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Grape and Wine Research and Development Corporation

Final Report

POLYURONIC ACIDS FROM VERAISON TO THE FINISHED WINE: THEIR ROLE IN CALCIUM TARTRATE STABILIZATION

(UM 94/2)

School of Chemistry

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Preamble

This report summarises research activities in investigating the composition of polyuronic acids in Australian Chardonnay wines. Sparkling and still wines were used in the study.

The major part of the research was carried out at The University of Melbourne. A significant component of the research was performed in the laboratories of the Institut des Produits de la Vigne at the Institut National de la Recherche Agronomique (INRA) in Montpellier, France.

Research staff and students involved in the project were

Dr Geoffrey R Scollary (School of Chemistry, The University of Melbourne)

Ms Deborah Hampson (PhD student, The University of Melbourne)

Dr Patrice Pellerin (INRA, Montpellier)

Dr Thierry Doco (INRA, Montpellier)

Dr Elizabeth Waters of The Australian Wine Research Institute assisted in the formative stages of this project.

Project Objectives

1. To examine the influence of grape processing and winemaking procedures on the polyuronic acids levels in wine.

2. To determine the efficiency of the grape-derived polyuronic acids in stabilizing wines with respect of calcium tartrate precipitation.

3. To identify the chemical composition of the grape-derived polyuronic acids which affect calcium tartrate stability.

Project Dates

Commencement Date	1 January, 1995
Completion Date	30 June, 1998

Background

This project followed on from Project UM1GW. In Project UM1GW, significant conceptual advances were made in understanding the phenomenon of calcium tartrate precipitation in wine. Using a model system, we identified the existence of soluble calcium tartrate and, based on nucleation studies, we described a mechanism for the spontaneous precipitation of calcium tartrate. The mechanism involved the aggregation of the soluble calcium tartrate molecules to generate a critical nucleus prior to the onset of precipitation and crystal growth.

The influence of common wine components on the spontaneous precipitation process was examined and it was observed that wine organic acids, specifically malic and citric acids, are effective inhibitors of the calcium tartrate precipitation process. Lactic acid is also an inhibitor but it is not as effective as malic acid. Succinic acid on the other hand had no effect on the induction process for the precipitation of calcium tartrate. From an exhaustive study, we concluded that wine organic acids with an hydroxyl group on the carbon atom adjacent to the carboxyl group were the most efficient inhibitors of the spontaneous precipitation process.

Uronic acids, including galacturonic and glucuronic acids, satisfy this structural requirement for effective inhibition. These monomeric uronic acids, however, have essentially no effect on the induction time for calcium tartrate crystallisation. On the other hand, a commercial sample of polygalacutronic acid proved to be an highly effective inhibitor, essentially as effective as the previously identified inhibitor carboxymethyl cellulose (CMC; CMC cannot be used in wine).

We also observed in project UM1GW that wine polyuronic acids are present at concentrations sufficient to inhibit calcium tartrate precipitation. Intriguingly, sparkling wines were shown to contain, on average, about one third the concentration of polyuronic acids in still white table wines. This observation is indicative of the inherent instability of calcium tartrate and correlates well with the general industry observation that sparkling wines are much more prone to throw calcium tartrate crystals than white table wines.

In project UM1GW, we were not able to identify particular wine polyuronic acids which may be more effective than others at inhibiting calcium tartrate precipitation. The results of this project (UM94/2) follow on from these previous studies. An attempt was made to identify the critical structural characteristics of wine polyuronic acids which bring about effective inhibition of calcium tartrate precipitation and to relate the winemaking procedures which enhance the presence of these natural inhibitors.

In carrying out this project, we had the opportunity to collaborate with Dr Patrice Pellerin of the Laboratoire de Chimie Analytique at the Institut Nationale Agronomique (INRA) in Montpellier, France. Dr Pellerin's group has isolated and identified a range of wine polymers which, in a separate study in France, were shown to have complex effects on the inhibition of the spontaneous precipitation of potassium hydrogen tartrate. The collaboration between Dr Pellerin's group and our own group allowed a detailed study of various wine polymers on the onset of calcium tartrate precipitation in the model wine system. Further, the collaboration allowed the detailed investigation of the carbohydrate composition of Australian Chardonnays in an attempt to identify the type and chemical makeup of acidic and neutral wine polymers in Australian wines.

