

Varietal differentiation of grape juices by protein fingerprinting

— an example of unexpected outcomes of benefit to label integrity programs arising from research into alternatives to bentonite fining

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ALL IN ACCORD with Louis Pasteur's famous dictum that *there is not pure science and applied science but only the application of science*, useful practical outcomes often start with a twinkle in a basic researcher's blurred, but alert, eye. Pasteur's own research reflects an impressive synthesis of goals of understanding to reach the applied goals of preventing spoilage in milk, vinegar, beer and wine, and of conquering flacherie in silkworms, anthrax in sheep and cattle, cholera in chickens, and rabies in animals and humans. The history of science is full of examples where an open mind has made important insights in areas not originally intended at the outset of the research. However, as Albert Einstein once said, *in science, chance favours the prepared mind*. In addition to the benefits of defined outcomes formulated by appropriate practitioner/researcher interactions (see Høj et al. 2003), the occasional serendipitous uncovering of phenomena is one of the important but often overlooked benefits of both public and industry investment in R&D. Alexander Flemming's discovery of penicillin represents a famous and monumental example of such serendipity at play. Another example of famous spin-offs from ongoing fundamental research was the development of the worldwide web which owes much of its existence to the need for physicists from a multitude of nations to communicate effectively in relation to the conduct of experiments at the CERN particle physics facilities in Europe.

During The Australian Wine Research Institute's ongoing research into the causes and potential prevention of protein hazes in wine, unintended discoveries, although on a much less monumental scale than that of penicillin and the Web, have been made in relation to haze-forming proteins. It has been discovered that haze-forming proteins have a potential use in varietal identification of grapes/juices and hence, by implication, also label integrity programs. Varietal authentication of grapes, juices, musts and wines is important, not only to grapegrowers and winemakers, but also to

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the marketing of wines, since wine labelling laws and trade regulations now demand that varieties be correctly identified (Pinder and Meredith 2003). This article describes a prime example of an effort that brings together informed judgments of scientific promise of basic research and of industry need for varietal identification.

The world's great wines are produced from a relatively small number of classic cultivars of a single grape species, *Vitis vinifera* L. This implies that these cultivars are evolutionary and closely related. Moreover, many of our modern and highly regarded winegrapes are the product of accidental crosses between varieties grown close to each other—probably in the same vineyard—a common practice in medieval times. For example, Cabernet Sauvignon is the offspring of Sauvignon Blanc and Cabernet Franc; Chardonnay originates from Pinot Noir and Gouais Blanc; Shiraz (or Syrah) is the chance offspring of two obscure grapes from south-eastern France: Dureza and Mondeuse Blanche (Pinder and Meredith 2003). It is, therefore, not surprising that it is often difficult to accurately identify these kaleidoscopic spectra of highly related winegrape cultivars.

IDENTIFICATION OF VARIETIES BY AMPELOGRAPHY

Traditionally, grapevine identification has relied on the skills of ampelographers who use up to 150 traits to identify varieties by their appearance (Galet 1979). Despite such detail, it is still possible for even experienced ampelographers to confuse varieties. In some cases, unknown vines need to be grown for several years and under the same conditions as known vines before identification can be achieved with a high degree of certainty. While ampelography has a rightful place in viticultural operations, it relies heavily on vine characteristics and clearly is not applicable to juice or wine or as a tool to identify grapes delivered at the weighbridge.

This article builds on a previous contribution made by Dr Elizabeth Waters to the Institute's Technical Review (Waters 2002) and Institute publication #663 (Hayasaka et al. 2001).

