Final report

Understanding methane reducing tannins in enteric fermentation using grape marc as a model tannin source

Project code: B.CCH.6410

Prepared by: Josh Hixon
The Australian Wine Research Institute Pernod Ricard Winemakers, (formerly Premium Wine Brands), Department of Environment and Primary Industries Victoria

Date published: August 2015

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Grape marc has been thoroughly tested for the presence of agrochemicals, tannins, nutritive profile and other compounds of interest to methanogenesis. The agrochemical survey highlighted iprodione as an area of further research, with high concentrations found in some unprocessed samples. Grape marc tannin was found to vary greatly in concentration and composition across the processing chain with skin only and seed only samples giving rise to the biggest compositional variations.

In vitro experiments highlighted the roles of both fat and tannin in reducing methanogenesis, although fat was also closely related to losses fermentation efficiency. Small tannin was found to be more effective at reducing methane production, with extractable tannin reducing methane without inhibiting fermentation.

Marc parcels that have undergone limited or no extraction will be beneficial due to the presence of small, extractable tannin, but also readily fermentable sugars. However, marc with limited processing needs to be screened for the presence of agrochemical residues.

Grape marc must be applied under the correct conditions, with reductions in total feed energy contributing to productivity losses that overshadow any anti- methanogenic potential. For marc to be used effectively on-farm a number of issues need to be addressed such as preservation of tannin and methods for handling that prevent mould formation.

Executive summary

Background

Tannins are a promising group of phenolic compounds for decreasing enteric CH4 emissions from ruminants when used as a dietary supplement, with both in vitro and in vivo studies pointing to reductions in methane production in the presence of supplementary tannins.

Grape marc, a solid grape-derived waste-product, is a rich source of simple phenolics and condensed tannins, is generated at scales of around 200,000 tonnes per annum in Australia. Preliminary studies have shown that feeding grape marc to cattle can reduce methanogenesis by up to 37% (unpublished data). The mechanism by which this occurs in the use of grape marc is not yet well understood, but it has been presumed to be due to the presence of high concentrations of condensed tannins.

Objectives

The objective of this project is to reduce Australia's agricultural methane emissions through:

- Quantifying grape marc compositional variation, including a survey of potential agrichemical residues and any other harmful or toxic chemical compounds, with a specific view to generating information about tannin levels and types in grape marc.
- Conducting in vitro screening of grape marc samples with known tannin variations, in partnership with DPI Victoria through project (B. CCH. 6460), This will determine how tannin variations (level and type/complex) effect methane emissions.
- Based on the findings of the tannin and marc characterisation work and the results of in vin, o screening, conduct in vivo assessment of grape marc as a feed additive, This will be completed in partnership with DPI Victoria through project B. CCH. 6460.
- Characterising grape marc tannins and elucidating to what extent they are the active ingredients responsible for reducing ruminant emissions -this could guide improvements across all agricultural feedstocks.

Methodology

In vitro experiments highlighted the roles of both fat and tannin in reducing methanogenesis, although fat was also closely related to losses fermentation efficiency. Small tannin was found to be more effective at reducing methane production, with extractable tannin reducing methane without inhibiting fermentation.

Results/key findings

Grape marc must be applied under the correct conditions, with reductions in total feed energy contributing to productivity losses that overshadow any anti-methanogenic potential. For marc to be used effectively on-farm a number of issues need to be addressed such as preservation of tannin and methods for handling that prevent mould formation.

Benefits to industry

Marc parcels that have undergone limited or no extraction will be beneficial due to the presence of small, extractable tannin, but also readily fermentable sugars. However, marc with limited processing needs to be screened for the presence of agrochemical residues. The use of grape marc feeding to achieve reductions in methane should be done during times of low energy requirements, such as the summer-autumn feed gap, during times of drought, or when a feed supplement of equivalent energy is being replaced.

Future research and recommendations

The supplementation of grape marc into high energy feeds has shown to result in reductions in fermentation performance or animal productivity. When grape marc is used in a lower energy ration, the effect of tannin and fat can be better observed. Additional feeding studies should be undertaken using low energy control diets, during the summer-autumn feed gap, or when animals are being fed at or just above maintenance. It is likely that the role of grape marc in feeding systems is during one of these scenarios, and that the extent of anti-methanogenic effect would be maximised.

