absence of reference materials (see below) is a major limiting factor in determining the sequence of the monomers in the original tannin molecule.

Mass spectrometry (MS)

Mass spectrometry can be used as a stand-alone instrument or as a detector in HPLC, often in combination with a DAD detector (LC-DAD-MS). Mass spectrometry is an ideal method for determining the molecular mass of a compound that allows an empirical/molecular formula to be estimated. High resolution MS (mass measured to four decimal places) allows the unique combination of carbon, hydrogen and oxygen atoms for a tannin molecule to be calculated. MS and nmr (see below) are ideal 'partner tools' as they provide detail of the mass and arrangement of atoms (ie, the structure) in the molecule.

There are a range of ionisation methods used in MS, all with advantages and limitations. Cheynier (2006) gives a good summary with multiple examples of the success of MS in general flavonoid analysis. For a compound to be detected by MS, it must be charged as the signal that is obtained is actually the mass/charge ratio. In some cases, multiple charged ions can be formed and this allows higher masses to be identified, with the proviso that signals of single and multiple charged ions often overlap.

Fragmentation patterns are also valuable to provide structural information (Cheynier, 2006). This approach uses the ionisation energy to fragment the molecule. Identification of the fragments can give an indication of the structure of the original tannin.

The AWRI is a world leader in mass spectral analysis. The characterisation of grape seed condensed tannins (Hayasaka et al., 2003) is one example of the quality of this work. It demonstrates the advantages of multiple charged ions for identifying tannins with higher mDP values (28 in this case). The strength and quality of this research is an area that must be supported.

Intriguingly, there does not appear to be any application of ultrahigh resolution mass spectrometry to condensed tannin analysis. The potential of FTICR-MS, as the technique is known, has been detailed by Gougeon et al. (2009) for wine analysis. One specific analysis examined the metabologeography expression of oak wood chemistry and showed that 10-year old wines expressed the signature of the forests from where the oak used in barrel

construction used to age the wines had been grown. The potential for this methodology to be used for tannin analysis needs to be investigated.

Nuclear magnetic resonance (nmr) is a potentially ideal technique for assessing structural characteristics of condensed tannin extracts (Cheynier and Fulcrand, 2003). For nmr to be successful however, the molecule must be pure, both chemically and isomerically. This requirement often limits the application of the methodology. Cheynier (2006) has reviewed some of the recent advances in nmr applications for the structural analysis of tannin and tannin-like compounds. Two-dimensional and multinuclear methods are capable of information on structures, *cis/trans* isomers and linkage isomers (C4-C8 or C4-C6, for example).

HPLC with an nmr detector (LC-nmr) is one evolving technique for general polyphenol compound analysis (Wolfender et al., 2003). Several complex structures have been determined, although little application to wine polyphenols has been described. The ability to gain on-line structural information is a major advantage of LC-nmr, particularly when LC-DAD-MS detection is ambiguous. The cost of LC-nmr, both establishment and operating, is high and it is more likely to be located in specialist nmr facilities, rather than become a routine analytical tool. The technique also requires a high concentration of the analytes under examination, especially for ^{13}C nmr and this tends to compromise the separation efficiency of the compounds on the chromatography column.

Future nmr developments will include the use of chemometrics for the analysis of instrumental data. For example, Masoum et al. (2006) applied partial least squares discriminant analysis and learning vector quantisation neural networks to the interpretation of two dimensional nmr spectra of phenolic compounds in Bordeaux wines. They were able to differentiate the wines based on grape variety, vintage and soil type. Recent advances in protein nmr would suggest that the application of the concepts to condensed tannin analysis may lead to greater insight of the complex structural arrangements that exist in the various tannin structures.

Limitations of existing analytical methods

Determination of mDP

The degree of polymerisation is defined as the ratio of the molecular mass of the polymer to the molecular mass of the repeat unit. This definition and its application are logical in traditional polymer chemistry where the determination of the respective molecular masses is relatively easy to determine. This is clearly not the case with condensed tannins.

The present common approach for condensed tannins is to calculate the mDP after acid hydrolysis of the condensed polymer. Cheynier and Fulcrand (2003) propose that mDP can be calculated as:

mDP = ([upper and extension units] + [end units])/[end units]

provided that each unit in the cleaved polymer can be identified and quantified. The calculation of mDP after acid hydrolysis by this method must be adapted for condensed tannins containing A-type linkages (see below). The A-type bonds are resistant to hydrolysis and need to be counted differently (Le Roux, 1988).

Further, the values obtained for mDP depends on the method of calculation and also the method of calibration. Table 2, extracted from Cheynier and Fulcrand (2003), and based on

data generated by Prieur et al. (1994) demonstrates variation in mDP values for condensed tannins extracted from grape seeds and subsequently fractionated.

Table 2. Method variation in mDP values (see text above for details).

Fraction	I	II	III	IV	V
Bate-Smith	1.8	2.4	2.7	2.8	3.4
VA (catechin as calibrant)	0.9	1.4	2.6	3.5	3.4
VA (B2 as calibrant)	1.2	1.4	2.6	3.5	6.9
DMACA (catechin as calibrant)	3.0	3.9	4.1	5.1	5.2
Thiolysis	2.3	3.6	5.4	7.8	15.1
Phloroglucinol	2.5	4.8	7.7	12.2	19.1
Gel permeation	2.5	4.6	7.2	12.9	18.9

VA = Vanillin Assay; DMACA = dimethylaminocinnamaldehyde; B2 = procyanidin B2

The colorimetric methods clearly give lower values for mDP, especially for the fractions of higher molecular mass. Phloroglucinol gives higher mDP values for fractions III, IV and V compared with thiolysis, but is generally in agreement with gel permeation.

Efficiency of the acid cleavage reaction

Thiolysis is subject to a number of variables including

- resistance of A-type linkages to degradation under thiolysis conditions (Karchesy and Hemingway, 1986). A-type linkages involve two bonds between flavan-3-ol monomers. This linkage probably results from oxidation of the tannin molecule. A-type molecules need to be counted as two when performing mDP calculations;
- low yields under certain conditions (McGraw et al., 1993);
- yield dependent on reaction conditions (Cheynier and Fulcrand, 2003);
- side reactions during the thiolysis step (Matthews, 1997);
- epimerisation of the flavan-3-ol units (Lazarus et al., 2003). The interconversion at different rates between (+)-catechin and (-)-epicatechin makes quantitative determination of the free terminal units difficult;
- the unfortunate aroma and potential toxicity of the reagents demands stringent working conditions.

On the positive side, thiolysis is useful for the determination of acetaldehyde-bridged (ie: ethyl-linked) flavan-3-ol oligomers. This has led to an assessment of the stability of linkages in the original ethyl-linked compounds (Es-Safi et al., 1999). However, not all tannin-like structures degrade under thiolysis conditions, including flavanol-ethyl-anthocyanin compounds. This again limits access to gaining structural information about the polymeric compounds.

Phloroglucinolysis is a simpler process than thiolysis, at least in terms of the chemical conditions required. Other aspects of this method are:

- Alcoholic media are preferred for the reaction, as water reduces the yield (Matthews et al., 1997);
- Lower yields than with thiolysis type reactions (Matthews et al., 1997)are obtained due to high instability of phloroglucinol derivatives;
- Ascorbic acid can limit the extent of side reactions (Kennedy and Jones, 2001).

Resolution of the more effective reagent would be beneficial.

Absence of standard reference materials

Herderich and Smith (2005) make the point that while depolymerisation reactions allow subunits to be identified, the sequence of these subunits cannot be determined by this approach. Different arrangements of (+)-catechin and (-)-epicatechin may well give rise to different chemical and sensory properties.

(+)-Catechin and (-)-epicatechin are diastereoisomers. The effect of this diastereorisomerism on pigmentation in white wine oxidation studies has been described by Labrouche et al. (2005): the molar absorptivity coefficient for the epicatechin-xanthylium pigment was 1.8 times higher than that for the equivalent catechin-xanthylium pigment. Preliminary molecular modelling indicates a different spatial arrangement of the rings in the two xanthylium pigments that would lead to this difference in molar absorptivity coefficients. Although removed from tannin chemistry, the work of Labrouche et al. (2005) clearly demonstrates that the different orientations in space around one carbon atom in the monomeric flavan-3-ols can lead to a marked change in properties.

The advances in analytical chemistry generally over the last two decades have been based to a significant extent on the availability of certified reference materials. The use of deuterated standards in wine flavour chemistry, for example, has become the norm in GC-MS analysis of flavour compounds. No such standards are available for condensed tannins. This leads to frustrations when researchers try to put together actual structures (or even models) for combinations of different monomeric flavonols.

While separating tannins based on the effective molecular mass (or mDP) might be achievable, there is no procedure presently available to quantify the arrangement of individual monomers with the tannin unit. While some ‘tannin standards’ may be available, the question remains as to whether they contain a consistent and replicated arrangement of monomers or whether they are mixtures (see also Herderich and Smith, 2005).

The absence of validated standards is clearly limiting progress in tannin chemistry. This must be rectified as a matter of urgency. Chemical synthesis would appear to be the most likely approach to produce standards of known sequences of monomeric flavonols. To be successful, considerable investment will be required. This investment may be balanced over time through royalties from international sales.

There are at least two approaches to the synthesis of tannin standards that need to be considered:

- classical organic chemistry synthesis is one approach. Starting with a known flavan-3-ol, chemical blocking of some sites while leaving others open for addition reactions can lead to highly regulated polymers. This methodology has proved successful in other areas of organic synthesis and is worthy of evaluation here. The procedure may, however, be limited to less than five linked monomer units.
- solid phase synthesis has been used successfully for synthesising peptides and relatively short fragments of RNA and DNA. More recently, solid phase synthesis has been applied to the preparation of oligosaccharides (Adinolfi et al., 1996; Plante et al., 2001). There are significant synthetic challenges, especially in controlling the linkage and stereochemical arrangements. Much greater control of the synthetic process can be obtained when the building block is attached to a solid support phase compared with reactions in solution.

Although untried for tannin synthesis, the solid phase approach has the potential to produce well-defined tannins with five or more linked monomers.

Molecular associations in the wine matrix

The concept of molecular association is related to the creation of some form of aggregation of molecules in the wine matrix. Molecular associations are well established in foods generally, with journals such as *Food Hydrocolloids* being one special focus publication. Only in the last 10 to 12 years has there been much interest in this concept regarding wine[5]. The author of this review has several pieces of anecdotal evidence for molecular associations in wine including:

- an increase in the fraction of ionised calcium concentration on dilution of wine – the increase indicates the dispersion of aggregates, similar to what occurs on dilution of milk, for example;
- the capacity of the acidic polysaccharide RG-II to bind lead ions is reduced by ultra-filtration, as the mechanical action pushes the RG-II monomer-dimer equilibrium to the monomer side (only the dimer binds lead ions). Over time, the dimer reforms and an increase in lead ion binding is observed.

Saucier et al. (1997) developed a model system for examining the potential colloidal state of wine polyphenols. Using the condensation reaction between (+)-catechin and acetaldehyde, the authors observed aggregation (haze formation) followed by precipitation. Scattered light intensity and mean particle diameter measurements showed that the polymers aggregate into colloidal intermediates before polymer precipitation. Evidence is presented to suggest that hydrophobicity is important in creating the colloid.

Dr Aude Vernhet and co-workers have examined in some detail the physical chemistry behaviour of polyphenolic compound aggregation and that the factors that influence the aggregation. The group argue that colloidal interactions are important in influencing wine stability, clarification and taste (Poncet-Legrand et al., 2003). In their first study (Poncet-Legrand et al., 2003), they used flavan-3-ol monomers and dimers extracted from grape seeds (as well as apple and pear) in a tartaric acid model wine base. Aggregation was studied by phase diagrams while the aggregates were examined by dynamic light scattering and cryo-transmission electron microscopy. The results showed:

- galloylation enhanced the formation of aggregates involving monomers, but it was not possible to confirm this for polymers;
- the relationship between mDP and aggregation is complex: more aggregation was observed with mDP values up to 8 -10;
- the higher solubility of condensed tannins with large mDPs (15 and higher) may be due to the ability of the large molecule to adopt a conformation that enhances solubility;
- an increase in ionic strength caused more dispersion of the particles, due to the effect of increased ion concentration on the size of the electrical double layer;
- increasing the ethanol concentration generated smaller and less polydispersed colloidal aggregates.

Adsorption isotherms were used by Cartalade and Vernhet (2006) for studying the adsorption of flavan-3-ol monomers and various grape seed procyanidin fractions (different mDPs) on

[5] The author of this review submitted a project on molecular association in wine in the preparation of the bid for Round 2 of the CRCV for Viticulture (1998). The proposal did not make the final bid.

surfaces of different polarities. The different mechanisms proposed for the build-up of adsorbed layers suggested that in some cases multiple bonds with the surface are formed. This type of study opens up the possibility of examining electron donor-electron acceptor interactions that could provide insight into mechanisms of sensory perceptions of astringency and related red wine taste parameters.

Zanchi et al. (2007) have examined the interactions between grape seed condensed tannins (mDP = 11) in aqueous ethanol solutions using small angle neutron scattering, light scattering and physical separation techniques. The results showed:

- the tannin fraction consisted of three components, labelled T1 (minor), T2a (33%) and T2b (65%);
- T1 is hydrophobic and forms colloidal particles in less than 60% ethanol – it is soluble at higher ethanol concentrations;
- T2a remains soluble to 12% ethanol concentrations, due to solvation by ethanol. It remains 'molecularly dissolved'; that is there were no stacks or micelles observed;
- T2b is hydrophilic and remains dissolved at all ethanol concentrations;
- the T1 colloids are metastable at low ionic strengths, due to the adsorption of organic acids on the surfaces of the particles.

The observation of three components of the tannin fraction was of some surprise to Zanchi et al. (2007). They comment that there may be three possible causes:

- the mDP11 fraction may well have actual DP values varying from 5 to 30 (see Mané et al., 2007);
- the fractions may have different chemical structures leading to differences in hydrophobic/hydrophilicity;
- the hydrophobic T1 fraction may be oxidised.

Irrespective of these limitations, this study by Zanchi et al. (2007) presents a way forward to understanding the relationship between tannin-tannin interactions and colloidal assemblies. The observation that the colloids can be stabilised by adsorption of organic acids, such as tartaric acid, is a significant conclusion of their work.

A further step towards understanding the basis for wine astringency has been made in the study of the influence of epigallocatechin gallate (EGCG) and condensed tannins on the aggregation of glycosylated proline-rich protein (PRP) (Pascal et al, 2008). Using dynamic light scattering and small angle X-ray scattering, Pascal et al. (2008) observed:

- for the monomer, there was a threshold EGCG/PRP ratio at which aggregation occurred. Aggregates were small and formed stable dispersions;
- when condensed tannins were used, aggregation occurred at lower PRP concentrations and lower tannin/PRP ratios. The aggregates were of discrete size and were stable;
- phase separation (precipitation) only occurred at 2% ethanol, but dependent on the tannin size;
- the relationship between the condensed tannin/PRP aggregation is probably the consequence of multiple bonding occurring, as proposed by Cartalade and Vernhet (2006) in their isotherm study;
- there is a complex relationship between condensed tannin mDP, tannin/PRP ratio and colloidal stability. Phase separation was not observed at 12% ethanol, an important observation for wine;

Most importantly, Pascal et al. (2008) were able to conclude that glycosylated PRPs are able to resist precipitation with tannins. However, they form complexes that most likely modify the rheological or flow properties. Small and finite particles might induce a friction-based taste sensation. The implications of this study for understanding sensory properties are significant.