Influence of wine polymers on the spontaneous precipitation of calcium tartrate

The wine polymers used in the study were isolated from wines prepared from Carignan Noir grapes. The grape derived polysaccharides were rhamnogalacturonan I and II (abbreviated to RGI and RGII), arabinogalactan-protein fractions (AGP0, AGP2 and AGP4) and yeast derived mannoproteins (MP0 and MP1). The different fractions within each class of polymer refer to the structural characteristics. The addition rates used in all experiments were equivalent to 100 mg/L. The model wine system used consisted of two solutions, one containing tartaric acid and relevant wine polymer which was added with efficient stirring to a calcium solution at 15°C. Both solutions were at pH 3.5 and the ethanol concentration was set to 11%.

Induction time (the critical time in determining the effectiveness of inhibition) was assessed by eye and the onset of cloudiness was used as an indicator of the induction time. In previous studies, a calcium ion selective electrode was used and the change in calcium concentration was taken as the indicator of induction time. However, it was found that the wine polymers interfered significantly with the response of the calcium electrode and accurate determinations of calcium concentrations could not be obtained.

The conformation of the crystals obtained in all experiments were shown by IR spectroscopy to be identical to an authentic sample of calcium L(+)-tartrate. Crystal morphology differed from experiment to experiment depending on the crystallisation rate but the model system was able to yield a compound identical to that isolated from actual wine samples.

Table I presents the induction times for a model wine at pH 3.5 and the model wine plus additives. It is obvious from the data in Table I that RGI is the most effective inhibitor and considerably more effective than the polygalacturonic acid used in the previous project (UM1GW). Intriguingly, RGII has essentially no ability to inhibit the onset of calcium tartrate precipitation. The AGP and MP fractions have little to no effect on the induction times for calcium tartrate (see Table I).

Table 1 Induction times (min) for the precipitation of calcium tartrate in a model wine system

Standard model	7
Standard model + RGI	180
Standard model + RGII	22
Standard model + AGP4	10
Standard model + MP0	12
Standard model + MP2	15
Standard model + PGA*	40

* PGA: commercial polygalacturonic acid, as used in the previous project UM1GW

It is well known in the chemical literature that calcium is capable of binding to carboxyl and phosphate groups and polyphosphates, in particular, are particularly effective binders of calcium. Further, the binding between calcium and various pectinic acids has been well documented but all previous studies have been carried out at close to neutral pH and not wine pH as in this study. It is obvious from the variations observed and presented in Table I that the structure of the wine polymer is critical in determining whether or not inhibition of calcium tartrate precipitation occurs.

RGI is a linear polymer with repeat units of galacturonic acid and rhamnose as the backbone. The galacturonic acid units provide the carboxyl groups as sites for interaction with calcium. The structural characteristics of the polymer have not been identified in detail but, of all wine polymers that are used in this study, RGI is known to have the highest amount of negative charge density.

RGII is a complex polysaccharide, characterised by five rare sugars and exhibits a high degree of branching on a short homogalacturonan backbone. Its negative charge density is related to the presence of acidic sugars in the side chains.

The arabinogalactan-proteins, AGP0, AGP2 and AGP4, show varying negative charge densities. Only AGP4 was examined in detail in this study as it has the highest

negative charge density of the three. The negative charge density originates from the ionisation of the carboxyl groups of the uronic acids in the polymers.

The mannoproteins (MP0, MP2) have a composition dominated by mannose with a low protein content. The negative charge density is probably related to their phosphate content.

The efficiency with which RGI inhibits the on-set of calcium tartrate precipitation is related to the simple linear structure and high negative charge density of the polymer. The structural complexity of the other polymers used would limit the ability of the polymer to interact with calcium ions in the model wine system.

Based on these observations, it can be concluded that RGI presents the possibility of establishing conditions for the "natural" inhibition of calcium tartrate precipitation in wine. That is, if winemaking conditions which affect the concentration of RGI can be identified, it may be possible to maximise the concentration of RGI in the wine and consequently minimise the occurrence of calcium tartrate precipitation.