Lastly, the logistics of using grape marc on farm needs to be better understood. Large scale storage and transportation methods, as a well as ways to limit mould formation all while preserving the anti-methanogenic properties of marc. As oxidative conditions are known to be responsible for degradation of tannin, methods for anaerobic long term storage must be the focus of future research, especially when the production of grape marc is limited to such a small window from February to April.

Table of contents

1. Background

1.1 Introduction

Tannins are a promising group of phenolic compounds for decreasing enteric CH4 emissions from ruminants when used as a dietary supplement, with both in vitro and in vivo studies pointing to reductions in methane production in the presence of supplementary tannins. (Jayengara 2012)

While some studies have shown a strong effect of tannin supplementation in the suppression of ruminal CH4 formation, others have not. Initial interest surrounded tannin quantity and the relationship to methane production, although with this factor alone failed to explain all experimental outcomes. (Beuchemin 2007); (Lorenz 2012)

The relationship between tannin structure and its associated functional impact is highlighted by a number of sources,(Mueller-Harvey 2006); (Aufrere 2013); (Lorenz 2013) and it is suggested it is as important as the tannin content in the feed. Therefore the type of phenolic or tannin may affect the mechanism, and the extent to which this effect occurs, may produce inconsistent results. This may assist in explaining why the anti-methanogenic properties of tannin are not always evident. (Beuchemin 2007)

Grape marc, a solid grape-derived waste-product, is a rich source of simple phenolics and condensed tannins, is generated at scales of around 200,000 tonnes per annum in Australia. Preliminary studies have shown that feeding grape marc to cattle can reduce methanogenesis by up to 37% (unpublished data). The mechanism by which this occurs in the use of grape marc is not yet well understood, but it has been presumed to be due to the presence of high concentrations of condensed tannins.

Whilst there is a substantial amount of condensed tannin in grape marc, the actual concentration and composition can differ greatly between grape varieties, as seen by differing sensory characteristics in wine produced from differing varieties. Furthermore, it is understood that the source of grape tannin can greatly affect tannin chemistry with grape seed tannin and grape skin tannin possessing unique compositional characteristics.

1.2 Anti-methanogenic potential of grape marc

While tannin is the focus of this study it has been noted that other grape marc components may potentially impact upon methanogenesis. It is therefore possible that a synergistic interaction may exist between grape marc components, and as such a detailed characterisation of grape marc composition is needed to better understand the observations.

In a 2011 review article on supplementary fats and their impact on methane production linoleic acid was identified as having potential in inhibit methane production. (Rasmussen 2011) Making up around 15-20% of grape seed mass, the average grape seed oil composition is approximately 65% linoleic (C18:2), 20% oleic (C18:1), 10% palmitic (C16:0), 5% stearic (C18:0), with trace amounts of linolenic (C18:3) and other longer chain fatty acids.8 In comparison, a study into seed oil and ruminant methane production included supplementary crushed sunflower seed with a fatty acid profile very similar to that of grape seed oil (70.1% C18:2, 17.9% C18:1, 7.0% C16:0, 4.1% C18:0), which resulted in a decrease in methane per kg of DMI, but a slight increase per kg of digestible DMI. (Beuchemin 2009).

Furthermore, there has been evidence that glycerol,(Lee 2011) copper sulphate, (Slyter 1967) catechin,(Becker 2013) and tartaric acid (Sirohi 2012) have potential to contribute towards any anti-methanogenic tendencies of grape marc. As such, all of these additional factors will need to be considered when assessing the underlying mechanisms by which grape marc affects methane production.