It is clear that that the detailed physical chemistry studies described here point a way forward to providing a mechanistic understanding of astringency and related wine descriptors. If particle sizes with diameters around 300 nm are being formed (Zanchi et al., 2007), the impacts on flow properties will be apparent. There are two techniques that are used in hydrocolloid analysis that have not been applied to wine and are worthy of investigation. Couette shear flow, in combination with fluorescence spectra, has been applied to the study of the aggregation of conjugated polymers (Chan et al., 2009). Similarly, fluorescence based analytical ultracentriguation can be used to determine the molar mass of polymers without the need for standards (Hao et al., 2009). The same methodology can also be used to study aggregation phenomena *in situ*.

The use of fluorescence spectra for analysis of wines has long been ignored as there is interference from the Raman spectrum of water and also from Rayleigh scattering severely limited the usable emission wavelength range. Recent work by Bouveresse et al. (2007) has developed a mathematical approach to removing these interferences. This in turn allows a wider excitation-emission wavelength range to be used.

The application of the analytical approaches of Couette shear flow and ultracentrifugation, in combination with fluorescence spectroscopy, as used in hydrocolloid science, opens up new possibilities for understanding the nature of colloid interactions in wine, as opposed to model, matrices.

Summary

Full characterisation of condensed tannins is faced with several significant analytical and structural challenges. The approach that has been generally adopted is to determine:

- the nature and number of monomeric flavan-3-ol units, sometimes referred to as constitutive units of terminal and extension units;
- the mean molecular mass of the polymer;
- the mean degree of polymerisation;
- the types and number of interflavanic links (that is, bonds between the flavan-3-ol monomers within the polymer.

Acid-catalysed cleavage of the carbon-carbon bonds in the presence of a nucleophilic agent (toluene-α-thiol or phloroglucinol), followed by reverse phase HPLC (RP-HPLC) has become standard practice for identifying the terminal and extension units in condensed tannins. Both procedures have their supporters and detractors but neither is totally successful for full characterisation of the condensed tannin.

Limitations of existing analytical methods include the determination of mDP and the efficiency of the acid cleavage reaction. There does not seem any research directed towards a more efficient cleavage reaction.

The major limitation is the absence of standard reference materials. This must be rectified as a matter of urgency.

Molecular associations in the wine matrix are receiving greater attention. There is increasing evidence that colloidal interactions are important in influencing wine stability, clarification and taste. Colloidal particles may be of sufficient size to modify the rheological or flow properties. Small and finite particles might induce a friction-based taste sensation.

The implications of molecular associations for understanding sensory properties are significant and demand further work on wine rather than on model systems. Analytical methods such as Couette shear flow and ultracentrifugation, in combination with fluorescence spectroscopy offer the potential to gain further insight into the colloidal structures within the wine matrix.

SECTION 6
Formation of stable polymeric pigments, especially polymers containing anthocyanins

The formation of stable pigments has been the subject of extensive research particularly since the report of Somers (1971) on non-dialysable coloured material that remains in aqueous solution after extraction of red wine with isoamyl alcohol. The research has been summarised in several book chapters and forms an integral component of advanced studies in oenology in university programs. Fascinatingly, more than one hundred derived pigments have been detected in red wine (or systems modelling red wine), although the extent to which most actually contribute to red wine colour is questionable.

This Section will address:

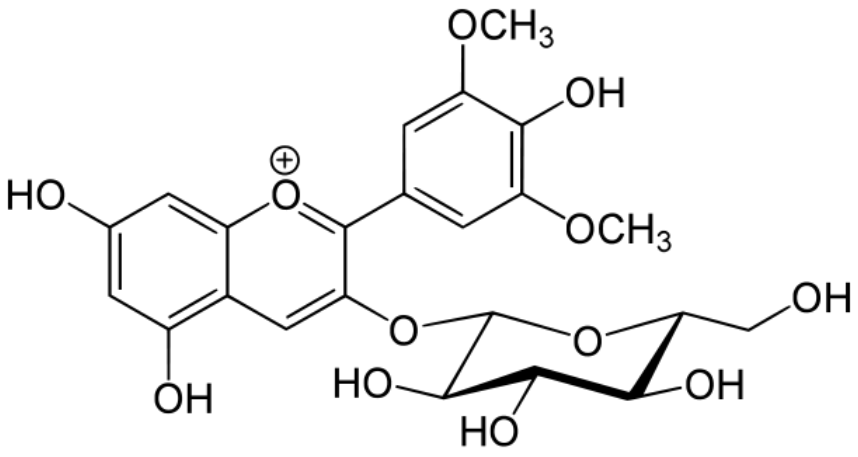
- the structures of the molecules involved in the pigmented polymer production;
- the mechanisms proposed for pigmented polymer production;
- polymeric pigment production and wine ageing;
- resistance to bleaching by sulfur dioxide;
- interactions in the wine matrix, including co-pigmentation, tannin-protein interactions and interactions involving polysaccharides;
- potential for further research.

Molecules involved pigmented polymer production

The main species involved in the production of the pigmented polymers are:

- anthocyanins – see Figure 23;
- flavan-3-ol, which may be a monomer such as (+)-catechin or a dimeric procyanidin species (Figure 24);
- acetaldehyde (Figure 25).

Figure 23. Representative structure of an anthocyanin: malvidin-3-glucoside



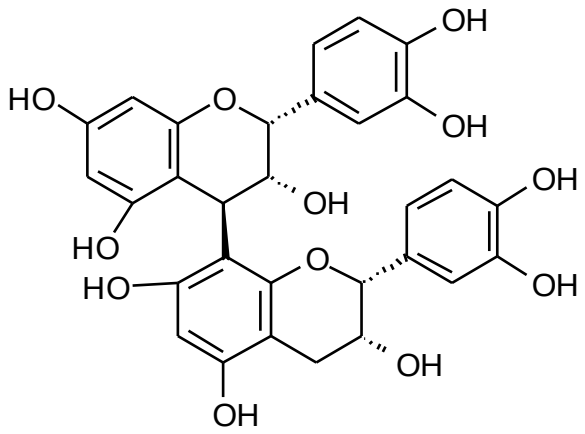
The critical structural features of anthocyanins are

- a residual positive charge – this give the *electrophilic* (electron seeking) character to the flavylium cation form of anthocyanins;
- the presence of a glucose unit at the 3 position;
- different forms due to hydration reactions that impact of reactivity;

- a reactivity with sulfur dioxide (more correctly, the hydrogen sulfite ion) that results in loss of colour.

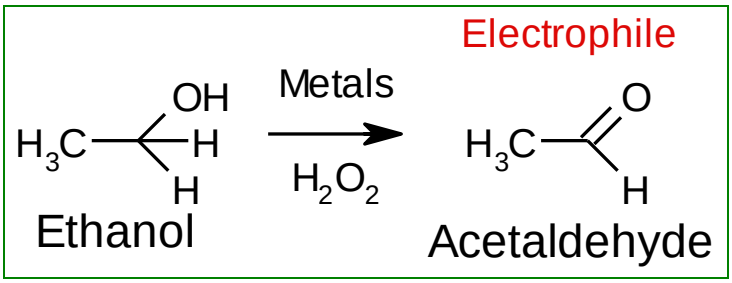
Flavan-3-ols, on the other hand, have a *nucleophilic* (positive seeking) character which makes them ideal to react with anthocyanins. Procyanidins are dimeric flavan-3-ols (Figure 24) that retain the nucleophilic character for assisting the reactivity with anthocyanins.

Figure 24. Structure of procyanidin B2



Acetaldehyde is produced by an oxidation reaction (Figure 25) or by liberation during yeast fermentation. It is also an electrophile that aids its reactivity with flavan-3-ols.

Figure 25. Representation of the production of acetaldehyde from the oxidation of ethanol.



Mechanism proposed for pigmented polymer production

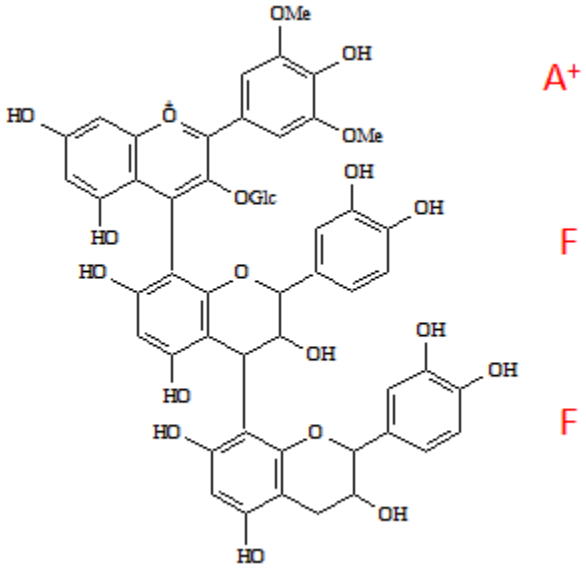
This section summarises the main types of mechanisms that lead to pigmented molecules. Mechanistic details are generally not included. Specific details can be found in, for example, Monagas and Bortolomé (2009). Structures of some of the coloured products are sometimes shown here to indicate the type of chemical compounds that are formed.

Mechanism a) Direct reaction of anthocyanin with flavan-3-ol-type phenolic compounds

This process requires the anthocyanin to be in its flavylium cation form. The reaction occurs between the 4-position of the flavylium cation and the 8-position (generally, but the 6-position is also possible) of the flavan-3-ol to produce a colourless flavene molecule. Subsequent oxidation produces the red coloured anthocyanin-flavanol compound. The oxidations step results in a positive charge residing on the anthocyanin portion of the molecule. This type of compound is often represented as (A^+-F) where the A^+ stands for the anthocyanin (flavylium cation) and F for the flavan-3-ol.

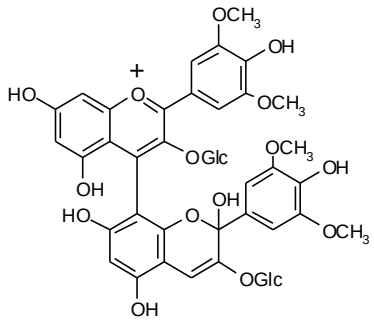
The same type of reaction can also occur with a procyanidin dimer. Figure 26 shows one possible structure. In this case, the structure is represented as (A^+-F-F) to indicate that two flavan-3-ol units are present.

Figure 26. Product of the reaction between an anthocyanin and a procyanidin dimer (A^+-F-F type)



In a variation of the above mechanism, the carbinol form of an anthocyanin (AOH) can react with the flavylium cation (A^+) form. That is, anthocyanins condense with themselves. The structure is represented in Figure 27. The mechanism is similar to that described above, resulting in a link between the 4-position of the flavylium cation form and the 8-position of the carbinol form.

Figure 27. Product of the reaction between the carbinol and flavylium cation forms of an anthocyanin, represented by A^+-AOH.



Vidal et al. (2004) has presented mass spectrometric evidence for anthocyanin dimers and trimers isolated from Shiraz grape skins. Structural options were presented, but not fully resolved. The anthocyanin oligomers were found to be resistant to acid cleavage, but resistance to SO_2 bleaching was not examined. Salas et al. (2005) also reported the presence of anthocyanin oligomers in their study, but did not fully identify the compounds.

The third variant on the anthocyanin-flavanol condensation mechanism results from the degradation of a condensed tannin in the acidic medium of wine. The cleavage of one flavan-3-ol generates a 'carbo-cation': the representation in Figure 28 shows the positive charge at C4. The carbo-cation can then react with the carbinol form of the anthocyanin to give the compound shown in Figure 29. This generates a so-called F-A^+ type pigment. The formation of the F-A^+ pigment does not require an oxidation step as does the A^+-F pigment. The generation of the carbo-cation (Figure 28) is thought to be relatively slow. The importance of this type of reaction in influencing wine colour is perhaps more relevant to wine ageing.

Figure 28. Cleavage of a condensed tannin to give a carbo-cation.

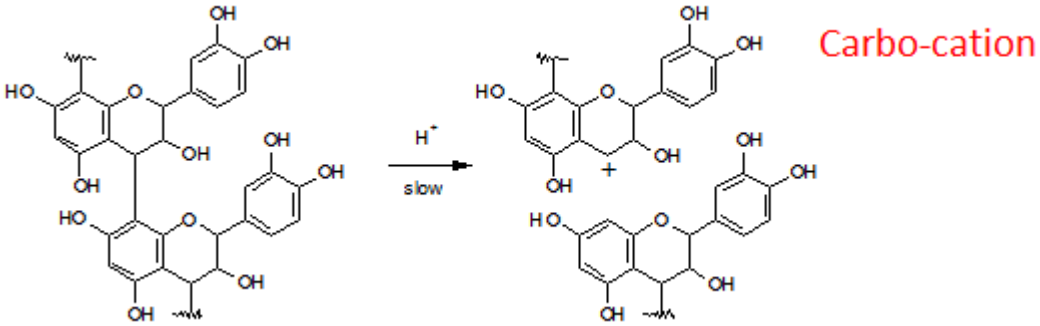
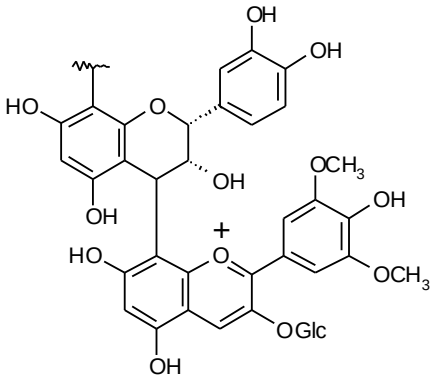


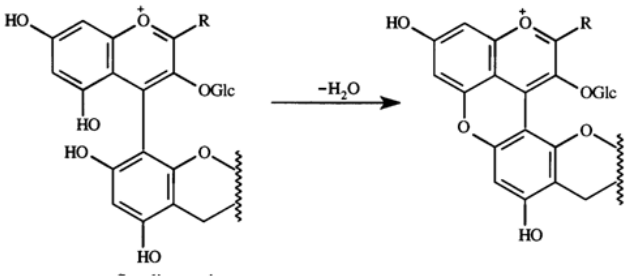
Figure 29. Representation of the F-A^+ type pigment: reaction of carbo-cation with a flavan-3-ol.



Internal re-arrangement reactions between polymeric pigments.
Internal re-arrangement of the A^+-F type pigments can generate xanthylium cations. The mechanism of production is shown in Figure 30. The reaction would appear to be more common at elevated temperatures and it seems only to occur with the A^+-F and not with A^+-F-F or higher polymers (Fulchrand et al., 2006).

This reaction sequence for xanthylium pigment production has been observed in model system. It is, however, a reaction that requires more examination as the xanthylium cation pigments are yellow, whereas the flavylium cation form of anthocyanin molecules is red. It is possible that this type of reaction is one contributor to the transition in red wine colour from red to orange with age.