Such a procedure would eliminate the need to use additive inhibitors, such as meta-tartaric acid, as is used in the production of many sparkling wines at present. The next phase of this study was designed to determine the influence of winemaking on the distribution of polyuronic acids. Chardonnay was chosen as the grape variety as it represents a major component of the white table and sparkling wine segments.

Isolation and characterisation of acidic and neutral sugars in Chardonnay

A major study was undertaken to determine the carbohydrate composition of four Chardonnay wines. From this carbohydrate composition, a profile of the polyuronic acid content can be assessed. Two of the wines studied had been prepared as base wines for sparkling wines and two had been prepared as dry table wines. The production methods are summarised in Table 2. Wine 4 originated from a different region to the other three wines. Juice for wines 1 and 2 was separated by whole bunch pressing with wine 1 being a cuvee-style and wine 2 being a firmer pressings style. Wine 3 was prepared from riper grapes, but from the same vineyard as wine 1. The juice for wines 1 to 3 was treated with a pectolytic enzyme (Pectinex SP Ultra) prior to settling and fermentation of the racked juice. Juice for wine 4 (different region) was not enzyme treated.

Analytical Procedure

Figure 1 summarises the initial separation process into 3 gross fractions A, B and C by ultrafiltration. Fraction A contains polymers of molecular mass in excess of 10kDa, fraction B has polymers between 10kDa and 1kDa while the components of fraction C are less than 1kDa in molecular mass.

Figure 1 – Ultrafiltration scheme

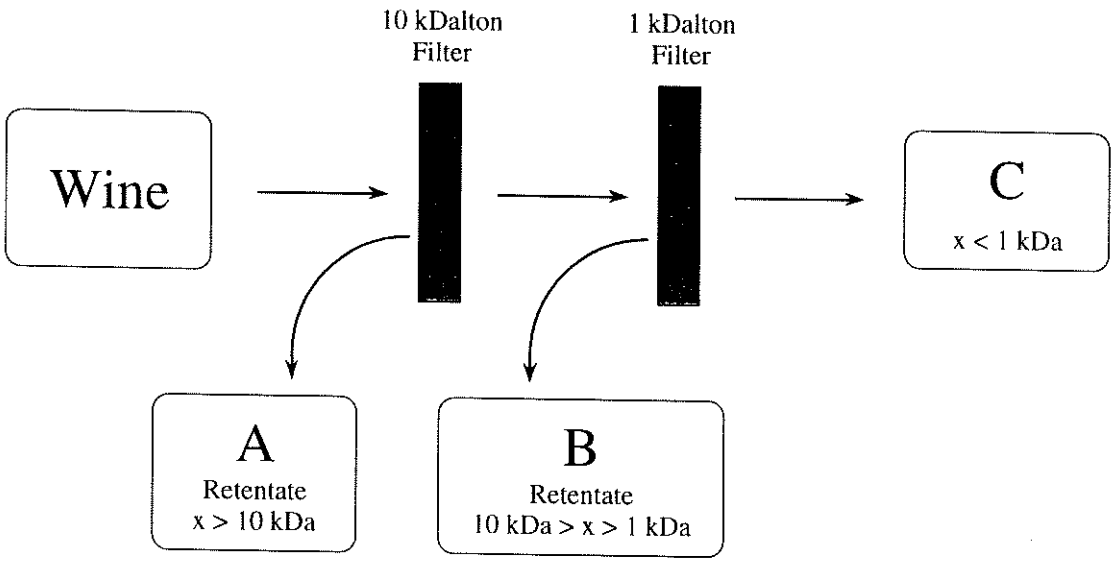
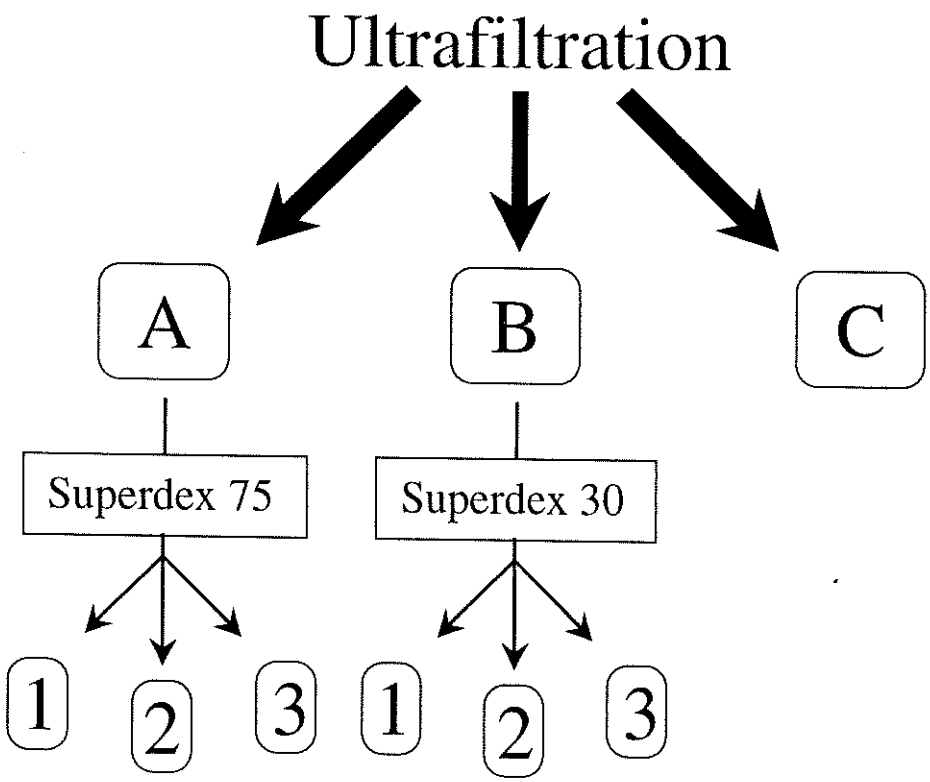