1.3 Marc generation and storage

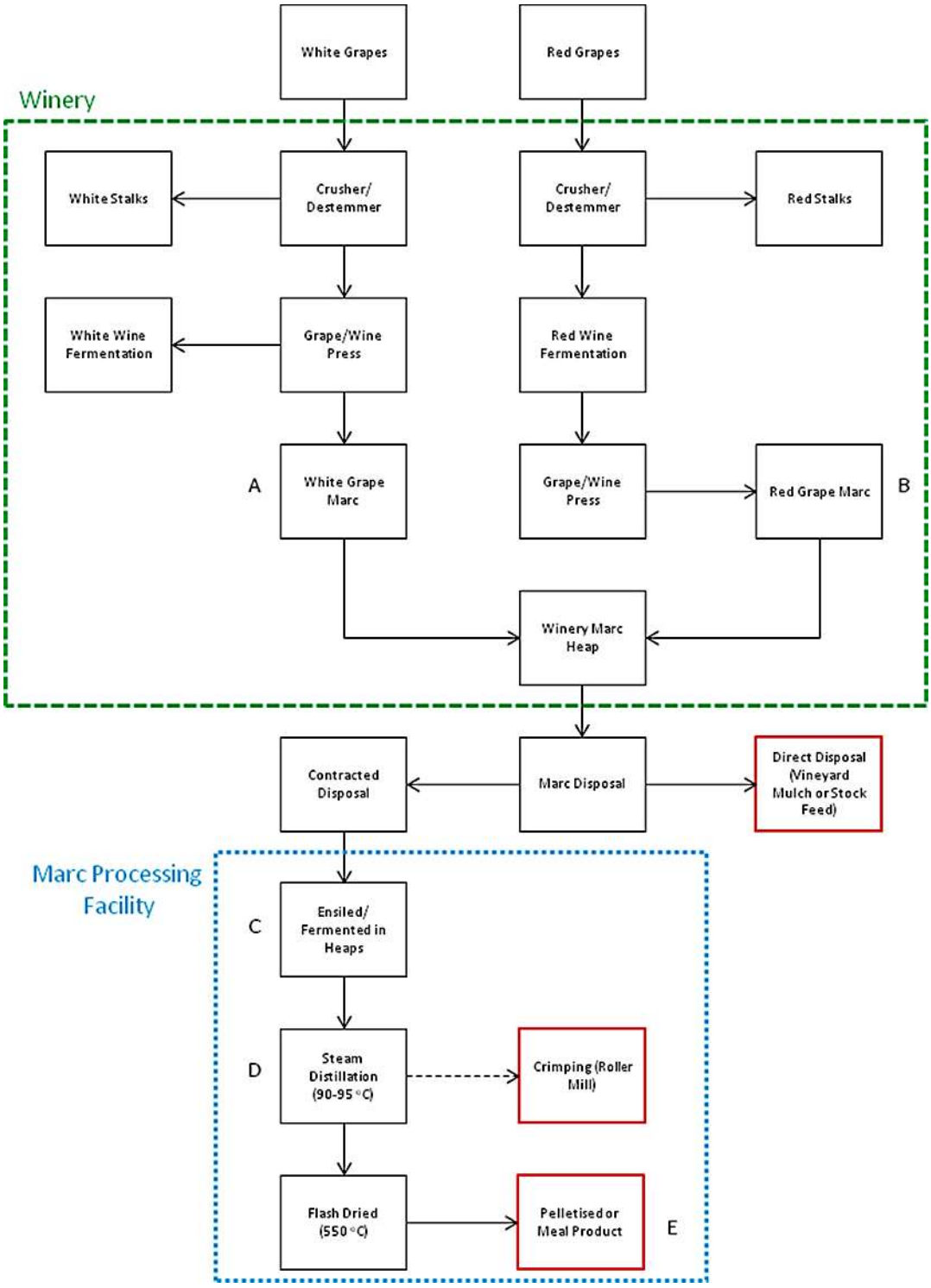
Grape marc is a waste product from the production of wine. Around 150,000 tonnes of grape marc is produced annually in Australia, subject to seasonal variation (Tarac 2015). Although a waste product, it is misleading to think of grape marc as being homogenous. Comprised of the left over fruit component of the winemaking process it generally includes grape skins, seeds and stalks but will have been through a variety of production processes. The degree of processing has a big impact on the availability and content of tannin. The current stream for marc processing is described below and shown in Figure 2.5:

- White fresh marc (stage A) – largely chemically unprocessed, white marc is essentially crushed white grapes including skins, seeds and occasionally stalks.
- Red fresh marc (stage B) – fermented skins, seeds and occasionally stalks. Red wine is made by fermenting crushed fruit including the skins and seeds. This contrasts with white wine where only solid free juice is fermented.
- Ensiled marc (stage C) – in the larger wine producing regions, marc is often collected and commercially processed. Marc is left in heaps to ferment, or ensile, for approximately 1 week to increase ethanol content (especially for white marc) prior to steam distillation.
- Steam distilled marc/spent marc (stage D) – ensiled marc is steam distilled to extract any remaining ethanol, often referred to as spent marc post-steam distillation.
- Dried marc (stage E) – following steam distillation, marc is dried and milled to create a marc meal, or pelletised, which can then be marketed as animal feed.

Currently, the application of marc into animal feeding is from two stages (indicated in red, Fig. 1): direct disposal of marc from a winery, and; post-processing as a dried product. A third process is being currently being investigated whereby spent marc is ‘crimped’ which involves removal of large material using a rotary screen, and the remaining marc is passed through a roller mill to crush the seeds. Furthermore, in previous in vivo trials (performed by DEPI Victoria) steam distilled marc was utilised in two forms, firstly a commercial dried marc meal (stage E) and secondly a spent marc (from stage D) that had undergone an additional ensilage process.

Although many options currently exist for marc processing, increasingly higher level of processing can lead to a reduction in the available tannin (through oxidation and interaction with cell wall material). And while there are multiple opportunities throughout marc production and processing for it to be accessed as a feed additive (including additional long-term ensiling and crimping at different stages), these additional preparation steps may also alter the available tannin content.

Figure 1. Grape marc production and processing



1.4 Changes in grape marc chemical composition

The inherent nature of winemaking processes responsible for generating white grape marc versus red grape marc results in compositional differences. Red grape marc has essentially undergone extraction process via maceration and fermentation of red juice on grape solids. As such, aqueous soluble compounds are found at lower concentrations in red grape marc than in white grape marc, including tannin, simple sugars (glucose and fructose), tartaric acid, and many others that may affect rumen fermentation.

In addition to differing between grape marc types, the different components of grape marc (skin vs seed vs stalk) results in important compositional changes. (Spanghero 2009); (Basalan 2011); (Molina-Alcaide 2008) Grape seed and grape skin tannin are chemically different, as well are the oils that are present in each fraction. Therefore, grape marc has exploitable differences as a result of colour and winemaking style but also the proportions of the base components that are present.

In earlier milestone reports we have identified a reduction in tannin concentration during grape marc processing and storage as these compounds are affected by oxygen and heat. This effect is consistent with the findings of other researchers, (Alipour 2007) along with observed increases to NDF, ADF and lignin values during ensiling. (Spanghero 2009)

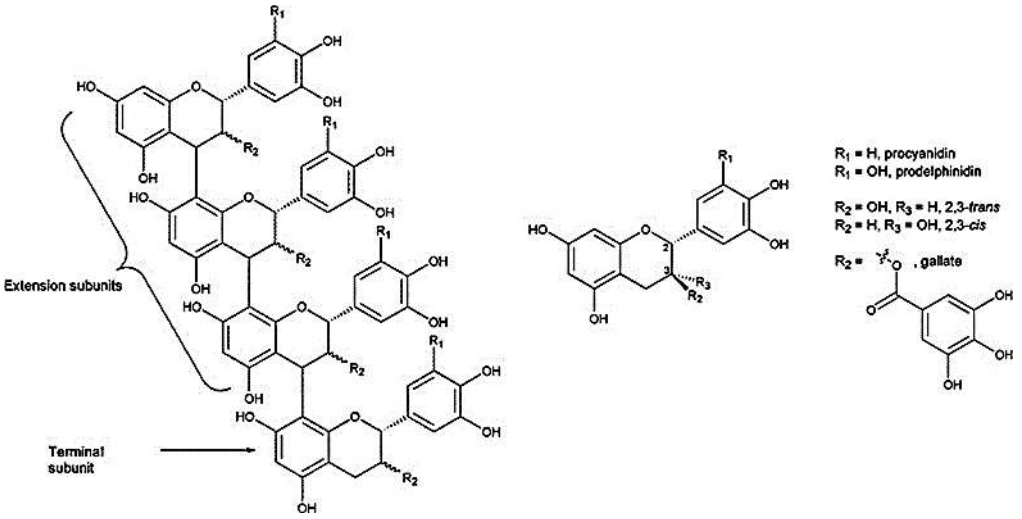
Along with changes in tannin and fibre during storage and ensiling, the nature of ensiling brings with it decreases in sugars and resultant increases in organic acids. (De Pina 1999)