ALTERNATIVES TO AMPELOGRAPHY

DNA typing: A powerful alternative to ampelography for varietal identification has been developed over the last 20 years. DNA-typing techniques have greatly enhanced the scope of ampelography and allowed genetic relationships among existing varieties to be defined (Bowers et al. 1993, Bowers and Meredith 1997, Sensi et al. 1996, Thomas et al. 1994). This method is successful when DNA is extracted from various vine tissues and stored appropriately to prevent degradation of the extracted nucleic acids. It is also possible to use DNA-typing techniques on freshly prepared juices where nucleases have yet to degrade the DNA. In general, however, it has proven very unreliable to use DNA-typing techniques on wine even a few days after fermentation (Garcia-Beneytez et al. 2002, Siret et al. 2002). In summary, therefore, it is possible to use the very powerful DNA-typing techniques to identify the varietal origin of grape samples received at the weighbridge, but highly impractical. However, once tissue disruption occurs, e.g. at crushing, and subsequent storage at ambient temperature has been initiated, the applicability of this technology becomes very time-sensitive (unless protective storage of samples by, for example, rapidly freezing at -80°C is ensured). Wine aroma compounds, pigments or other trace organic compounds can be used, in some cases, to indicate the variety of grape used to produce a wine (Rapp 1998, Wenzel et al. 1987 and Wittkowski 1998).

Protein profiling by mass spectrometry: In recent years, collaborative research (see Høj et al. 2001 and Tattersall et al. 2001 for overviews) between The Australian Wine Research

Institute and The University of Adelaide on haze-forming wine proteins, has uncovered another possible tool for varietal identification. All grape cultivars tested by us synthesise a set of pathogenesis-related (PR) proteins following veraison (these proteins are identical to those forming a haze in wine) (Pocock et al. 2000, Pocock et al. 1998, Pocock and Waters 1998, Tattersall et al. 1997, Waters et al. 1996). These proteins, unlike DNA, are stable in juice and wine and a number of isoforms exist within individual varieties (Busam et al. 1997, Derckel et al. 1996, Jacobs et al. 1999, Robinson et al. 1997, Waters et al. 1998, Waters and Williams 1996). The molecular masses of these isoforms differ slightly across varietal boundaries, due to very small differences in the sequences of their respective genes across varieties.

We have observed that these proteins remain stable in juice, even after prolonged storage and indeed survive vinification to a large extent. This would form a necessary criterion for identification of the varietal origin of grapes, for the varietal origin of pure grape juices and indeed for analysis of wine prior to bentonite fining. Indeed, as low but detectable amounts of some of these stable PR-proteins persist in wine even after bentonite fining, grape proteins may also assist to identify the grape varieties which have been employed to produce a finished wine.

Previous attempts to differentiate grapes and their products by electrophoretic analyses of their protein complement have been reported and have been partially successful (González-Lara et al. 1989, Moreno-Arribas et al. 1999, Murphey et al. 1989, Polo et al. 1989, Pueyo et al. 1993, Tedesco et al. 1997, Weiss et al. 1998). Since electrospray ionisation mass spectrometry is likely to exhibit far greater resolving power than electrophoretic techniques, we set out to explore its feasibility as a method for varietal differentiation of grapes and wines and have previously reported the 'hard science' underpinning the approach.

We have acquired mass spectral data of unstable proteins in juices from berries from 20 different varieties (*Vitis vinifera* cv.) harvested in at least two different seasons from seven different vineyards including those at the Waite Campus of The University of Adelaide, and various commercial vineyards at Padthaway, Summertown, Adelaide Hills, Langhorne Creek, Coonawarra and Barossa Valley. The identity of grapevines in the Waite Campus vineyard was determined by ampelography and confirmed by DNA fingerprinting. The protein profiles in these different juices showed significant differences and these differences persisted through different harvest years and in fruit grown in different locations (for an example of profiles of white grape varieties in the Waite Campus vineyard in different years see Figure 1 and Table 1). Based on the defi-