Table 2 Wine Production Summary

Characteristic	Wine 1	Wine 2	Wine 3	Wine 4
Source	Yarra Valley	Yarra Valley	Yarra Valley	Eden Valley
Variety	Chardonnay single vineyard	Chardonnay multiple sources	Chardonnay single vineyard	Chardonnay single vineyard
Baume at harvest	11.1	11.2	12.1	
Harvest method	Machine	Machine	Machine	Machine
Grape processing	Whole bunch press P initial = 0.2 Bar P final = 1.0-1.1	Whole bunch press P initial = 0.6 Bar P final = 1.4	Crushed and destemmed; pumped to membrane press	Tank press; first juice expressed direct to barrel.
Juice yield	480-500 litre/tonne	150 litre/tonne	740-750 litre/tonne	
Juice filtration	Diatomaceous earth	Diatomaceous earth	Diatomaceous earth	
Enzyme	Pectinex SP Ultra (30 mg/Litre)	Pectinex SP Ultra (30 mg/Litre)	Pectinex SP Ultra (30 mg/Litre)	Not used
Cold settling	Yes	Yes	Yes	No
Sulfur dioxide Press Settling Prior to sampling	 No Yes Yes	 No Yes Yes	 Yes Yes	 No Yes
TA	8.9	8.5-9.0	7.2	7.2
PH	3.20	3.35	3.08	3.47
Yeast	Active dry yeast (Prise de Mousse)	Active dry yeast (Prise de Mousse)	Active dry yeast (AWRI 796)	Not known
Bentonite	0.1 g/Litre	0.1 g/Litre	0.1 g/Litre	No
Malo-lactic fermentation	No	No	No	Yes

Fractions A and B were sub-fractionated as shown in Figure 2. Emphasis was placed on studying the polymer components of these two fractions as, based on molecular mass, RGI (if present) should be found in either or both fractions. The components of fraction C were too small to be of any interest to this study and were not examined any further. Subsequent analyses were performed on bulk fractions A and B and sub-fractions A1, A2, A3 and B1, B2 and B3.