Figure 30. Schematic representation of internal re-arrangement of a flavylium cation to give a xanthylium cation.

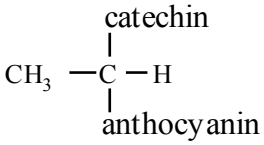


Bleaching by sulfur dioxide
Loss of colour by anthocyanins in the presence of sulfur dioxide (the bleaching effect) is due to the formation of the bisulfite addition product at the C4 position of the anthocyanin. Bisulfite addition at the C2 position may occur, also resulting in loss of colour.

The polymers formed by mechanism a), that is the direct anthocyanin-flavanol condensation reaction, generally have substitution at position C4 of the anthocyanin molecule, protecting it from bleaching by sulfur dioxide. The exception is the F-A^+ type pigment where the C4 position does not have a substituent (Figure 29). However, this type of molecule appears to be largely resistant to SO_2 bleaching (Cheynier, 2006).

Bleaching effects are discussed in more detail later in this Section.

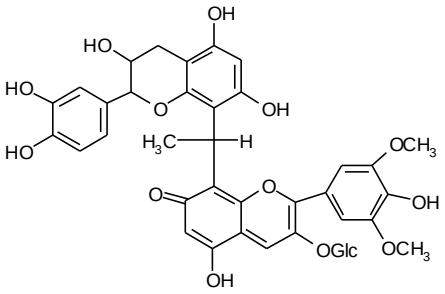
Mechanism b) Condensation reactions involving ethyl bridges
The presence of the two-carbon ethyl-bridge between a flavan-3-ol and an anthocyanin is generally the consequence of an acetaldehyde mediated reaction. Under the acidic conditions of wine, acetaldehyde becomes protonated and this increased its electrophilic character. The protonated acetaldehyde can then react with the electron-rich C8 position of a catechin-type flavan-3-ol. This 'acetaldehyde-flavanol' molecule can then react with the C8 position of an anthocyanin (assumed to be in its carbinol form). The generalised structure can be represented as



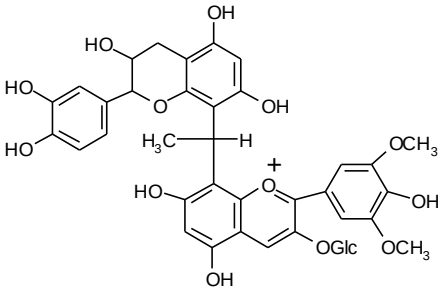
This reaction mechanism has been studied in great detail for malvidin-3-glucoside (Cheynier, 2006; Fulcrand et al., 2006). The colour is generally violet or purple. It is more intense (higher molar absorptivity) that the original anthocyanin and it is less sensitive to changes in pH. Figure 31 shows the quinoidal and flavylium cation forms of this type of pigment.

An ethyl link between two flavonols can occur via the same process as described here for linking a flavan-3-ol with an anthocyanin.

Figure 31. Quinoidal base and flavylium cation forms of the ethyl bridged anthocyanin-flavanol pigment.



Quinoidal form



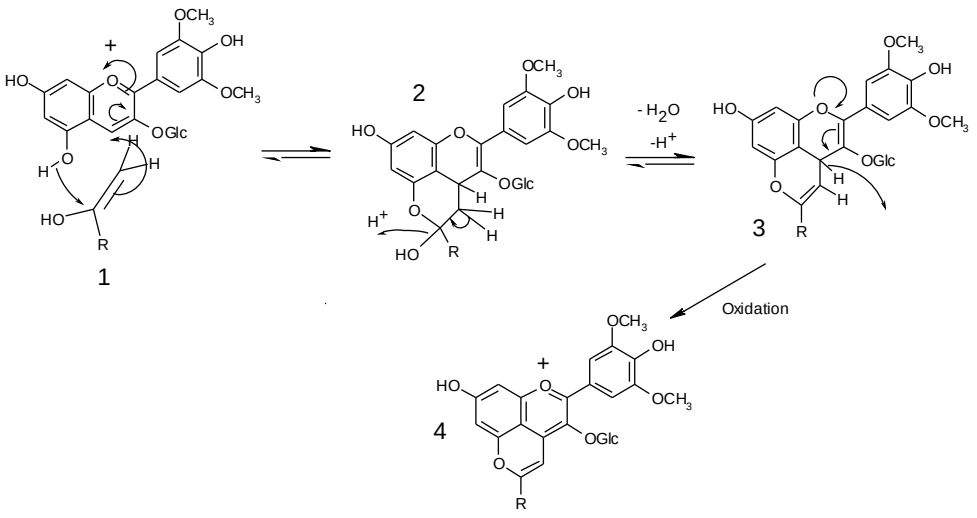
Flavylium cation form

Mechanism c) Cycloaddition reaction

Some wine constituents and yeast metabolites can react directly with the flavylium cation form of an anthocyanin to generate a new pigment. An example is shown in Figure 32 for the condensation reaction between acetaldehyde in its enol form and a flavylium cation. Note that the new ring (cyclisation) has occurred between the C4 position and the hydroxyl group at C5 of the anthocyanin.

The new pigment, known as vitisin-B, is brick-red and its colour is extremely resistant to sulfur dioxide bleaching as well as to changes in pH (Bakker and Timberlake, 1997). It is important to note that vitisin-B is a monomeric species and yet is resistant to pH. So often in the wine literature, it is assumed that only polymeric pigments resist SO_2 bleaching.

Figure 32. Mechanism for the reaction between the enol form of acetaldehyde (hydroxyethene) and a flavylium cation of malvidin-3-glucoside.



This type of pigment is classified as a pyranoanthocyanin, as the new ring that is formed between the C4 and C5 positions is a pyran ring in organic chemistry nomenclature.

A wide range of compounds of this general type has been prepared and examined. The requirement appears to be that the reactive molecule must possess a polarisable double bond, similar to that shown in Figure 32 for the enol form of acetaldehyde. These include the enol form of pyruvic acid (yeast metabolite), caffeic acid (a hydroxycinnamic acid), vinyl phenol and vinyl flavonols (Fulcrand et al., 2006). The actual pyranoanthocyanins formed appear to depend on grape cultivar and winemaking conditions (Fulcrand et al., 2006). Generally, the

molecules exhibit a red colour, although a blue 'portisin' has also been identified (Mateus et al., 2003). More recently, Oliveira et al. (2010) has described a turquoise blue methyne linked pyranoanthocyanin dimer found in port wine. The colour properties of portisins or vinylpyranoanthocyanins have also been published (Carvalho et al., 2010). Resistance to SO_2 bleaching is a major characteristic of these pyranoanthocyanins.

Mechanism d) Pigments from flavan-3-ols and wood aldehydes

Aldehydes derived from oak, such as sinapaldehyde and coniferaldehyde, have been found to react with flavonols to give a new class of pigments, known as oaklins. The mechanism of formation of these compounds has been addressed by de Freitas et al. (2004) and Sousa et al. (2005). In essence, condensation and new ring formation occurs between the C8 position and C7 hydroxy group of the flavan-3-ol with a residual positive charge residing on the oxygen atom that was originally at the C7 position of the flavan-3-ol. While most work has been carried out using model systems, at least one pigment has been detected in wine (Oliveira et al., 2006). The pigments exhibit an orange-red colour, compared with the colourless flavan-3-ol starting product. The pigments resist SO_2 bleaching and are stable towards changes in pH (Oliveira et al., 2006).

Summary of mechanisms

The four mechanisms described above are generally well understood. However, kinetic parameters for the various mechanisms have not been examined in detail as it has always been assumed that reactions between anthocyanins and tannins occur slowly as a wine ages. Work by Morel-Salmi et al. (2006) using flash release of grape phenolics followed by pressing and fermenting clear red juice showed about a 50% loss of anthocyanins and 40% loss of condensed tannins after 5 days of fermentation. This suggests that, even allowing for anthocyanin losses through adsorption onto yeast lees, the reactions between anthocyanins and condensed tannins may be initiated very early in the wine making process.

A detailed kinetic study of reaction mechanisms would provide insight into the formation of the pigmented species as the wine evolves. Data of the evolution of some pyranoanthocyanins has been published by Rentzsch et al. (2007a) and the pathways to their formation has been reviewed (Rentzsch et al., 2007b)

Polymeric pigment production and wine ageing

The shift in colour from the purple-red hue of young wines to a more red or red-brown hue is well recognised. As noted in the introduction to this Section, the colour change has long been associated with the production of polymeric pigments on the basis that they were not dialyzable (Somers, 1971). The colour change with age is also associated with the production of pigments that are more resistant to bleaching by sulfur dioxide and the pigment colour is markedly less sensitive to changes in pH when compared with anthocyanins. Over the last 20 years, there has been extensive research on pigment production and an amazing article by Alcalde-Eon et al. (2006) lists 129 compounds that were identified in a study on Tempranillo wines.

In terms of the mechanisms described above, the following comments can be made:

- the formation of F-A^+ pigments is more favourable than A^+-F pigments at pH values less than 3.8 (Fulcrand et al., 2006);
- higher temperatures seem to favour the A^+-F pigments (Fulcrand et al., 2006);
- pyranoanthocyanins are extremely stable and long-lived: Vitisin-A has been found in 20 or more-year old Tempranillo wines (Rentzsch et al., 2010);

- Vitisin-A is more resistant to oxidation that ethyl-linked pigments (Rentzsch et al., 2010);
- ethyl-bridged species undergo acid hydrolysis with a consequent loss of the purple colour associated with these pigments (Mateus et al., 2003);
- the rate of degradation of the ethyl-linked pigments increases with temperature and decreasing pH;
- the 'vinyl phenol' degradation product from the degradation of the ethyl-bridged pigments can re-react with an anthocyanin to give a new pyranoanthocyanin (Mateus et al., 2003)

The particular stability of pyranoanthocyanins within the wine matrix suggests that their chemistry should be a major focus for understanding the relationship between pigments, wine age and colour. This is one of the major conclusions of the study by Alcalde-Eon et al. (2006) and reinforced by Rentzsch et al. (2007b).

Separation and nature of pigmented material

Bourzeix et al. (1980) used column chromatography to fractionate red wines into 'red polymers' and 'brown polymers'. Bourzeix et al. (1980) estimated the molecular mass of the red polymers to be around 560, implying that no more than 2 flavonoid units could be present. They did not investigate the brown polymers.

There have been several more recent attempts to fractionate the 'humps' observed in the RP-HPLC chromatograms of red wine, with perhaps Salas et al. (2005) achieving the most success. Using high speed counter current chromatography, five fractions were separated. Ethyl-bridged flavanol anthocyanin dimers were located in fraction 4, while direct flavanol-anthocyanin dimers were found in fractions 2 and 3. Colourless compounds were also found in the various fractions. Pyranoanthocyanins and anthocyanin dimers were found in fraction 1. Fraction 1 was not fully characterised, although thiolysis suggested the existence of condensed tannin-anthocyanin oligomers (Salas et al., 2005). Thiolysis data coupled with glucose determination suggested a ratio of three flavan-3-ol molecules to one anthocyanin.

According to Santos-Buelga and de Freitas (2009), this tetramer (1 anthocyanin to 3 flavan-3-ols) is the largest anthocyanin-condensed tannin reported. However, Santos-Buelga and de Freitas (2009) appear to have overlooked the work of Hayasaka and Kennedy (2003) who reported pigmented polymers up to 8 units: one anthocyanin and seven flavan-3-ol units.

Speculation about the existence of larger pigmented polymers is reasonably common in the literature (see, for example, Santos-Buelga and de Freitas (2009) and Salas et al. (2005)). In fact, citing their own unpublished data, Santos-Buelga and de Freitas (2009) suggest that methodological issues may be significant in the interpretation of the results. For example, diluting the red wine showed the appearance of monomeric anthocyanins that were not present in the original chromatographic hump. This in turn implies some form of dispersion of an assembly of molecules (see also Section 5). The possibility of anthocyanins being retained within a molecular assembly was first proposed by Somers (1966). Clearly, this issue requires further research. The related question in examining the mechanism of pigmented polymer formation is how does the oxidation step (see mechanism a, page 69) occur in the presence of SO_2? Whether there is a critical level of SO_2 that impedes this oxidation has not been determined.

Summary of research into changes during ageing

Some of the critical issues to be resolved are:

- are there actually polymeric pigments *per se* or are anthocyanins physically, rather than chemically, attached to molecular assemblies in the wine matrix?
- are pyranoanthocyanins major contributors to the pigment pool, especially as the wine ages? Alcalde-Eon et al (2006) report that, in a 2-year old wine, pyranoanthocyanins are about 70% of the total derived pigments with the value decreasing to about 50% in a 5-year old wine (Boido et al., 2006);
- are the concentrations of ethyl-bridged anthocyanin-flavanol compounds and direct condensation anthocyanin-flavanol compounds high enough to impact on colour, especially if their concentrations decrease over time (Alcalde-Eon et al., 2006)?

Santos-Buelga and de Freitas (2009) summarise the issues succinctly as:

> *...a significant part of the colour expressed by aged red wines is still not satisfactorily explained and even in the case of the identified pigment families the actual contribution of each of them is not yet well established. Thus, further studies are required to elucidate the actual contribution of each of them to the definition of the colour of red wines.*

Resistance to sulfur dioxide bleaching

The bleaching effect of sulfur dioxide on red wine is well-known and, as mentioned earlier in this Section, is a consequence of the addition of hydrogen sulfite to the C ring of the flavylium cation (at positions 2 or 4).

Fascinatingly, the majority of studies on SO_2 bleaching have been carried out at pH values lower than occur in wine, for example pH 2.5 or even 1.5. Low pH values are required for the anthocyanin to exist predominately in the red coloured flavylium cation form. At pH 3.5, malvidin-3-glucoside, the anthocyanin most commonly used in model studies, is 89% in its colourless form, according to the data of Broulliard and Delaporte (1977).

Salas et al. (2005) compared SO_2 bleaching effects on the 'fraction 1' isolated from a red wine (see above page 72). Using a very high concentration of SO_2, the authors found that at pH 2.5, 50% of the pigments were resistant to bleaching compared with 60% at pH 3.5. Considerably less bleaching occurred when 'oenological' levels (not quantified) were used.

It is apparent that some pigmented species are resistant to SO_2 bleaching effects at wine pH where monomeric anthocyanin bleaching is known to occur. SO_2 bleaching resistance is exhibited by pyranoanthocyanins (Sarni-Manchado et al., 1996) and ethyl-bridged anthocyanin-flavanol dimers (Escribano-Bailon et al., 2001). Resistance to SO_2 bleaching by anthocyanin oligomers and directly linked anthocyanin-flavanol oligomers and polymers appears not to have been examined.

A re-examination of the resistance to SO_2 bleaching by individual pigments most commonly found in wine is therefore required. The issue to be addressed is that the expression of colour at wine pH, where monomeric anthocyanins are mostly colourless, may not be due to 'polymeric pigments' but rather a reflection of the contribution to colour by a wide range of monomeric and dimeric compounds.

Interactions in the wine matrix

Three types of interactions will be considered:

- copigmentation;
- protein-tannin interactions;
- polysaccharide interactions.

This component needs to be read in conjunction with the component on molecular assembly in Section 5.