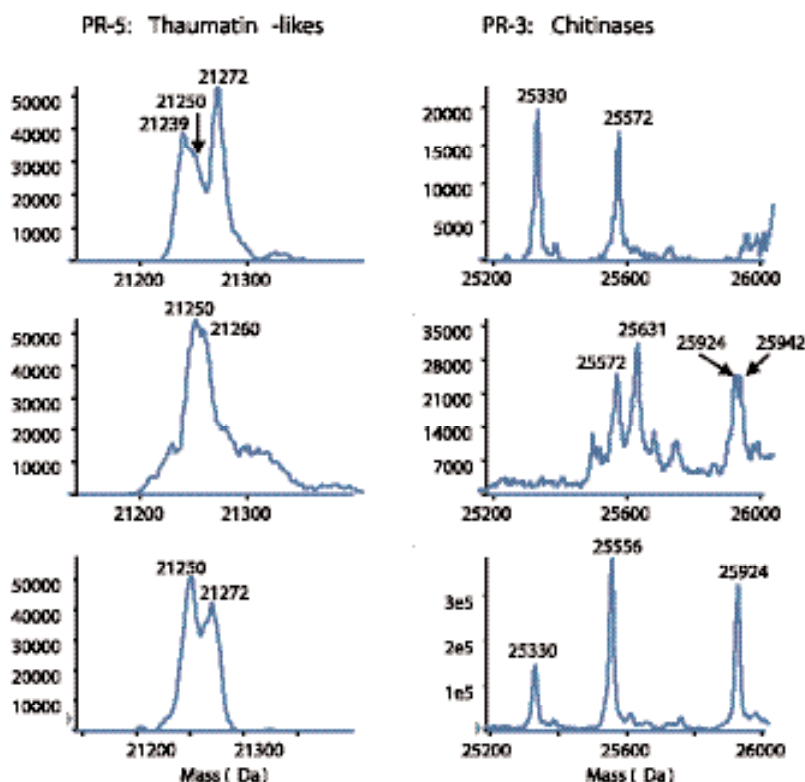


Figure 1. A typical example of the mass-patterns obtained by electrospray mass spectrometry of juice from from three different grape varieties.

nition of four different masses for the PR-5 (thaumatin-like) proteins and 12 different masses for the PR-3 proteins (chitinases) and using statistical analysis, the methods developed can be used for varietal differentiation of grapes on the basis of the protein composition of the juice. Thus, the use of mass spectrometry to differentiate grape varieties is feasible. The technology is most likely not as robust as DNA-typing technology but has the advantage of being applicable to liquid samples stored at commercially realistic temperatures for an extended period of time. This work has been published (Hayasaka et al. 2001) and is available from the Institute as staff reprint #663.

ANALYSES OF JUICES

The use of bentonite fining of white wines to achieve protein stability is universal. The search for proteases that can degrade haze-forming proteins as an alternative to the cumbersome use of bentonite has been frustrating, due to the resistance of these haze-forming proteins to most commercial proteases under normal winemaking conditions. While this characteristic of haze-forming proteins is unfortunate from a production perspective, it does indicate that protein profiling of juices (and most likely fermented wines) for varietal 'identification' should be possible, even after long-term storage under commercially-realistic conditions.

In light of the Institute's research into these new technologies (Hayasaka et al. 2001, Waters 2002) and with the strong commitment to label integrity by Australian winemakers, it has not been surprising that some juice samples have been submitted to the Institute for analyses — either to test our claims or to ensure, for one reason or other, that

Protein type	Mr	Chard		Sauv Blanc		Ries		Gordo		Sultana		Doradillo		Crouchen		Semil		Mus'delle			
		98	99	97	98	99	98	99	98	99	97	98	99	97	98	99	98	99	97	98	99
PR-5 (thaumatin-likes)	21272	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	
	21250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	21239	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	
	21260	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
PR-3 (chitinases)	25924	+	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	-	-	-	-
	25942	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+
	25330	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
	25341	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	25556	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
	25572	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	+	+	+	+
	25588	-	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-
	25631	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
	25410	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
	25423	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
	25456	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+

Table 1. Protein composition of the juice samples from white grape varieties from the Waite Campus Vineyard harvested in different years. The Mr values of the proteins were determined by trap-MS, and the presence or absence of the protein in the juice are shown as + or -.

record keeping from vineyard to winery is accurate prior to the commencement of fermentation and blending actions. What we have learnt from these activities is that it indeed is possible to obtain very good protein spectra of stored juice and that the application of this technology will ensure that Australian winemakers are in a stronger position than their overseas competitors to back up claims of integrity from vineyard to bottle. It would, therefore, be prudent to consider employing this technology at various stages of the winemaking process starting at the weighbridge and extending through to the pre-bentonite fining stage—not as a substitution for current label integrity measures but as a useful additional tool in the quest to ensure that consumers can have even greater confidence that Australian wines are true to label.