Figure 2 – Sub fractionation of Ultrafiltered fractions

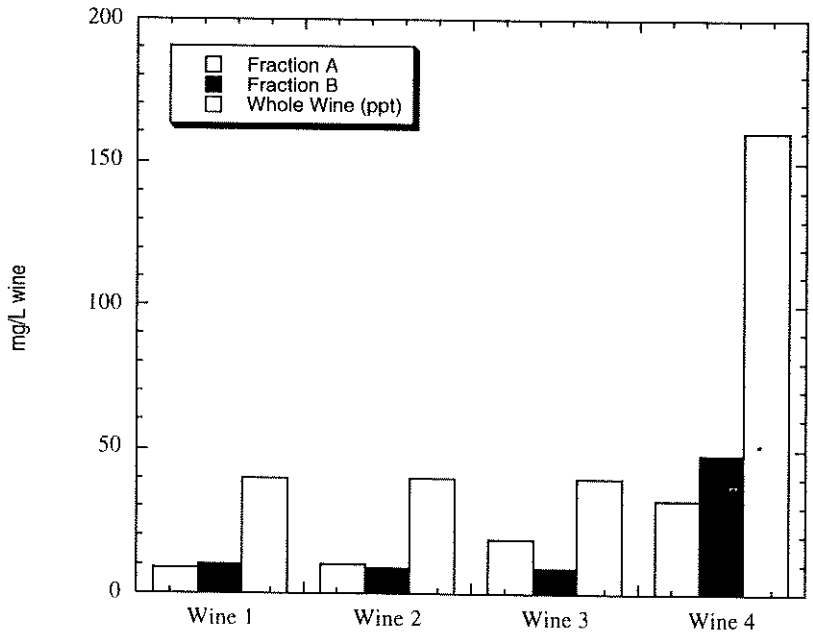


Uronic Acid Content

The uronic acid content on bulk fractions A and B as well as on the whole (that is, not ultra-filtered) wine were determined by the classical colorimetric (*m*-hydroxydiphenyl) method. The results are plotted in Figure 3.

It is apparent from Figure 3 that the concentration of polyuronic acids is affected by some of the methods of grape processing. Wine 4 (non-enzyme treated) has by far the highest concentration in the whole wine as well as the two fractions. Wine 3 has more polyuronic acids in fraction A compared to fraction B, while the amount in the both fractions for wines 1 and 2 is similar.

Figure 3 Total polyuronic acids in Fractions A and B and total wine for wines 1 to 4



The need to fractionate the wines for a meaningful determination of the polyuronic acid content by this colorimetric procedure can be seen in Figure 3. The value obtained for the whole wine overestimates the sum of that in fractions A and B, which implies that there may be other components in the non-ultrafiltered wine which also respond to the colorimetric assay.

Analysis of Fraction A

The subfractions of Fraction A were assayed for their macromolecule content. The results are listed in Table 3 for wines 1 and 3 only. In all fractions, only three classes of macromolecules could be found – arabinogalactan (AG), mannoprotein (MP), and rhamnogalacturonan II (RGII). RGI was not detected in any sub fraction.

Table 3 Macromolecules in Fraction A (mg/L)

Wine Fraction	1			3		
Sub-fraction	1A	2A	3A	1A	2A	3A
AG	22	7.1	4.6	53	16	14
RGII		5	6.3		16	10
MP	99	13	24	91	12	9

Analysis of Fraction B

Analysis of the three sub-fractions of Fraction B for each wine showed the following:

Subfraction B1: macromolecules, similar to fraction A3, due to inefficiencies in ultrafiltration.

Subfraction B2: oligomeric fractions containing uronic acids.

Subfraction B3: sugars with low degree of polymerisation (eg glucose and inositol monomers).

RGI could not be identified in this fraction.

The oligomeric components of subfraction B2 were of a size suggesting that some or all may have a relationship to RGI. These oligomers were further investigated after separation by high performance anion exchange chromatography with detection by electrospray mass spectrometry. Results for wine 4 are listed in Table 4. Wine 4 was chosen for this analysis as it had not been treated with pectolytic enzymes so any oligomers detected would be due to natural breakdown processes in the wine.

Table 4 Characterisation of Oligomers in Subfraction B2 of Wine 4

Component	Mass (Daltons)	Identity*
1	983.4	$[Rha\text{-}GalU]_3$
2	737.2	2 Hexose + 3 Pentose
3	721.2	$[GalU]_4$
4	661	$[Rha\text{-}GalU]_2$
5	605.2	2 Hexose + 2 Pentose
6	545	$[GalU]_3$

*Rha = rhamnose; GalU = galacturonic acid

This analysis shows that the oligomers have a degree of polymerisation between 4 and 6 and, although not indicated in Table 4, methylated chains were also present.