The range of grape marc that has been accessed in this project includes red and white grape marc, components of each of these as well as samples from different stages of processing. With much compositional variation existing in grape marc, this allows us to consider tannin chemistry and quantity, but also allows for an investigation into many other variables that have been identified as having some effect of methanogenesis.

1.5 Tannin chemistry and analysis

Condensed tannins, or proanthocyanidins (PAs), are polymers of a number of structurally similar flavan-3-ol subunits (Fig. 2). Grape tannins differ in composition between grape skin and grape seed with skin derived tannin possessing a large proportion of tri-hydroxylated (%Tri-OH, R1 = OH) or prodelphinidin subunits, while procyanidin subunits lack one of these hydroxyl groups (R1 = H). Seed derived tannin generally has a higher degree of gallic acid substitution (at R2), referred to as percentage galloylation (%Gall), and also consists, on average, of fewer subunits per polymer chain, or a lower mean degree of polymerisation (mDP). Along with size, number of hydroxyl groups and gallic acid substitution, grape tannin composition can differ in subunit stereochemistry between the catechin based subunits (2,3-trans stereochemistry) and the epicatechin based subunits (2,3-cis stereochemistry).

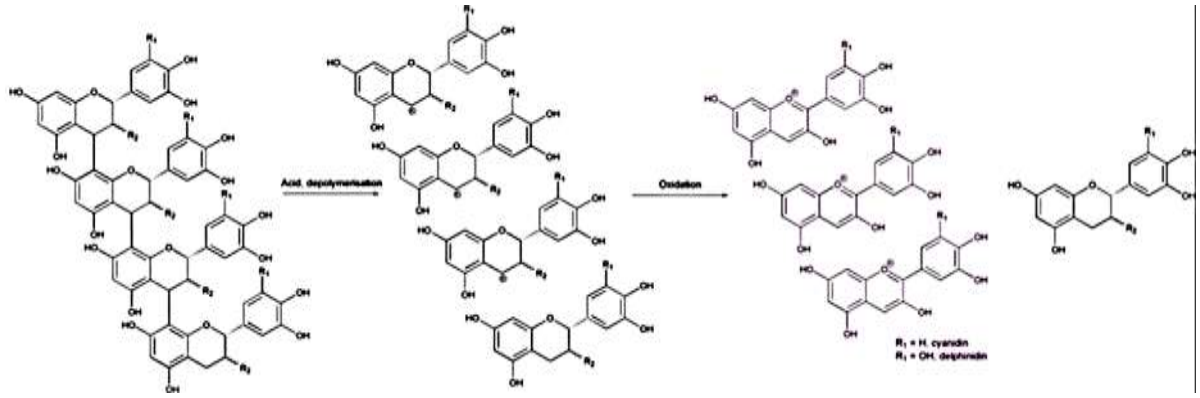
Figure 2: General structure of tannin and flavan-3-ol subunits.



The current analytical standard for tannin applied in agricultural laboratories generally, is a simple colourimetric analysis based around the acidic depolymerisation of tannin followed by oxidation of the released flavan-3-ol extension units to the anthocyanidins, cyandin and delphinidin, either directly from plant fibres or on extracts (Fig. 3).