Copigmentation

Copigmentation can be described as the hydrophobic interactions between the planar forms of anthocyanins with other organic molecules, referred to as copigments. Generally the copigments are flavonols. Boulton (2001) has reviewed the basic concepts of the copigmentation phenomenon. The copigmentation effect is usually estimated from the effect of dilution on the absorbance values in the visible range and some reports suggest that the effect may contribute 30 to 50% of the colour in young red wines (Boulton, 2001).

The copigmentation phenomenon is well-established in plant pigments, but its importance (even occurrence) in wine is the subject of considerable debate. The controversy is related to the lower anthocyanin concentration in wine compared to that in plant vacuoles and to the dissociating influence of ethanol on the proposed copigmentation structure (Dangles and Brouillard, 1992). A further contributing factor to the controversy is that many studies have been carried out using model systems in such a way that the results are not directly applicable to wine.

The lifetime of the copigmentation effect is one area where the published work appears confused. There is general acceptance that a decrease in the copigmentation effect occurs with wine age. For example, Hermosin et al (2005) in a study of several varieties including Tempranillo, Cabernet Sauvignon and Syrah, reported a copigmentation contribution to colour of 32 – 45% in new wines, decreasing to 20 – 34% after 3 months with a further decline to 0 – 5% after 9 months. Others (Darias-Martin et al., 2007; Lorenzo et al., 2005) claim around 20% copigmentation after 1 or even 2 years. Whether this is a varietal or winemaking effect has never been resolved.

While ethanol is known to enhance the dissociation of copigmentation complexes, Hermosin (2003) has presented data that shows a copigmentation effect at 12 – 14% ethanol. The bathochromic shift observed in the presence of ethanol in model studies was not observed in the wines used by Hermosín Gutiérrez (2003) until the ethanol concentration reached over 20%. This again points to the conflict between model studies and actual wines.

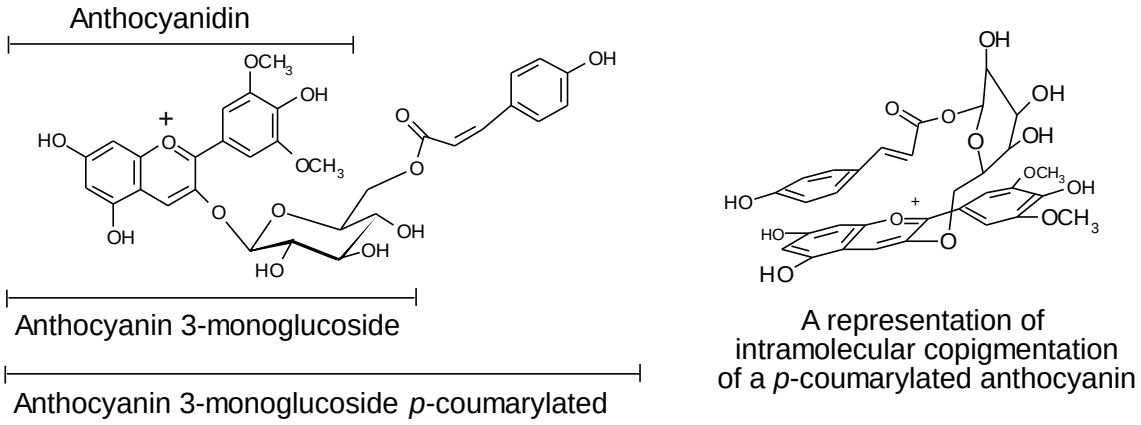
The nature of the cofactor is one aspect of copigmentation that is often overlooked. While there is strong evidence that flavonols, such as quercetin, are important, there is on-going research examining the ability of a range of phenolic compounds to act as effective co-pigments. Some of the research has delved into structure and quantum mechanical calculations to determine the most appropriate molecular orientations for effective copigmentation (see Kunsági-Máté et al., 2006, for example). This type of fundamental physical chemistry work is still an active area of research: a comparison of vinyl catechin dimers with the catechin dimer, procyanidin B3, in terms of effectiveness as copigments has recently been published (Cruz et al, 2010). While this fundamental work is fascinating from a chemistry perspective, it does point out that there is insufficient information on wine components that can act as effective copigments.

There are different mechanisms for copigmentation that are postulated:

- intermolecular copigmentation: anthocyanins associating with other molecules (copigments);
- intramolecular copigmentation: interactions between residues or substituents within the anthocyanin molecule;
- self-association: a form of molecular assembly, as described in Section 5.

Intramolecular associations have the possibility of occurring when the glucose unit of the anthocyanin monoglucoside is esterified with p-coumaric acid. Figure 33 presents a schematic representation of the process and shows how the aromatic rings are able to 'stack' over one another. There is a suggestion that the absence of p-coumarylated anthocyanins in Pinot Noir may contribute to its lack of colour intensity in comparison with Cabernet Sauvignon (Schwarz et al., 2005).

Figure 33. Schematic representation of intramolecular association



Self-association is generally assessed using visible spectroscopy and noting deviations from Beer's Law, although Boulton (2001), using circular dichroism, claimed self-association is of little relevance. On the other hand, young red wines in which the classic copigments were absent showed deviations from Beer's Law, thereby providing evidence for self-association (di Stefano et al., 2005). Further, González-Manzano et al. (2009) present data from model systems to support the contribution of self-association to colour expression.

In essence, mechanisms of copigmentation have been developed using model systems and speculation on the relative contribution of each mechanism to their influence in red wines has followed. The mix of anthocyanins, copigments and ethanol in wine makes isolation of individual mechanisms complex. A greater understanding of all interactions in the wine matrix is required before a full understanding of copigmentation is possible.

Protein-tannin interactions

Interactions between proteins and tannins are well-recognised as playing a role in wine sensory properties, especially astringency perception, as well as haze formation and inhibition of enzyme activity. There would appear to be little specificity in terms of either the protein or the flavonoids, although proteins rich in proline (PRPs) have a high affinity for interacting with tannins. Flavonols and non-galloylated flavan-3-ol monomers appear to have some affinity for proteins, but there is no evidence of aggregate formation (Terrier et al., 2009).

Nephelometry (light scattering measurement) has been used to study the interactions between protein and tannins. In some cases, bovine serum albumin (BSA) was used as a model protein (Carvelho et al., 2004) while in others, salivary proteins were used (de Freitas and Mateus, 2001a). PRPs were more effective than BSA and α-amylase in binding condensed tannins extracted from grape seeds. The ability of condensed tannins to bind PRPs and α-amylase increased with the average molecular mass of the condensed tannin fraction. In addition to molecular mass, there would appear to be a structural influence as flavan-3-ol dimers linked C4-C8 were more effective than those with a C4-C6 linkage (de Freitas and Mateus, 2001b). Similar results were obtained using fluorescence quenching (Soares et al, 2007).

The interaction between proteins and condensed tannins is understood to be a combination of hydrogen-bonding and hydrophobic effects. The hydrophobic effects are more probably van der Waals-London interactions. These interactions have been documented by Oh et al. (1980), Murray et al. (1994) and Charlton et al. (1996) (see also Santos-Buelga and de Freitas, 2009). Murray et al. (1994) used nmr to identify stacking between the phenolic rings of the tannins and proline residues with hydrogen bonding between the hydroxyl groups on the phenolic B-ring and hydrogen acceptor sites of the peptide bond. Further evidence for the two types of bonding was obtained in an isothermal calorimetry study of the interactions between flavan-3-ols and poly-L-proline (Poncet-Legrand et al., 2007a). Entropy changes were interpreted as reflecting hydrophobic effects and conformational changes with enthalpic changes reflected hydrogen bonding effects.

It is important to recognise that interactions between tannins and proteins do not always lead to precipitation. Terrier et al. (2009), in summarising the aggregation of flavan-3-ols with proteins, note the following:

- there would appear to be three stages in the aggregation process;
- the first step is ligand binding and saturation of binding sites;
- this may be coupled with protein conformation change, especially in the case of unstructured proteins such as salivary PRP;
- this is followed by the formation of small protein-flavonoid aggregates that tend to be homogeneous;
- further aggregation can lead to precipitation.

Much of this work has been developed in model studies, essential to understand the processes involved. When applied to wine, the situation becomes more complicated. There has been some work, mainly originating from Montpellier, on structure-function relationships. Gelatin, albumins, caseins, proteins isolated from lupins or wheat and salivary proteins have been reported to exhibit selective precipitation of higher molecular mass condensed tannins (Ricardo da Silva et al., 1991; Sarni-Manchado et al., 1999; Maury et al., 2001). Intriguingly, Maury et al. (2003) observed that only a small proportion of the total flavonoid composition was present in the solid fraction after fining. In fact, the wine composition was essentially unchanged pre- and post-fining. This raises the interesting question posed by Terrier et al. (2009) that the loss of astringency after fining may be due in part to the inclusion of the astringent flavan-3-ols in soluble complexes or aggregates.

Some of the other structure/function relationships that have been reported include:

- formation of insoluble complexes between polymeric flavan-3-ols and proteins increases with the degree of flavan-3-ol polymerisation and galloyation (Haslam and Lilley, 1988);
- isothermal calorimetry experiments using poly-L-proline (a model system) did not detect binding with (+)-catechin or (-)-epicatechin whereas interactions were found with epigallocatechin gallate and epicatechin gallate (Poncet-Legrand et al., 2007a);
- some lower molecular mass gelatins and glutens are more selective than larger ones (Maury et al., 2001; Maury et al., 2003).

The issue of structure/function relationship clearly requires further study. The role of polysaccharides in influencing the interactions between condensed tannins and proteins is discussed in the next component of this Section.

Polysaccharide-tannin interactions

The capacity of polysaccharides to inhibit protein-tannin interactions was established some time ago (Ozawa et al., 1987; Luck et al., 1994). Luck et al. (1994) referred to the capacity of polysaccharides to moderate the astringent response in foods and beverages.

There have been two approaches to understanding the role of polysaccharides in affecting protein-tannin interactions and hence astringent response in wine. Work by the group of Professor de Freitas of Porto, Portugal, has used nephelometry to examine polysaccharide/protein/tannin aggregations. De Freitas et al. (2003) found that polysaccharides induced a solubilisation of protein-tannin complexes with pectin, xanthan, polygalacturonic acid and gum arabic being effective. There is also a structure/function relationship between the effectiveness of the polysaccharide in inhibiting the protein-tannin aggregation, although the conclusions from various studies are not always in agreement (Mateus et al., 2004; Carvalho et al., 2006a). In further investigations using salivary proteins and condensed tannins isolated from grape seeds, Carvalho et al. (2006b) found different responses from acidic polysaccharides with respect to α-amylase and IB8c, a synthetic proline-rich protein.

The INRA research group in Montpellier, France, has utilised its general knowledge base on polysaccharides in wine to examine polysaccharide-tannin interactions. Riou et al. (2002) used dynamic light scattering (DLS) to examine the influence of polysaccharides on tannin aggregation: no protein was present in this study. In general, it was observed that polysaccharides did not inhibit tannin aggregation; rather they affected particle size evolution. Neutral arabinogalactanprotein (AGP0) and rhamnogalacturonan-II (RG-II) monomer had no effect while manoproteins (MPs) and acidic AGP4 showed a strong inhibition effect. The RG-II dimer actually enhanced the mean particle diameter, suggesting the possibility of co-aggregation.

The efficiency of MPs as stabilising species decreased as their molecular mass increased which led Poncet-Legrand et al. (2007b) to conclude that steric factors might be important. From these observations, Poncet-Legrand et al. (2007b) proposed a mechanism where high molecular mass polysaccharides generate ‘bridging flocculation’ whereas smaller molecular mass polysaccharides generate high surface coverage which limits the interaction between the tannin compounds resulting in smaller particles. This mechanism is highly speculative, to say the least. As noted above, proteins were not part of the Montpellier studies.

Based on their studies on the polysaccharide/protein/tannin systems in fruits and beverages generally, Haslam and co-workers have suggested two possible mechanisms for the role of polysaccharides in inhibiting the protein-tannin interactions (Luck et al., 1994; Haslam 1998; see also Mateus et al., 2004):

- charged (polyelectrolyte) polysaccharides could form ternary complexes with protein-tannin aggregates to enhance solubility;
- polysaccharides could encapsulate tannins and thereby interfere with their ability to aggregate with tannins.

Increasing ionic character of the polysaccharides enhances their effectiveness in inhibiting protein-tannin aggregation (Luck et al., 1994; Carvalho et al., 2006b), supporting the first proposal. Recent work by Soares et al. (2009) on polysaccharide inhibition of α-amalyse-condensed tannin interactions provides further evidence for the first of the two proposed mechanisms.

On the other hand, the work of Poncet-Legrand et al. (2007b) is perhaps more supportive of the second proposal. Clarification of the mechanism is essential for understanding how changes in the colloidal state can affect the sensory properties of the wine.

Summary
This Section has identified the need for several areas of additional research.

The critical issues of research into changes during wine ageing are presented on page 76 and are not repeated here.

Additional areas are:

- information of the kinetics of the various mechanisms for polymer formation to provide insight into the formation and/or re-arrangement of pigmented polymers as the wine ages;
- an examination of the impact of reduced oxygen availability from efficient bottling and packaging on the development of colour, especially pigment production that requires an oxidation step;
- a re-examination of the resistance towards SO_2 bleaching under wine conditions to determine the relative importance of pigment types to colour expression;
- a better understanding of the mechanisms and relevance of copigmentation as a contributor to wine colour expression;
- a clearer understanding of the structure/function relationships that determine the interactions between proteins and tannins;
- a more detailed assessment of changes in wine composition that occur as a consequence of fining with proteins: that is, are more astringent molecules removed from solution or encapsulated in some way that minimises their impact on sensory responses;
- clarification of the mechanism of polysaccharide inhibition of protein-tannin interaction.

The real challenge now is to understand how the various reactions that have been described in this ection actually occur in the wine matrix. Model studies have provided unquestionable advances in our knowledge of these processes and the recent work by Stranks et al. (2009) may provide a mathematical approach for describing aggregation processes involving proteins. Extrapolation from model studies to the real context of wine chemistry needs to be done with care, especially when predictions regarding the impact of individual wine components on wine sensory descriptors are drawn. This point in discussed in more detail in Section 11.

SECTION 7
Extraction of tannins into wine

Aspects of the extraction of condensed tannins and other phenolic compounds during fermentation are well known, with some reports dating back to 1964. However, there are several questions that remain unanswered. This Section begins with a summary of the state of information and follows this with some areas where further research is needed.

The review by Cheynier et al. (2006) and the book chapter by Terrier et al. (2009) present useful summaries of the state of knowledge. Some of the critical points are:

- extraction of condensed tannins during the fermentation continues throughout contact between the fermenting liquid/wine and grape components (must) (Singleton and Draper, 1964);
- extraction of anthocyanins, however, reaches a maximum early in the fermentation (Nagel and Wulf, 1979);
- the difference in extraction times is due in part to the differential solubilities – anthocyanins are soluble in an aqueous medium (see Figure 1), whereas condensed tannin extraction requires alcohol;
- the total anthocyanin concentration decreases after the maximum is reached as the rate of conversion to other species exceeds the rate of extraction (Cheynier et al., 1997);
- some anthocyanins are lost by adsorption onto yeast or other grape solids, but recoveries from the solids after pressing do not account for losses measured from the amount in grapes to the amount in wine (Cheynier et al., 2006);
- pre-fermentation maceration increases levels of anthocyanins while post-fermentation maceration increases the concentration of condensed tannins that arise mostly from seeds (Cheynier et al., 2006);
- the extraction of condensed tannins from skins approximates that of anthocyanins while that from seeds is slower (Morel-Salmi et al., 2006).