Like grape berries and juices, protein unstable (i.e. unfined) white wines contain the profile of proteins characteristic of the variety of grapes they were produced from. We have further established that protein profiling of red grape juice is feasible, however, only a very few red wines have been

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examined to date. It was not possible to obtain 'conclusive' mass spectra from these red wines and we predict that this method will not be applicable to bottled red wines. At present, some other characteristics and acknowledged shortcomings of the technology that are relevant include:

- The technology is primarily of a qualitative nature and is quantitative only to a limited extent. Thus, while the characteristic masses of the proteins from different varieties remain constant, the levels of proteins in individual juices will be dependent on grape ripeness (Pocock et al. 2000 and Tattersall et al. 1997) and variety in particular (e.g. Sauvignon Blanc is known to contain high levels of protein) but may also be influenced by harvesting practices (Pocock et al. 1998a, 1998b) and, potentially, environmental conditions;
- Although well documented through publication in an international refereed journal, the technology is relatively new and the experience with this technology is limited, as applied to commercial samples. The Institute will consider introducing this as a commercial service based on the interest and demand for such a technology;
- Currently, the technology is particularly suited to detecting the presence of certain components rather than the absence of particular components. For example, if a typical set of proteins in a juice fits a 'Variety X' pattern, the conclusion can be drawn that 'Variety X' is present in the sample. If the proteins typical of 'Variety Y' are absent, it is highly unlikely that 'Variety Y' constitutes the majority of the juice, unless heavily bentonite fined or 'unripe' 'Variety Y' juice has been blended with non-fined 'Variety X' juice; and
- If protein patterns typical of two varieties coexist in a juice, our experience indicates that it can be established with a good degree of confidence that the juice did not originate from one variety only, however, the relative proportion of the two juice components cannot be quantified readily. This is because varieties differ in their protein content, a fact well-known to the experienced practitioner who has observed the differing bentonite requirements for fining, for example, a typical Sauvignon Blanc and a typical Chardonnay wine. As mentioned above, the ripeness of grapes at harvesting and the degree of juice skin contact can also influence the amount of proteins derived from a given batch of grapes to a considerable extent.

ANALYSES OF BENTONITE-FINED WINES

The technique we are describing here clearly depends on the presence of protein in the analysed sample. Bentonite fining, therefore, presents a particular challenge to this method. In our experience, white wines stabilised with bentonite contain low but detectable levels of the PR-5 (thaumatin-like) proteins but practically undetectable levels of the PR-3 proteins (chitinases). The PR-3 proteins are the most varietally variable group of wine haze proteins. The differences in the molecular mass of thaumatin-like proteins alone are probably not sufficient for varietal differen-

tiation but could be used to exclude the presence of certain varieties in a wine. For example, the technique applied to a bentonite-fined wine could potentially categorise that bentonite-fined wine as being in one of the following groups:

1. Chardonnay, Riesling or Sauvignon Blanc
2. Muscat Gordo Blanco or Sultana
3. Doradillo or Crouchen
4. Muscadelle
5. Semillon

Thus the method could potentially confirm that a bentonite-fined wine considered to be Riesling was not Muscat Gordo Blanco, Sultana, Doradillo, Crouchen, Muscadelle or Semillon, but it could not confirm that the wine was not Sauvignon Blanc or Chardonnay.

The collective data gathered so far clearly demonstrate the potential for mass spectrometry of proteins to differentiate varieties of *Vitis vinifera* with a good degree of robustness. The extent to which the discriminating power can be extended to wine is limited if bentonite fining has occurred. However, because DNA-typing does not appear to be practically possible for authenticating wine samples, the extension of our findings might represent one of very few avenues by which varietal authentication of wine can be executed with some degree of certainty. This could be achieved, if necessary, through a combination of analyses of secondary metabolites and sensory analyses for varietal typicity.

NOTE

The Institute is currently not offering the protein-profiling as a commercial service. Considerations to introduce the methodology as a commercial service are underway, but this will depend on the projected demand for the service and our ability to access the expensive instrumentation required to carry out the mass-spectrometrical analysis on a routine basis. We reiterate that the technology described here is a powerful complement to existing approaches to ensure varietal label integrity, however, it is by no means a replacement for existing good manufacturing practices.

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