Components [Rha-GalU]$_3$ and [Rha-GalU]$_2$ may well have originated from RGI as the repeat unit in RGI is [Rha-GalU]. That is, while RGI itself could not be found, components originating from it were identified.

Summary

RGI obviously is a difficult molecule to find in the wines prepared for this study. One major contributing factor may well have been the use of pectolytic enzymes in wines 1, 2 and 3. However even wine 4, which was prepared without the use of enzymes, did not yield any RGI. This suggests that RGI is not a particularly stable molecule.

In previous studies on RGI, the wines were prepared from red grapes with extensive skin contact. It may be that the RGI is localised in the skins and fermentation on skins may be needed to generate the presence of RGI in the wine. This, however, is not an option for premium white wine production.

Impact of grape and wine processing on wine calcium concentration

A series of wines were prepared using Chardonnay grapes from the one region (Yarra Valley). Eight wines were prepared with only one major variable (juice extraction, pectolytic enzyme addition, settling) being changed in each wine. Table 5 summarises the matrix of samples used.

Table 5 Wines used in calcium distribution study

Wine	Juice extraction	Enzyme addition	Settling before fermentation
1	Whole bunch press	Yes	Yes
2	Whole bunch press	Yes	No
3	Whole bunch press	No	Yes
4	Whole bunch press	No	No
5	Crush and press	Yes	Yes
6	Crush and press	Yes	No
7	Crush and press	No	Yes
8	Crush and press	No	No

At the end of fermentation, the wines were clarified by settling and bottled in 750 mL bottles until analysed. Before calcium concentrations were determined, the wines were filtered through a 0.45 μm filter to remove gross particulate material.

Each wine was analysed for total calcium by flame AAS and ionised calcium using a calcium ion selective electrode. The results of the analyses are given in Table 6.

Table 6 Calcium concentration in wines as determined by grape processing

Wine	Total Ca (mg/L)	Ionised Ca (mg/L)
1	112	43
2	113	43
3	115	44
4	114	45
5	80	22
6	77	23
7	74	25
8	75	25

It is obvious from the data in Table 6 that the only variable which has a significant effect on the calcium concentration is the method of juice extraction from the grapes. Wines 1 to 4 have a higher calcium concentration than wines 5 to 8. This observation is surprising as it implies that juice produced by whole bunch pressing has a higher calcium concentration than that produced by crushing and pressing. Little is known about the distribution of calcium in the grape berry. These results suggest that the calcium is either located in the pulp or that the crushing process opens up calcium binding sites on the skins so that some calcium is lost with the skins.

The important consequence for calcium tartrate stability is that premium sparkling wines produced by whole bunch processing will have a higher calcium concentration and consequently will be more at risk of calcium tartrate precipitation. This result would in fact appear to be critical in explaining the industry observation that premium sparkling wines have a higher incidence of calcium tartrate crystallisation compared to still table wines.

The total polyuronic acid content of the wines in this study, as assayed by the colorimetric procedure, was essentially invariant with a mean value of about 100 mg/L. This value is above the normal range for sparkling wines and at the low end for table wines (project UM1GW). It is surprising that grape processing has little impact on the total polyuronic acid content.

It is also surprising that the mean polyuronic acid concentration is intermediate between the ranges for sparkling and table wines. The grapes used in this study were picked at about 12.5 Bé, intermediate between the normal levels for sparkling (11.0 to 11.5 Bé) and table (13.0 to 13.5 Bé) wines in the region from which the grapes originated. That is, the intermediate concentration of polyuronic acids may be a consequence of the degree of berry ripeness. There is evidence from the work of Hardie [Aust. J. Grape Wine Res., 2, 1996, 97-142] that the polyuronic acids are located in the cell wall, but with the collapse of the cell wall in mature berries, the polyuronic acids move into the pulp. This provides a further explanation for the apparent inherent instability of sparkling wines with respect to calcium tartrate. Whole bunch pressing of less mature grape berries implies, on the basis of the observations here, that the resultant wine will be high in calcium and low in

polyuronic acids (the source of natural inhibitors). These conditions are ideal for the inherent calcium tartrate instability in premium sparkling wines.