There would appear to be both a degree of ripeness effect and an ethanol concentration effect in understanding the differential rates of extraction of seed and skin condensed tannins. There are two major studies that have examined these effects, but both have used model extraction systems rather than actual fermentations.

Canals et al. (2005) have described the influence of ethanol on the extraction of anthocyanins and phenolic compounds from the skins and seeds of Tempranillo grapes from Tarragona (Spain) at different stages of ripening. They used tartaric acid wine base at 0%, 6% and 13% ethanol. Extraction/maceration on skins or seeds was carried out for 7 days. There was no sugar present in the extracting system, so osmotic influences on the extraction process would have been markedly different to actual fermentation conditions. Their data suggest the following:

- the initial rate of extraction of skin condensed tannins was not dependent on the alcohol concentration, but the final amount extracted was higher at higher ethanol concentrations;
- there was a delay in the onset of seed condensed tannin extraction and significantly higher levels of extracted condensed tannins were achieved at 13% ethanol;
- there was a general trend of increased extraction with ripening, contrary to that with acetone or ethanol extraction (see Section 3), although tannins were quantified by measuring the absorbance at 280 nm which is not a very specific measurement;

- the extraction data was used to estimate the amount of condensed tannin-polysaccharide complexes for skins only – these complexes were found to increase during ripening;
- astringency was assessed by ovoalbumin precipitation – this measured parameter increased in the first stages of ripening and decreased in the final stages, to which Canals et al., (2005) comment that this may be related to an increase in the amount of condensed tannin-polysaccharide complexes;
- at each stage of maturity, the measured astringency was higher at higher ethanol concentrations.

Terrier et al. (2009), in reviewing this work, comment that the differences in extraction rates between skin and seed condensed tannins may be a consequence of the different chemical structures, a reflection of the increased hydrophobicity of seed tannins, or simply to different characteristics of the berry components that leads to differential access to the condensed tannins. This question has not yet been resolved.

Fournand et al. (2006) also used a hydroalcoholic solution to examine the extraction of skin condensed tannins, as well as anthocyanins, as a function of ripening from Shiraz grown in Montpellier (France). Extraction occurred at 27^{o}C for 5 hours. As with Canals et al. (2005), sugar was not present in the extracting solution. As part of this study, the extraction solutions and residual solid parts were analysed for condensed tannins by HPLC after phloroglucinolysis. Their work showed:

- extracted tannins amounted to about 23% and non-extracted tannins 62% of the total amount;
- mDP was much higher in the non-extracted fraction:19 (extracted) versus 55 (non-extracted);
- the galloylation rate was slightly higher in the non-extracted fraction: 3.7% (extracted) versus 4.8% (non-extracted);
- there was very little change in these parameters as a function of berry ripening.

Fournand et al (2006) conclude that the minor compositional changes they observed cannot account for the loss of astringency usually observed during ripening, although they did not assess astringency in this study. They speculate that the astringency change may be due to increased polysaccharide extraction from cell wall degradation as berries ripen. It is unfortunate that this study did not assess the polysaccharide content. An extraction time longer than 5 hours may have been more meaningful in an attempt to simulate fermentation conditions.

The studies by Canals et al. (2005) and Fournand et al. (2006) provide interesting insights into what may occur during fermentation, but both are limited for the reasons mentioned above. In fact, it is reasonable to suggest that protocols that claim to simulate extraction during the maceration and fermentation (see Sections 2 and 3) will always be limited as they are unable to reproduce the effect of increasing alcohol concentration, temperature fluctuations and duration of the maceration/fermentation. This then opens up a new avenue for research.

Work on fermenting red wine systems is complicated, if only in attempting to determine how best to take accurate samples in the highly variable matrix. The study by Morel-Salmi et al. (2006), mentioned in Section 6, may provide an approach to examining red wine fermentations: flash release of phenolic compounds followed by pressing and then

fermentation in the absence of solids. Their data suggests that anthocyanin and tannin reactions may actually start early in the wine making process rather than occur slowly during ageing and, as mentioned in Section 6, this needs more detailed investigation.

Morel-Salmi et al (2006) also determined the concentrations of condensed tannins and anthocyanins in Grenache wines grown in Gruissan (France). At the end of fermentation, the condensed tannin concentration in the wine was 40% of the total grape concentration, increasing to 60% for the flash release sample. Extraction of the pomace, after pressing, allowed recovery of the remaining amount in each case. In comparison, anthocyanins in the control wine amounted to just under 20% and increased to just over 25% of the total after extraction from the pomace. For the flash release sample, the values were 30% (wine) and 40% (after pomace extraction). While the loss of anthocyanins after the initial peak during fermentation is not new (Nagel and Wulf, 1979), Morel-Salmi et al. (2006) suggested that the tannin-anthocyanin ratio and the formation of F-A+ dimers (T-A adducts in their terminology) might be important indicators of colour stability. The results however were varietal dependent, but the approach does suggest a possible new way to assess colour potential. Tracking the fate of the anthocyanins is clearly an important issue.

Summary

The review of published work has opened up several possible lines for further enquiry:

a) understanding the factors that determine the differential extraction of condensed tannins from skins and seeds – is this a chemical structural (hydrophobicity) issue or a grape tissue structural issue?

b) validation of the rapid extraction and tannin assessment protocols against what happens in maceration/fermentation;

c) examination of the relationship between berry ripeness and the release of polysaccharides – this links with work in Section 3, Section 5 and Section 6;

d) examining in more detail the flash photolysis/pressing/fermentation without skins study referred to above as a means of examining the reaction processes that involve condensed tannins and anthocyanins;

e) this medium would also allow acetaldehyde bridging reactions to be followed more effectively, as solid components would not interfere with the sampling analysis process - in essence, the fermenting system would be akin to a white wine fermentation;

f) an examination of the tannin-anthocyanin ratio as a possible predictor of colour stability;

g) tracking the fate of anthocyanins with the aim of maintaining higher concentrations, preferably stabilised, in the finished wine;

h) developing approaches to release more condensed tannins from grapes into wine (see also Section 3).

SECTION 8
Role of oxygen in tannin modification in grapes and wine

Oxygen access to red wine can occur at several stages during the wine's life. Various winemaking strategies during the fermentation of skins allow mixing between the fermenting juice/wine and air, for example délestage. Perhaps the best example of introducing oxygen is in the traditional foot crushing practice of the Douro Valley or cap plunging by foot in Burgundy. There is oxygen ingress during barrel storage and at rackings. The bottling process and ingress post-bottling (dependent on closure or seal used) also contribute to the possibility of oxygen pickup. While oxygen transmission rates can be measured in some instances, the amount of oxygen pick-up during wine processing is generally unknown.

The deliberate addition of oxygen in measured and known amounts, through micro-oxygenation, is discussed in Section 9.

The majority of research on wine and oxygen has focussed on white wine. Several mechanisms have been presented for pigment development in white wine and more recently for aroma changes. The recent review by Karbowiak et al. (2010) summarises the mechanisms for wine oxidation with a specific focus on white, rather than red, wine. This review by Karbowiak et al. (2010) also addresses theoretical and practical aspects of oxygen ingress after bottling, discussing particularly the role of the closure.

Detailed oxidation studies describing tannin structural modification are rare. Generally, the focus has been on colour and sensory changes, rather than on the chemical changes to tannin composition (see, for example, Caillé et al., 2010). This effect is described in more detail in Section 11.

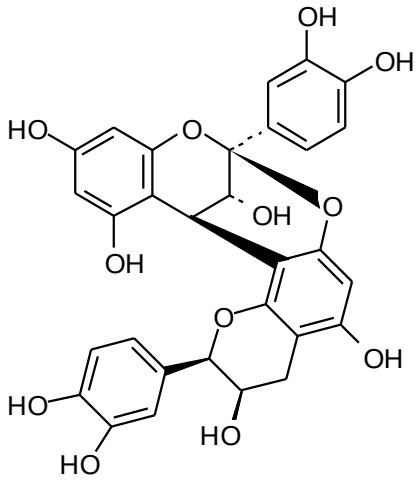
Atanasova et al. (2002) have, however, examined in some detail the effect of oxygenation on polyphenolic compounds. The wine was a blend of Cabernet Sauvignon (60%) and Tannat (40%) to which pure oxygen was added at a rate of 5 mL L^{-1}. The period of oxygenation is not given in the paper. Sulfur dioxide appears not to have been used. In comparing the control (saturated with nitrogen) and the oxygenated wines, Atanasova et al. (2002) found:

- the oxygenated wines showed a significant increase in the concentrations of pyranoanthocyanins, ethyl-linked compounds and several new derived pigments;
- acetaldehyde production through oxygenation was a key factor in pigment formation;
- at 1 month after treatments, the wines exhibited higher levels of pigments that could be bleached by SO_2;
- after 7 months, pyranoanthocyanins and other more stable (that is resistant to SO_2 bleaching) pigments were found to be accumulating;
- the formation of $(epicatechin)_n$-pyranomalvidin-3-glucoside (n = 1 or 2) opens up the possibility for new stable pigment production.

The work by Atanasova et al. (2002) has clearly opened up a range of issues relating to the development of red wine in the presence of oxygen. The limitation of their work however is the amount of oxygen that seems to have been added to the wines – this far exceeds normal wine ageing (barrel storage, rackings, bottle development). None-the-less, the analytical approaches that they adopted are widely applicable and could be readily applied to winemaking and wine ageing trials that are designed to resemble more closely the conditions under which wines age.

So-called A-type procyanidins (Figure 33) are thought to be formed as a result of phenolic oxidation. The A-type procyanidins contain two bonds between the two flavan-3-ol monomers, compared with only one bond in the B-type procyanidins (Figure 24). There appears to be little published information about the formation and impact of A-type procyanidins in wine, although they have been studied in other fruits (see Le Roux et al., 1988).

Figure 33. Structure of procyanidin A2



A-type procyanidins have been detected in wine and they are known to impact on the calculation of mDP acid by thiolysis (see Section 5).

A more detailed study of these A-type procyanidins may provide insight into oxidation processes, not only in wine, but also in the grape berry. The potential for A-type procyanidins to be markers for oxidation is an area to be studied.

Mechanisms of oxidation

Reviews by Waterhouse and Laurie (2006) and Karbowiak et al. (2010) summarise the general mechanisms that are thought to apply to wine oxidation. Much of the published work tends to reflect the well-established enzymatic processes. Waterhouse and Laurie (2006) have proposed a 'comprehensive scheme' that includes Fenton chemistry and hydroxyl radical production. Elias and Waterhouse (2010) have recently re-visited this mechanism, proposing a way to control the Fenton chemistry and thereby protecting wine from this type of oxidative spoilage.

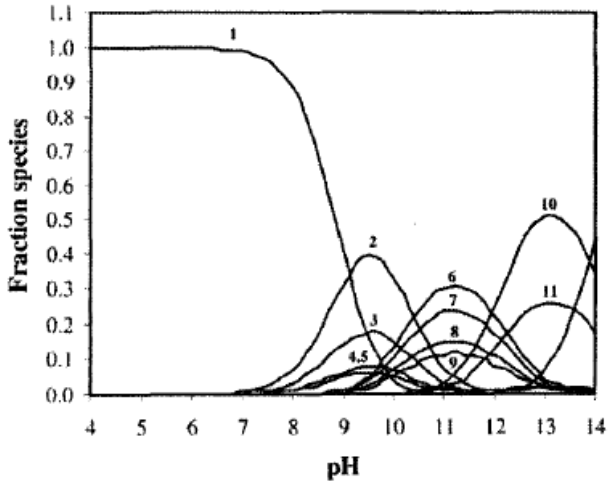
Much of the published work on non-enzymatic or chemical oxidation has focussed on the role of metal ions (ions of iron and copper in particular) as catalysts or mediators of the oxidation process. While metal ions play an important role, one unanswered question is the chemical form in which the ions exist. Metal ions can form coordination complexes with organic molecules (ligands) of which organic acids are a good example. That is, iron(III) for example could exist in wine as a complex with tartrate, rather than as an aquated ion, commonly represented as Fe^{3+}.

Clark et al. (2007) recognised this in their study of light induced degradation of tartaric acid. Iron(III) tartrate is photoactive in the low visible wavelength range (it was used in photography) and its photoactivation can lead to hydroxyl radical production. The results of

photo-degradation were the same as those induced by Fenton chemistry, but occurred at lower iron concentrations (Clark et al., 2007). Similarly, Clark et al. (2003) have demonstrated that copper ions mediate the glyoxylic acid bridging of two catechin-type phenolic compounds. Although these results were outputs from modelling white wine oxidation, they demonstrate the complexity of studies on oxidation mechanisms.

To date there is no convincing evidence of metal ion binding to wine phenolic compounds (especially the B-ring), although this has been the source of considerable speculation in some mechanisms. Further, some mechanisms suggest quinone formation of the *ortho*-hydroxy groups of the B-ring of catechin as a precursor of oxidation. The reactivity of the B-ring is well-established at higher pH values and many oxidation studies have been carried out at pH values around 7. The difficulty here is that the chemistry changes with pH. At wine pH, the hydroxyl groups of the B-ring are fully protonated (see curve **1** in Figure 34) and ionisation does not occur until the pH is over 6.5 (**2** in Figure 34). More detailed studies of oxidation mechanisms at wine pH are called for.

Figure 34. pH distribution of ionised forms of (+)-catechin. Data extracted from Herrero-Martínez et al. (2005).



Kinetics of wine oxidation reactions

Acetaldehyde is generally seen as a mediator of several of the major structural changes in the polyphenolic compounds resulting from oxidation. But herein lies a major complexity in understanding the chemistry of the oxidation process. If sulfur dioxide is present in the wine, it is reasonable to expect that it will bind with acetaldehyde, possibly in preference to acetaldehyde mediated condensation reactions.

There are several competing reactions in the wine matrix during oxidation. These include:

- the scavenging of hydrogen peroxide formed from oxygen reduction and its reaction with SO_2 will lead to a reduction in the total SO_2 concentration;
- hydrogen peroxide may oxidise ethanol to form acetaldehyde;
- reaction between SO_2 and acetaldehyde which will increase the bound SO_2 concentration;
- possible rapid depletion of SO_2 at a point of oxygen ingress would allow for an increase of the acetaldehyde concentration in this localised environment;

- condensation reactions of acetaldehyde with flavonoids, including anthocyanins, resulting in phenolic structural change;
- depletion of free SO_2 by hydrogen peroxide, leading to dissociation of the acetaldehyde/bisulfite addition product and the subsequent involvement of the liberated acetaldehyde in condensation reactions

The absence of kinetic data for the individual reactions that can occur in the wine matrix during oxidation makes decisions regarding the reaction sequences/reaction mechanisms difficult, if not impossible. Improving the knowledge base of kinetic data for the various competing reactions is critical.

Summary

This analysis of published information on oxygen and tannin modification suggests the following areas requiring further research are:

- polyphenolic structural and compositional changes that occur as a result of oxygen ingress that is representative of winemaking and wine ageing conditions;
- a better understanding of the formation and role played by A-type procyanidins;
- further development of wine oxidation processes/mechanisms including knowledge of the chemical speciation of metal ions;
- rigorous kinetic data for the competing reactions that could be involved in wine oxidation.

Structural changes to tannins through oxidation will almost certainly influence sensory perception and a greater understanding of the chemical changes is required. Research in this area is important.

SECTION 9
Micro-oxygenation

Microoxygenation (MOX) is best described as the controlled addition of oxygen into wine. The importance of oxygen in red winemaking has been known for a considerable period (Singleton, 1987). Previously, oxygen additions could be considered to be uncontrolled as the oxygen increase occurred during racking (sometimes with a spray ball), pumping over and ingress through staves, for example. The introduction of the micro-bullage system (Docournau and Laplace, 1993) and the semi-permeable tubular membrane system (Kelly and Wollan, 2002) established technologies for proper control of the amount of gaseous oxygen being introduced into a tank or barrel. The relatively recent introduction of high density polyethylene (HDPE) tanks, (Flextanks; see Flecknoe-Brown, (2005) for example) has added a new variant to the controlled introduction of oxygen.

The introduction of MOX created a wave of enthusiasm amongst winemakers across the world, even making an entry in the documentary movie *Mondovino*[6]. Some of the claimed advantages of MOX for red wine are

- Improving the body, structure and fruitfulness of red wines;
- Allowing controlled ingress of oxygen into wine without the need to put wine in barrels for long periods of time;
- Inducing acetaldehyde cross linking of tannins, producing stable pigments;
- Lower winemaking costs, as barrels can be replaced with tanks, with oak character generated from the addition of chips or staves.

The enthusiasm seems to have waned of late, although there are still a significant number of research papers appearing. On the other hand, correspondence from winemakers suggests that the initial euphoria regarding the value of MOX is now somewhat tempered. Schmidtke et al. (2010) have recently reviewed various aspects of MOX. This review summarises the MOX equipment used in winemaking, analyses reported applications of MOX and identifies inconsistencies, especially with the transfer of information for research trials to commercial applications[7].

Introduction of oxygen into wine

a) Micro-bullage system

The micro-bullage system was first introduced into the market in 1993. The system used a ceramic sinter for the introduction of oxygen: see Figure 35. The sinter is constructed from a ceramic material with a pore size between 1-10 µm and is enclosed in a stainless steel housing. The approximate dimensions of the sinter are 5 cm high by 4 cm diameter and it is normally placed near the bottom and in the centre of the tank.

Most of the trial work on MOX has been carried out in stainless steel tanks which are about 3 metre in height and 35 cm in diameter. These narrow volume tanks with a total volume of 305 litres were specifically designed for experimentation: replication is easy and the costs (tank and wine costs) are prohibitive for proper experimental replication in commercial volume tanks. The volume of the 305 litre experimental tanks is also close to that of a barrique.

[6] ***Mondovino*** is a 2004 documentary film on the impact of globalization on the world's different wine regions written and directed by American film maker Jonathan Nossiter.

[7] The review has been accepted for publication and a pdf version of the text can be made available to the GWRDC, if requested.

Figure 35. Images of the sinter used for MOX in the micro-bullage system (photos courtesy of L Schmidtke, CSU).





Tanks used in commercial operations are considerably larger, with dimensions (diameter x height) ranging from 8 kL (2 m x 4.2m) to 272 kL (5m x 13 m). Clearly, the process of oxygen movement in the commercial tanks will be significantly different to what occurs within the smaller volume, narrow diameter experimental tanks. Dykes (2007) developed a fluid mechanical model for the movement of oxygen from the sinter through the tank. In his work, however, tank volumes were only 100 litres.

In essence, the wine in the commercial tanks will form a stagnant fluid system, with minimal or no lateral dispersion of the oxygen plume. Agitation has been used by some winemakers in an attempt to improve oxygen dispersion (personal communications with winemakers), but evidence to support the advantages of this approach is not available.

b) Semi-permeable membrane system
This approach uses a pressurised semi-permeable membrane that is pervious to oxygen, to disperse the gas through the wine container. Depending on the layout of the tube with the wine container, much greater dispersion of oxygen can be achieved in comparison to the micro-bullage system. A 10 m tube is required for a 4 kL tank (Kelly and Wollan, 2002, 2003). Maintaining integrity of the system with longer tube lengths and larger tank volumes is a challenge.

c) HDPE tanks
The HDPE tanks have well defined 'linear' molecular structures, with enhanced and consistent gas permeability, allowing a known amount of oxygen migration from the exterior to the interior over a set period of time. The majority of the trials with HDPE tanks have been within-company evaluations, generally with positive outcomes in terms of improved sensory properties (Flecknoe-Brown, 2005). A more structural trial is presently underway in Tasmania, funded by the AusIndustry program (Smith, 2009). The outcomes of this project will be interesting and could open up new approaches for controlled oxygenation of red wines.

Monitoring the MOX process
a) Measurement of dissolved oxygen
A range of oxygen measuring systems have been used, not always successfully. The recent development of sensors based on luminescence and fluorescence (Nevares, I. and del Álamo, 2008) appear to provide greater reliability in the measurement of dissolved oxygen.

The positioning of an oxygen sensor in relation to the ingress points for oxygen can lead to substantial errors in oxygen readings. Similarly, the position from which a sample is taken is also critical. Further, the proper measurement of the distribution of dissolved oxygen in a large volume tank would need an array of sensors covering the lateral and vertical distribution of oxygen. Nevares et al. (2010) have measured oxygen dispersion in a 15% aqueous alcohol solution, but only in a narrow diameter tank.

Accurate measurement of the DO concentration remains an analytical challenge. Trends in the DO concentration may be observed provided the sensor remains stable over the time course of the trial. Matrix effects on the calibration of the sensors will always influence the accuracy of the measurement.

b) Sensorial monitoring
Regular sensorial monitoring is claimed to be important in ensuring effective outcomes from MOX (Lemaire, 2002). There are however a range of issues that must be considered when adopting this approach:

- Daily monitoring should occur;
- A trained panel is required;
- As wines continue to change after MOX is terminated, experience is required to assess how a wine will taste, say 3 months after the completion of MOX;
- There is a lack of standard reference materials for panel training.

These requirements are significant, both in terms of time and cost, limiting the successful application of this form of monitoring.

c) Other monitoring approaches
Assessment of acetaldehyde development, either sensorially or by GC-MS, has been proposed. However, the build-up of acetaldehyde would suggest that the system is being over-oxygenated, given that the purpose of MOX is to generate acetaldehyde for polymer production (see below).

Similarly, free and bound SO_2 measurements have been proposed as a means of monitoring MOX when it is applied post malo-lactic fermentation. A rapid loss of free SO_2 may indicate over-oxygenation of the wine (Tao et al., 2007). At best, this measurement is only a *de facto* indicator and detection of the rapid loss of free SO_2 may be too late to take remedial action.

Chemical modifications arising from MOX
A wide range of MOX applications have been described in the literature (see Schmidtke et al., 2010). The published applications vary in:

- grape variety;
- whether MOX is applied pre- or post-MLF;
- amount of and rate at which oxygen is infused into the wine;
- duration of MOX;
- tank volume (which is often not stated);
- SO_2 regime (not always mentioned).

This large number of variables makes drawing conclusions difficult, although there is general agreement that polymeric structural changes occur during and after MOX and that these structural changes often involve acetaldehyde.

As discussed in Section 8, one of the most significant reports of structural changes occurring after oxygenation of wine has been published by Atanasova et al. (2002). This study gives an indication of what may happen during the oxygenation, although the conditions used by Atanasova et al. (2002) would appear to be more extreme than occur during routine applications of MOX.

Acetaldehyde is generally seen as a mediator of the structural change in the polyphenolic compounds that result from MOX (Schmidtke et al., 2010). This point has been discussed in Section 8 and the general conclusions on the need for more information of competing reactions also apply here. Specifically in MOX, the possible rapid depletion of SO_2 in the oxygen bubble plume might create conditions for an increase of the acetaldehyde concentration in this localised environment. This in turn could lead to enhanced acetaldehyde-mediated bridging and cyclo-addition reactions. Further examination of this point is required.

MOX experiments have confirmed the gradual loss of free and total sulfur dioxide in wine and a delay in the formation of pigments that are resistant to sulfur dioxide bleaching until the free and total sulfur dioxide concentration falls to around 10 and 40 to 50 mg L^{-1} respectively (Tao et al., 2007). It should be noted, however, that this work was performed in 15 litre research tanks.

The critical role of SO_2 in influencing the chemistry of MOX outcomes has been demonstrated in two recent publications. Rudnitskaya et al. (2009) have described the influence on MOX on wine composition over three vintages. The winemaking protocol used in this study set a very tight specification for molecular SO_2 of 0.6 mg L^{-1}. The SO_2 concentration was continually adjusted during the MOX process to maintain this specification. After completion of MOX and stabilisation of the wines, the wines were analysed for condensed tannins (MCP method, Section 2), Somers colour parameters and CIELab values. The response of a 26-sensor electronic tongue to the phenolic composition was also determined. A chemometrics treatment of the data found the major influence on phenolic composition was vintage, as this accounted for 70% of the variance in the chemical parameters and 33% in the electronic tongue data. A similar conclusion was drawn from the analysis of the mid-infrared spectra of the same wines (Climaco-Pinto et al., 2009).

Del Álamo et al. (2010) focused on a comparison of MOX for a single vintage wine that contained oak chips or staves. The SO_2 concentration was monitored regularly and kept 'low': around 25 mg L^{-1} and adjusted at each sampling time. The results showed that the oxygenation strategy depended on whether chips or staves from various sources were used. For example, wine containing staves consumed more oxygen than chips and chips and staves from French oak required a higher oxygen amount for the same outcome in terms of wine compositional change.

It is difficult to reconcile the differences between the studies of Rudnitskaya et al. (2009) and del Álamo et al. (2010), based on published information. However, Schmidtke[8] (data submitted for publication) found variations between several chemical parameters as well as sensory scores within a vintage, but the between-vintage variation dominated the within-vintage changes. Further study is required to validate the relative influences of between-vintage and within-vintage. This may require the development of a predictive model.

MOX and other outcomes

MOX and the effects of oak chips and staves and the origin of the oak has been the subject of several studies, but outside the main aim of this review. Similarly, there are several microbiological changes, both beneficial and harmful, associated with MOX, but this is also outside the scope of this review.

Summary

MOX may have been originally conceived in response to a perceived need to improve the structure of red wines, especially those which have a high tannin content. The technique may have been promoted as a means for accelerating the ageing of wine, but the reality is that it was seen to be a more economic means for the elevation of wines in tanks, rather than barrels.

While research trials on MOX and red wine have been extensive, the concept of MOX presents a classic example of the difficulty of transferring information from research trials to commercial practice. That is, research trials in small volume (250 to 300 L) tanks do not represent the spatial distribution processes that need to occur in commercial tanks of up to 200 kilolitre or more.

Most of the published trials have understandably focused on phenolic chemistry. However, there is a distinct lack of kinetic data relevant to the competing reactions that can occur within the wine matrix. The next phase in understanding the MOX process must be the production of kinetic data for the various competing reactions that can occur with the wine matrix following the addition of oxygen. Until this kinetic information is available, knowledge of the MOX process and the potential for more efficient and effective control will be limited.

If further studies confirm that the between-vintage variation outweighs the within-vintage variation, it will be necessary to develop a predictive model for the general useability of MOX. The predictive model will need to include:

- marker compounds in the untreated wines that reflect potential for change as a result of MOX;
- marker compounds in the finished wines that reflect the positive and negative changes arising from MOX;
- oxygenation rates, both ingress and consumption;
- the critical role played by SO_2;
- the influence of oak additives on the consumption of oxygen and the subsequent impact on oxygenation addition rates.

[8] Leigh Schmidtke of CSU produced the wines for the study published with Rudnitskaya et al. (2009). Further publications and a PhD thesis are in preparation.

A start towards such a model has been made by del Álamo et al. (2010). The application of chemometrics as a means of understanding the convergence of various analytical approaches, similar to that being developed by Schmidtke (personal communication), will play an important part of this predictive model concept.

SECTION 10
Role that the addition of exogenous tannins plays in modifying colour stability, mouthfeel and astringency of wine

Exogenous tannins are compounds present in wine that were not present in the original grape material used to prepare the wine. The sources can be from oak used in winemaking or by selected addition. Obradovic (2006) has suggested a classification of exogenous tannins as

- gallotannins
- ellagitannins
- grape derived tannins (red skin; white skin, seed, stalk)
- mixtures of the above.

Gallotannins and ellagitannins are so-called hydrolysable tannins (see page 22) and are based on gallic acid or ellagic acid respectively. Grape derived tannins are based on flavan-3-ol units and are condensed tannins.

Hydrolysable tannins are present in oak and can be extracted when wine is in barrel or following the addition of chips, staves or other oak products. Several hydrolysable tannins are also available commercially. Grape derived tannins can also be purchased commercially and, as noted above, mixtures with claimed different functions are also available. Obradovic (2006) has suggested the following roles for exogenous tannin use:

- to ensure palate balance and complexity;
- to stabilise colour of red wines;
- to participate in wine ageing reactions;
- to inhibit laccase activity in *Botrytis*-affected fruit.

Enhancing anti-oxidant capacity and a lowering of reductive characters are two additional roles that can be added to the above list (Dr P Bowyer, personal communication).

The commercial nature of these materials, and the associated commercial-in-confidence issues, means that the chemical composition of these products is not generally available. With a few exceptions, research on these products has been carried out within companies or has been contracted research leading to technical publications. There are few peer-reviewed research papers available.

It would of course be possible to fund an independent assessment of the commercial products so that the structure and possible chemical function can be determined. However, continual slight composition changes in the commercial products would negate the value of this type of work.

As an example of the applied research, Bowyer (2009) has published the results of an industry trial on the use of a commercial product, VR SUPRA, and oak chips. The results of this trial argued strongly in favour of the advantages of using exogenous tannin additions.

Parker et al. (2007) examined the effect of grape-derived tannin (condensed tannin) pre- and post-ferment additions. The authors concluded that under the conditions of this particular study, the addition of tannin did not affect wine colour properties and had only a minor impact on perceived astringency.

Bautista-Ortín et al. (2005) examined the role of ‘enological’ tannin addition to Monastrell. One tannin was a gallotannin and the other was a condensed tannin. While the study design is

not particularly clear and the trials also used macerating enzymes, the conclusions are similar to those of Parker et al. (2007):

- there was no improvement in the colour or sensory characteristics following tannin addition;
- some 8 months after bottling, the wines with added tannin showed slightly more yellow colour;
- the wines with added tannin showed higher astringency, dryness and bitter sensory characteristics.

Parker et al. (2007) conclude that their work indicates that:

> *The value of wine companies conducting their own trials before making commercial decisions regarding the use of a particular tannin product cannot be underestimated and is strongly encouraged.*

Bautista-Ortín et al. (2005) present a similar conclusion. The outputs of these two studies suggest that on-site trials and not isolated research trials are required.