SUMMARY OF MAJOR FINDINGS

1. Rhamnogalacturonan I (RGI) is a highly efficient inhibitor of calcium tartrate precipitation.
2. Other wine macromolecules including rhamnogalacturonanII (RGII), arabinogalactan-proteins (AGP) and mannoproteins (MP) are inefficient inhibitors of calcium tartrate precipitation.
3. Fractionation of Chardonnay wines by size showed that the fraction greater than 10 kDa contained RGII, AGP's and MP's. The size fraction between 10 kDa and 1 kDa contained some RGII, but mainly oligomeric neutral and acidic sugars. The size fraction less than 1 kDa contained mainly monomeric sugars.
4. The critical macromolecule, RGI, was not found in any fraction.
5. A sub fraction of the 10 kDa to 1 kDa fraction was found to contain oligomers of the repeat unit ([rhamnose-galacturonic acid]) of RGI. A dimer and trimer of this repeat unit were identified.
6. The calcium concentration of wines prepared from whole bunch pressed grapes is significantly higher than that prepared from grapes which have been crushed and pressed.
7. The total polyuronic acid concentration is not affected by the method of juice extraction from the grape, use of pectolytic enzymes or by fermentation with or without solids.

Implications for wine production

1. RGI would be a highly effective natural inhibitor of calcium tartrate crystallisation.
2. The inability to detect RGI in any wine in this study negates further consideration of maximising its concentration by manipulating the wine making process.
3. The detection of fragments (dimer, trimer) of RGI in a subfraction suggests that RGI is an unstable molecule.
4. Pectinases are known to hydrolyse RGI. However, the inability to find RGI in non-enzyme treated wines is further evidence for the inherent instability of RGI.

5. High calcium concentrations in wines prepared by whole bunch grape pressing will enhance the instability of a wine with respect to calcium tartrate precipitation.

6. There is little impact of grape and wine processing on the total polyuronic acid concentration. Rather, there is tentative evidence from this project and project UM1GW that grape berry ripeness may be important in determining the final concentration of polyuronic acids in the wine. The implication of the results of both projects is that less mature berries will lead to wines of lower total polyuronic acid concentrations.

7. The consequent implication is that the criteria essential for the production of premium sparkling wines (whole bunch pressing, less mature berries) will generate wines which are high in calcium and low in potential natural inhibitors.

   These two conditions (high calcium, low polyuronic acid concentration) maximise the potential for calcium tartrate instability.

8. The feasibility of using RGI as a means of natural inhibition of calcium tartrate crystallisation would appear not to be a realistic option.

   Rather, it is essential that the winemaking protocols proposed in project UM1GW (low pH, malolactic fermentation of base wines) and the addition of metatartaric acid are used as effective means of controlling calcium tartrate instability.

OUTPUTS

1. Conference and other presentations by D Hampson

Calcium Tartrate Crystallisation in Sparkling White Wines
RACI Research and Development Topics Meeting, Newcastle, NSW, 1995.

Polyuronic Acids: Influence of Winemaking on Structure and Distribution in Wine
Oral Presentation, National Grape and Wine Industry Centre Seminar, Charles Sturt University, Wagga Wagga, 1997.

Polyuronic Acids: Influence of Winemaking on Structure and Distribution in Wine
Oral Presentation, Australian Wine Research Institute Seminar, Adelaide, SA, 1997.

Preliminary Investigation of the Uronic Acid-containing Oligomers of Australian Sparkling and Still White Wines : Influence of the Vinification Procedures.
Oral Presentation, Departmental Seminar, IPV, INRA, Montpellier, FRANCE, 1997.

Investigation of the Uronic Acid Containing Oligomers Found in Australian Sparkling and Still White Wines.
Poster Presentation (Prize Winner), RACI 14th Symposium on Analytical Chemistry, Adelaide, SA, 1997.

Investigation of the Uronic Acid Containing Oligomers Found in Australian Sparkling and Still White Wines.
Poster Presentation, In Vino Analytica Scientia, Bordeaux, FRANCE, 1997.

2. Refereed publications

The following manuscripts are in preparation for the Australian Journal of Grape and Wine Research.

a) Influence of wine polymers on the spontaneous precipitation of calcium tartrate.
b) Isolation and characterisation of acidic and neutral sugars in Chardonnay.
c) Impact of grape and wine processing on the calcium concentration in the finished wine.