MOX and exogenous tannins

There is considerable speculation about the possible advantages of exogenous tannin addition and MOX, but very few structured research trials have been reported. In their review of micro-oxygenation, Schmidtke et al. (2010) have noted the following potential benefits from exogenous tannins when MOX is used:

- the presence of oak that contains extractable hydrolysable tannins such as gallotannins and ellagitannins may enhance the formation of polymeric compounds, particularly pyranoanthocyanins. Hydrolysable tannins generally have a high gallolated content that is more efficiently oxidised than the majority of the grape-derived phenolic compounds which are non-gallolated (Danilewicz, 2003);
- Loch (2002) has speculated that MOX in the presence of oak may encourage reactions between hydrolysable tannin extracts and monomeric anthocyanins alleviating the requirement for exogenous tannin additions, leading to an improvement of wood flavour and less undesirable herbaceous green characters;
- the most favourable outcomes of MOX are reported to arise when the ratio of tannin to monomeric anthocyanins is 1:4 (Lemaire, 2002). Some winemakers make exogenous tannin additions to wine for pre-MLF MOX with reported improved oak and lessened herbaceous sensorial characters (Loch, 2002). However, the optimised concentration of hydrolysable tannins for MOX has not been reported.

There are only a limited number of research trials that address the impacts of combining MOX and exogenous tannins:

- Canuti et al. (2006) added grape tannin (150 mg L^{-1}) to Sangiovese in combination with a low oxygen addition rate post-MLF. They reported enhancement of the wine colour intensity and copigmentation as well as the loss of free monomeric anthocyanins;
- high MOX rates applied prior to MLF in a Shiraz wine with commercially available ‘naturally derived’ grape seed and grape skin tannin additions was found to improve wine colour intensity and improve organoleptic qualities of the wine (Kinley, 2008).

The specificity of the wine type and style again calls into question whether meaningful research trials on MOX and exogenous tannins can be established that would allow generalised conclusions to be drawn.

Exogenous tannin addition and protein removal
The interactions between tannins and proteins are well established. It has been proposed[9] that the addition of exogenous tannin may preferentially bind or remove the proteins, leaving the 'natural' grape derived tannin essentially untouched.

Research on the interactions between proteins and tannins extracted from grape seeds is underway at the University of Bordeaux. Some preliminary data was presented at the *In Vino Analytica Scientia* conference in July 2009. Grape proteins were not used in this reported study, but the researchers were able to determine a tannin to protein ratio for effective (implying total) binding. The kinetic behaviour of the process was also studied. The methodology used by the researchers allows the kinetics of the tannin/protein binding to be examined in the first few seconds of the interaction. This has the possibility to link to the study of astringency sensations.

A watch on this study needs to be undertaken, as it may open up new areas of examining tannin to protein interactions.

Exogenous tannin additions and colour stabilisation
The use of exogenous tannins to stabilise colour has been the subject of discussion and speculation for some time. While the mechanism of pigmentation processes has often been a point of heated debate, it is apparent that the extraction of anthocyanins (water soluble) commences before phenolic extraction (ethanol soluble) in the fermentation process. Thus, it has been argued that the availability of flavan-3-ols through addition will be advantageous in helping stabilise the wine colour. The research that has been performed has been generally observational: that is, improvements in colour stabilisation and preservation have been recorded, but the mechanism has not been addressed.

Recent work from Bordeaux (Chassaing et al, 2010) has opened the possibility of exploring mechanistic effects in anthocyanin- exogenous tannin pigments. The authors performed a series of physiochemical studies on the anthocyano-ellagitannin pigment, 1-deoxyvescalagin-(1β→8)-oenin. Structures were analysed by nmr and several kinetic and thermodynamic parameters measured. Importantly, a bathochromic shift in the visible spectra of the pigments with respect to the free oenin was observed. Chassaing et al (2010) speculate that the bathochromic shift may be a consequence of intramolecular π-stacking between the two parts of the pigment.

Chassaing et al. (2010) claim that their data 'constitutes a first plausible molecular-level of the red-to-purple colour evolution' that occurs during the early stages of red wine maturation in oak barrels or in the presence of oak products.

The claim may well be without full justification (as the authors themselves suggest). The work of Chassaing et al. (2010) does show the value in studies that attempt to understand the chemical and/or physical processes, the mechanistic processes in fact, that will allow a proper molecular understanding of colour stabilisation and preservation.

[9] This proposal results from the author's discussions with providers of exogenous tannins

Exogenous tannin addition and consumer preference studies

There is a clear market sector that seeks wines that are high in tannin without an astringent or aggressive sensory response. On the other hand, there is a sector that seeks relatively simple wines with low tannin concentration. The addition of so-called 'fermentation' exogenous tannins is a strategy that appears to be in use already so that the tannin levels and mouthfeel response can be tailored to specific market sectors. Generally, work in this area is in the realm of company marketing/consumer profiling.

A possible research area, however, would be determining mechanisms of sensory responses to selected exogenous tannins. This would be potentially advantageous information, as it could provide a basis for decision making when matching choice of exogenous tannin with wine style.

Summary

- The use of exogenous tannins as a winemaking aid now falls more in the area of company specific trials to ascertain the more effective product for a given wine style.

- The same is essentially the case for the link between exogenous tannin addition and micro-oxidation. It is not realistic to expect a general outcome, given the large number of variables that can be manipulated. In essence, companies need to develop their own strategies.

- The work that is being performed at Bordeaux on tannin/protein interactions and the possibility of using exogenous tannins in a sacrificial way to preserve the natural grape-derived tannins is an area that could develop into a new research field. The methodology that is being developed in Bordeaux coupled with knowledge in Australia regarding grape proteins may well generate useful outcomes for industry.

- The mechanistic work, also being performed in Bordeaux, to ascertain the molecular basis for colour stabilisation in the presence exogenous tannins opens up a new research area.

- Understanding the mechanism of the sensory response to exogenous tannins is a potential new research area as it could provide a basis for matching exogenous tannin type to wine style.

SECTION 11
Links between tannin content and composition and sensory perception of astringency and mouthfeel in wines (including interactions with polysaccharides)

There has been extensive research on sensory aspects over the last 20 years or more and the same questions keep arising:

- what are the main drivers of mouthfeel responses, including astringency, drying and so on?
- is astringency perception only a consequence of precipitation with proteins, especially salivary proteins?
- can the mDP of condensed tannins be correlated with sensory perception?
- what is the influence of tannin modification, including formation of acetaldehyde linked compounds, during wine ageing on sensory perception?

More recent questions link to a broader appreciation that components of the wine matrix other than condensed tannins could possibly influence the response of the taster. Gawel (1998) raised this issue in his review published just over 10 years ago. The issues that are now being addressed include:

- is there an influence on sensory response from colloidal particles that may form in wine from condensed tannin-protein interactions?
- to what extent do polysaccharides influence sensory response?
- does fining wine with proteins actually remove the compounds responsible for astringency from the wine?
- can rapid tannin assays be used to predict sensory response?
- can one or more analytical markers be found as indicators of sensory responses?
- what is the link between winemaker perception of astringency and the response of consumers?

Mechanism of astringency response

Phenolic compounds can exhibit bitterness and astringency. Bitterness is a taste response linked to specific receptors on the tongue. The response is generally assumed to be restricted to small molecules; that is the molecules require the appropriate size and shape to interact with the receptor site (Cheynier et al., 2006).

Astringency is a tactile response felt around the mouth and transduced by the trigeminal nerve (Green, 1993). There is a range of astringency primary descriptors that include dryness, roughness and constriction. Gawel et al. (2000) have developed a hierarchy of sensory descriptors in the form of a ‘mouth-feel wheel’. This was a major development towards the development of a common vocabulary, although some (see for example, Santos-Buelga and de Freitas, 2009) query whether the vocabulary is in fact too extensive.

Lesschaeve and Noble (2005), in reviewing factors that influence sensory perception, used time-intensity curves to show that astringency intensity reaches a maximum around 6 to 8 seconds after ingestion. There is also a well-established individual sensory response to astringency that depends on salivary protein composition as well as saliva flow rates (Lesschaeve and Noble, 2005). This latter point is significant when assessing broad consumer response studies.

Although it is widely accepted that astringency results from the interaction between condensed tannins and salivary proteins, Santos-Buelga and de Freitas, (2009) note that the detailed physiochemical mechanism needs further refinement. It is now clear, and as discussed below, there are several interactions in the wine matrix that may influence the 'availability' of tannins to respond to salivary proteins. Various wine components (eg: ethanol, glycerol and residual sugar) are known to influence the response of the taster to astringency (Gawel, 1998). In essence this implies that model systems cannot replicate the complexity of the wine matrix and the interpretation of results from model studies should be developed with caution.

Response to astringency is a clearly a complex problem. Consumers of red wine, in contrast to other food and beverages, appear to want to know more about its behaviour (Santos-Buelga and de Freitas, 2009). Lesschaeve and Noble (2005) also comment:

> *Learning to like astringent red wines may require repeated exposure and is enhanced by peer pressure and consumption under positive conditions.*

These points are important when designing consumer studies.

Structure-function relationships in astringency perception.
There is a diverse literature on this topic and the following points attempt to summarise the main issues:

- astringency of flavan-3-ols increases from the monomer to the trimer (Noble, 2002);
- (-)-epicatechin is more astringent than its diastereoisomer, (+)-catechin (Thorngate and Noble, 1995);
- the linking mode of flavan-3-ols affects astringency: catechin-(4→8)-catechin was less astringent than catechin-(4→8)-epicatechin (Noble, 2002);
- astringency is reported to increase as the mDP of condensed tannin fractions increases (Vidal et al., 2003);
- increased galloylation led to increased 'coarseness' whereas trihydroxylation of the B-ring of the flavan-3-ol decreased 'coarseness' (Vidal et al., 2003);
- ethyl-linked catechin oligomers were of similar astringency to condensed tannins of similar size (Vidal et al., 2004a);
- there is some speculation about the astringency of pigmented polymers, but the results are not conclusive (Vidal et al., 2004a);
- Generally however, data on sensory properties of tannins formed during wine ageing ('derived' tannins) are scarce (Cheynier et al., 2006).

Lea (1990) claimed that condensed tannins containing more than 10 sub-units are insoluble and therefore could not contribute to astringency. Vidal et al. (2003) suggest the reverse as their data showed that condensed tannins extracted for apples (mDP 70) and grapes (mDP 20) were highly astringent, the fractions being readily soluble in aqueous ethanol. Further evidence for the solubility of condensed tannins of high mDPs can be found in the fining with protein studies of Sarni-Manchado et al. (1999) and Maury et al. (2001).

Cheynier et al. (2006) reflecting on the above and presumably also their general observations suggest:

- flavan-3-ol polymerisation reactions enhance rather than reduce astringency;
- this appears to be regardless of the type of polymer formed: flavan-3-ol polymers, ethyl-linked flavan-3-ols or oxidation products;

- the acid cleavage of condensed tannins (see page 71), assumed to be a slow process, may generate smaller and hence less astringent species;
- astringency can be reduced by oligomeric flavanol-anthocyanin polymers;
- there is no information about the astringent response of other polymers including ethyl-linked flavanol-anthocyanin polymers and flavanol-pyranoanthocyanin polymers.

Clearly, much remains to be done in our understanding of the astringent responses of different classes of polymers, both in model systems and also within the wine matrix.

Skins versus seeds

Noble (2002) commented that there is considerable controversy regarding the contributions of condensed tannins from skins and seeds to the perception of astringency.

Broussaud et al. (2001) compared the astringent response of skin and seed condensed tannins isolated from Cabernet Franc grapes grown in the Loire Valley. The mDP of the seed and skin extracts were 5.7 and 39.8 respectively. Using intensity-time curves, the astringency of the two fractions was found to be essentially identical. One possible interpretation of these results posed by Broussaud et al. (2001) was that the lower galloylation of skin tannins may have compensated for the higher mDP. Unfortunately, a full compositional (sub-unit) analysis of the two fractions was not carried out.

In contrast, a similar study on Cabernet Sauvignon grapes grown in the Napa Valley showed a markedly different result (Kennedy, 1999). In this case, the mDP values were 5.5 and 33 for the seed and skin fractions, respectively. The skin condensed tannin extract showed a significantly higher level of astringency than the seed tannin extract, although the ripeness level of the grapes differed for the two extracts.

Del Llaudy et al. (2008) have attempted to model the relationship between skin and seed tannin astringency as a function of ripeness and also maceration post-fermentation for Cabernet Sauvignon from Spain. They interpret their data as suggesting that the contribution to astringency from skin tannins increases with ripeness while that of seed tannins decreased. Astringency was assessed by protein precipitation and not by sensory analysis.

These three studies have opened up the need for studies that compare the relative astringency of skin and seed tannins by addressing:

- varietal influence;
- maturity influence;
- regional or location influence;
- consistent approach to the assessment of astringency.

Astringency and wine fining

The work of Maury et al (2001) on the impact of fining with proteins on the composition of wine was described in Section 6 (page 79). In essence the results showed that there was minimal change to wine composition before and after fining, however there was a decrease in astringency after fining. Cheynier et al. (2006) comment that this observation may be due to residual protein remaining after fining incorporating potentially astringent tannin molecules in a colloidal form so that they are 'unavailable' to generate an astringent response.

The results of Maury et al. (2001) and the interpretation placed on them by Cheynier et al. (2006) open up a potential new pathway for understanding astringency and its modification. This work deserves much greater attention than it has received to now.

Interactions in the wine matrix

The concept of molecular assembly was introduced in Section 5. Tannin-protein and tannin-protein-polysaccharide interactions were described in Section 6. Most of the studies have used model systems to try and understand the interactions, an essential first step. It is now necessary to move forward and examine these assemblies/interactions in the actual wine matrix.

Molecular assembly

The role of colloidal dispersions (Sections 5 and 6) on sensory properties needs to be examined and either accepted or discounted. Zanchi et al. (2007) reported that in solutions containing condensed tannins of mDP 11 particles of 300nm diameter could be formed. The potential for colloidal particles of this diameter to change rheological or flow properties as well as induce frictional effects must be examined. In essence, the colloidal influences may require a re-estimation of the basis of mouthfeel effects such as astringency, drying and bitterness. Further research in this area is critical.

Tannin-protein and tannin-protein-polysaccharide interactions

While the tannin-protein precipitation process has longed been established, there is increasing evidence that this process alone is not sufficient to explain the mouthfeel properties of wine (Santos-Buelga and de Freitas, 2009).

There is some recent model system work that looks at the chemical and physical structures of tannin-protein complexes in an attempt to relate the structures to sensory effects (Section 6). The work at this stage (mostly from Montpellier in France) is somewhat esoteric, but may well develop into an approach of value in understanding the mechanisms of mouthfeel properties.

The work of Maury et al (2001) mentioned above regarding the minimal impact on wine composition after fining with protein compounds highlights?the complexity of this tannin-protein-astringency story. To add to the general confusion in interpreting published data in some cases, astringency is assessed by sensory analysis and in others by chemical analysis. There is no detailed evidence of any correlation between the two measures.

Polysaccharides are now known to influence tannin-protein interactions, although there is still speculation on the mechanism (Section 6). Cheynier et al. (2006) present data that show how polysaccharides reduce the amount of precipitated tannins that had been added to a model wine. They interpreted these data as reflecting competition between polysaccharides and proteins for tannins, although the data may also reflect the ternary complex model described in Mateus et al. (2004).

Vidal et al. (2004b), using model systems, examined the impact of two polysaccharide fractions on astringency. The acidic fraction, containing rhamnogalacturonan-II, significantly decreased sensory attributes associated with astringency. The neutral polysaccharide fraction, based on mannoproteins and arabinogalactan-proteins, had less effect. Both fractions did increase the ‘fullness’ attribute, however. Vidal et al (2004b) concluded that their data showed that the structure and composition of the polysaccharide would appear to be the

important determinant of the ability of polysaccharides to decrease the astringent response. The study did not examine the relationship between particle size in the presence of the polysaccharides and the astringent response.

In Section 3, the possibility of using pectolytic enzymes to degrade cell walls and thereby allow greater release of skin condensed tannin in particular, was mentioned (Ducasse et al., 2010). This process will of course change the polysaccharide composition with a possible subsequent effect on sensory perception. The advantage of releasing more tannin versus the changing mouthfeel properties must be considered when this approach to winemaking is considered.

The best summary of this general area of sensory response and wine component interactions is presented by Vidal et al. (2004c). After describing the results of an experimental design approach to the impact of tannins, ethanol, anthocyanins and polysaccharides on mouthfeel in model systems, they concluded that

> *Our results also indicate that the construction of mouthfeel perception is a highly complex process depending on the presence of each individual component but also on interactions between components and on the structure of the resulting molecular assemblies. Further experiments are needed to study these complex phenomena and relate them to sensory perception.*

The complexity of the experiments is apparent, but resolution of factors impinging on mouthfeel perception is vital. Experiments that link sensory studies with particle dynamics (molecular assembly/colloidal behaviour) would assist in resolving the nature of the link between aggregation processes and astringent response.

Markers for mouthfeel response

There have been several research activities trying to established markers or indicators for wine mouthfeel response.

Rapid analytical markers

Both the MCP and Adams-Harbertson assays have been examined to determine if correlations between the assays values and sensory scores can be established for wine. Mercurio and Smith (2008) reported the correlations (r^2) between the MCP and Adams-Harbertson assays with wine astringency to be 0.83 and 0.90 respectively. These strong correlations were obtained, even though the agreement between the two methods in terms of assessing tannin concentration differed by a factor of 3. The advantages and limitations of these two methods have been described in Section 2. While some success has been achieved, the predictive capacity of the assays requires further evaluation.

Predicting mouthfeel attributes of a finished wine from grape homogenate (or berry component) analysis would not be achievable, given the manipulation that can occur during the conversion of grapes into wine.

Wine fractionation and reconstitution studies

There have been a few studies[10] where wine has been fractionated (up to nine fractions in one case) and the sensory parameters of the original wine compared with the descriptors obtained

[10] Results have been presented at conferences. Little work has appeared in the peer-reviewed literature.

for the individual fractions. Some fractions have been recombined in an attempt to reconstitute the wine. Generally, meaningful results have not been obtained. The concept of fractionation and subsequent sensory analysis would be fundamentally flawed if the colloidal structures described in Section 5 contribute to the wine's sensory response.

Analytical surrogates

Some recent work on Hunter Valley Semillon examined the 'common dimensions' between sensory descriptors and analytical measurements (Blackman et al., 2010). This is a chemometrics/statistical approach that might have value for defining analytical surrogates for mouthfeel. For example, if analytical measures for one or more compounds were found to fall into a common dimension with bitterness, then the analytical measurement itself could be used to predict the sensory property.

An early approach by Prey et al. (2006) in applying the common dimensions and specific weights analysis approach to red wines suggested that there was a potential relationship between flavonol aglycones and bitterness. Further investigation of this methodology would appear to be advantageous.

Application of metabolomics

Metabolomics can be described as 'the systematic study of the unique chemical fingerprints'. These fingerprints can result from cellular processes (eg: fermentation in the case of wine) or more expansively to chemical transformations that result in the composition of the finished product. Many compounds contribute to the chemical fingerprint and high-powered analytical techniques associated with good statistical analysis (chemometrics) are required.

GC-MS and nmr methods have been applied to white wines in an attempt to find correlations with sensory attributes of wine body (Skogerson et al, 2009). Partial least squares modelling suggested strong correlations between the instrumental metabolite profiles and panel scores of 'viscosity'.

Rochfort et al. (2010) have applied nmr metabolomics to discriminating sensory attributes of wines made from berry shaded Shiraz and Cabernet Sauvignon grapes. The results demonstrated that nmr metabolomics has the capacity to differentiate the wines based on shade treatment similar to that obtained by sensory profiling. Further, statistical modelling suggested that nmr may well be able to be developed into a tool for identifying critical metabolites that contribute to wine quality. While sensory differentiation of the wines used in the study of Rochfort et al. (2010) appears to have been fairly straightforward, the study has opened up the possibility of nmr (and other instrumental) metabolomic profiling being used as a surrogate for sensory assessment/differentiation of wines.

Bioassays

Preliminary work on the binding of grape seed condensed tannins to oral epithelial cells has opened up the possibility of another approach to examining the mechanism for astringency (Payne et al., 2009). Binding of astringent condensed tannins to oral epithelial cells was demonstrated and the dependence of the binding on temperature, pH and the concentration of condensed tannins was examined. Ethanol concentrations up to 13% did not affect the binding. This exploratory study opens up another approach to developing markers for rapid assessment of astringency.

Wine typicality

Wine typicality is a concept where sensory analysis and various forms of data treatments are used to define characteristics that represent wines of a region. One of the most recent studies in this area is by Cadot et al. (2010). They used Quantitative Descriptive Analysis, Just About Right Analysis and typicality assessment to determine the terroir influences on wine typicality. The study is interesting in its approach and may become relevant to the Australian situation if there is to be a greater emphasis on regionality.

Consumer studies

Lattey et al. (2009) have examined the sensory attributes that appear to drive consumer acceptance of young Cabernet Sauvignon and Shiraz wines. The results of the study suggested that consumer testing, in combination with trained sensory panel data, can provide insights into 'what consumers like'. As perhaps expected, there were different groupings within the 203 consumers, but 'positive' and 'negative' attributes were identified.

There are only a limited number of well structured consumer studies on red wine. In one recent study, Parpinello et al. (2009) have described the relationship between sensory descriptors, consumer preferences and colour parameters for Italian Novello wines with perhaps the not-surprising outcome that consumers preferred highly coloured wines. Barreiro-Hurlé et al. (2008) examined consumer willingness to pay for resveratrol-enriched red wines, concluding that consumers regarded this functional-value being as important as the ageing of wine.

The borderline between consumer preference studies as a general guide to winemaking and wine marketing consumer preferences is narrow. Perhaps some of the classic examples of wine marketing consumer preference studies are the creation of the 'Pink' and 'Bella' sparkling wines.

The outcomes of the study by Lattey et al (2009) show the relevance of well-structured consumer studies. In this study, they found that quality concepts expressed by wine makers did not align closely with consumer preferences. One of their conclusions is particularly pertinent to the on-going debate regarding the funding of consumer preference research:

> *(This study)...has highlighted the difference that exists between consumers compared with highly trained and experienced experts, and the importance of testing consumers rather than making product development or research decisions based on winemaker judgements exclusively.*

Summary

This analysis has revealed several areas where additional research is required:

- a comprehensive understanding of the mechanism of mouthfeel response;
- a better understanding of condensed tannin structure/function activity in relation to astringency – does astringency increase with polymer size and is the loss of astringency in ageing due to hydrolysis of flavan-3-ol polymers;
- a re-examination of the relative astringency of skin versus seed condensed tannins in a winemaking context;
- are there colloidal particles of sufficient size in the actual wine matrix to cause changes in flow properties with the possible induction of frictional responses affecting mouthfeel;

- a comprehensive understanding of the mechanisms of tannin/protein/polysaccharide interactions, both in model systems (to understand the effect) and in the wine matrix;
- a search for markers of mouthfeel as potential methods of rapid assessment in place of lengthy and expensive trained sensory panel analysis.

SUMMARY AND RECOMMENDATIONS

The field of grape and wine tannin research is extremely broad and this scientific status report has focussed on those areas that are relevant to the questions posed by the GWRDC in commissioning the review.

Much of the published work is observational and there is a clear lack of mechanistic understanding of the various processes from the production of condensed tannins in grapes through extraction in winemaking to ageing and the expression of colour and mouthfeel. The recommendations made for further research address the perceived lack of knowledge on mechanistic processes.

During the analysis of published literature and from discussions with researchers, it became apparent that there are differences in interpretation of wine chemistry data, particularly the contributions of different types of molecules to the colour expression and the potential significance of molecular assembly processes. An explanation for these differences cannot be provided here, but it may be simply a reflection of the varieties and growing conditions used in the various studies on the expression of tannins and other phenolic compounds.

The major areas where additional research is recommended are:

- what are the mechanisms that regulate the formation of condensed tannin polymers
    - are these enzymatically or chemically driven?
    - how can gene expression and regulation be achieved?

- understanding the cell wall and its role in binding condensed tannins, proteins and polysaccharides;

- the influence of enzyme treatment on the release of grape condensed tannins into wine;

- the relationship between environmental factors and gene expression to identify the link between condensed tannin production and environmental stresses;

- a better definition of the processes occurring in the MCP and Adams-Harbertson methods for tannin assessment
    - do the solvents used reflect what is happening in the winemaking process?
    - round-robin studies with wine laboratories to validate precision;
    - can the methods predict astringency in wine (predictor modelling)?

- the production of tannin reference standards for analytical work and sensory analysis;

- a rigorous examination of molecular association processes that might be occurring in wine to determine the extent and size of colloidal particle formation;

- a better understanding of the interactions occurring between condensed tannins, proteins and polysaccharides;

- an examination of the relationship between molecular assembly, colloidal structure and sensory responses;

- a more detailed understanding of the mechanisms of the reactions associated with pigment production;

- a clearer understanding of the contribution to colour expression of the various types of pigmented molecules over the life of a wine;

- clarification of whether there are actually polymeric pigments *per se* in wine (not models) or whether the pigmented molecules are physically, rather than chemically, attached to molecular assemblies;

- the mechanism of various competing reactions that could be involved in tannin oxidation and/or polymerisation;

- a comprehensive understanding of the mechanism of mouthfeel response, including tannin structure/function in relation to astringency and the contribution of colloidal particles to friction effects;

- a search for markers of mouthfeel (chemometrics, metabolomics) as potential surrogates for astringency responses.

TANNIN RESEARCH – INTERNATIONAL PERSPECTIVE

The following notes are extracted from email correspondence with researchers and technical managers in various countries.

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Researcher 1

- We still do not have an adequate understanding of the chemical basis of changes in astringency and mouthfeel, even though it is an area where some good research has been undertaken in recent years.
  The work of Vidal I found very good for establishing that longer tannins are more astringent, and others have highlighted differences that galloyl groups make for astringency effects, but we still wouldn't be able to a take a chemical measure (e.g. the mean degree of polymerisation of a certain tannin fraction) and be able to predict the sensory astringency of the wine. I remember visiting the estate of Denis Dubourdieu a few years back and he gave me a (young) red wine to taste, which was very smooth from a mouthfeel point of view - and he commented that this wine was in fact very high in tannins and yet was not harsh and astringent, and that we simply do not know why that is.

- Research into polyphenol-polysaccharide interactions and the importance of non-polyphenol compounds on mouthfeel look relevant here as well.

- Related to this would be the lack of easy to use measures of tannins for industry.
  Both the methyl cellulose and BSA based assays are promising in this regard, and we teach these to our students (they all run the MC assay on red wines as a lab alongside spectrophotometric colour measures), but these may not yet be advanced enough for industry to widely use them in a reliable way.

Researcher 2

- We are working on their (*tannin*) biosynthesis in grapes and this is in my opinion where the major gaps in our knowledge lie:
  - how are the polymers formed,
  - is it enzymatically or chemically driven/controlled;
  - what are the regulation mechanisms, the vacuolar transport mechanisms ?

- Another important topic is how diverse are the molecules: in term of DP, branching etc and how does this affect their properties

- Also we need to understand better the basis of astringency perception: does this require precipitation of the salivary proteins? What is the respective role of PRP, glycosylated PRP, mucins, etc.

Researcher 3
1) Evolution of tannins during grape cluster maturation. We are not all together in agreeing that those compounds decrease during ripening in all parts of the grape cluster. The same is true for the variations in mDP during ripening.

2) Bitterness and astringency of wine tannins. What about the effect of mDP?

3) What is the real relevance of the wine derived tannin molecules, in respect of the astringency, colour, bitterness, etc?

Researcher 4
Regarding your question about 'research gaps' in the knowledge of grape and wine tannins, even if relevant improvements in the analytical techniques and understanding of tannin structure have been made, I would say that the key research issues have hardly changed:

A reference method for the specific and accurate quantification of total tannins in wine is still lacking. There is not a universal and precise methodology for the assessment of the (mean) degree of polymerisation of wine tannins, either.

Analysis of individual tannins in wine (and grape) is not well solved. Compounds greater than trimers cannot be properly separated and identified. It is not clear the actual composition and distribution of tannins in wines (e.g., up to what extent large polymers (condensed tannins) are extracted from grape and remain in wine). Similarly, an insufficient knowledge on extractability of grape tannins (and from different parts of the grape) into wine still exists.

There is an insufficient knowledge of hydrolysable tannins in wood-aged wines.

Actual contribution of different tannins and tannin mixtures to astringency perception in wines is not well understood. Astringency of derived tannins and newly-formed pigments is to be established. Also, how involvement of tannin in copigmentation processes affects astringency requires further studies.

The actual components of red wine colour have not been fully elucidated, in particular in which refers to matured and aged wines. Influence of tannins (and derived tannins and novel pigments) in colour stability and definition is incomplete, many gaps still exist on the influence of different tannins on copigmentation, involvement in the formation of new pigments and role of each type of compounds and their mixtures on the final colour of red wines.

Technical manager 1
Date of harvest: we still lack good decision making tool to pick grapes at the optimum phenolic “ripening”.

Astringency: even if quick method had been developed to be used in the industry to measure astringency (UC Davis, AWRI) I still think they don’t really help winemakers to make the right decision.

Analytical challenges: so far we can only analyse one part of the tannin fraction easily but we miss the hidden part of the iceberg. Mass spec techniques must be developed further to have a broader range of molecules analysed, that is a compulsory step towards a better understanding.

Technical manager 2

In terms of research gaps relating to tannins, here is my perception:

- Chemical base of sensory stimuli astringency and structure as related to human taste physiology
- Contribution of tannin to the sensory space of wine as perceived by the human palate
- Role of tannin in stabilizing color intensity of young wines in presence and absence of oxygen
- Technology for non-destructive assessment of tannins in grapes
- Biomarker of «tannin maturity» in grapes
- Quick method for evaluation of total tannin content of grapes and wines and oenological-active tannin fractions of grapes and wines
- Quick method for estimation of anti-oxidant and anti-microbial potentials of tannins in wine as a tool for a more rational management of sulfur
- Quick method for the non-destructive assessment of total tannin and oenological-active tannin fractions in closured wine bottles

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