Based on a simple assay utilising butanol and hydrochloric acid,(Bale-Smith, 1973); (Porter 1986) the inclusion of acetone into the assay has proved successful in ensuring complete removal of tannin from plant cell wall material. (Grabber 2013) Even so, this method relies heavily on using the correct tannin standard to create a calibration curve as the mDP of the standard will determine the number of colour producing subunits per weight of tannin and the ratio of procyanidin (R1 = H) to prodelphinidin (R1 = OH) will affect the final ratio of coloured products, and in turn the amount of colour.

Figure 3. **Tannin depolymerisation and evolution of colour during butanol-HCl assays.**

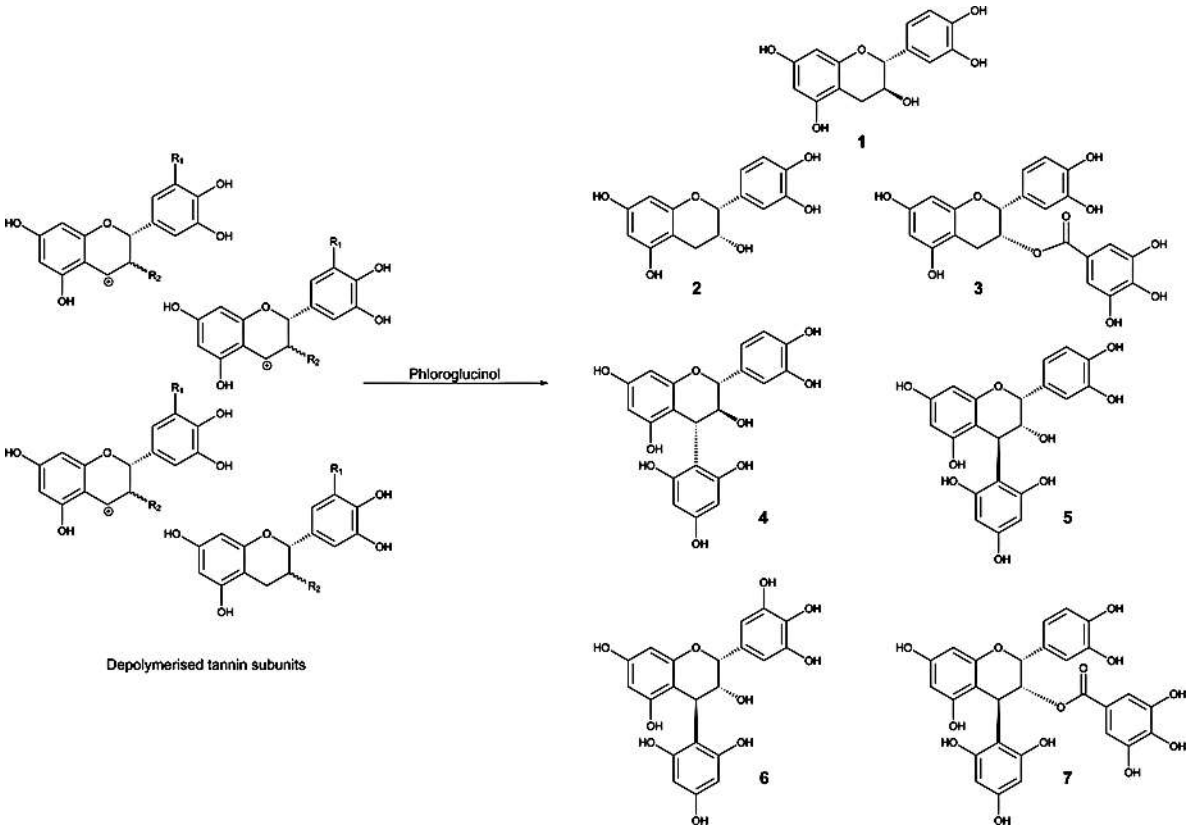


Two methods used commonly for analysis of wine derived tannin, that are applicable to grape marc, are: the methyl cellulose precipitation assay (MCP), (Mercurio 2007) and; acid catalysed depolymerisation in the presence of phloroglucinol (phloroglucinolysis). (Kennedy 2001) MCP takes advantage of the precipitation abilities of tannin and through interaction between tannin and methyl cellulose removes tannin from solution. A comparative absorbance measurement can be made

between this and a blank (having undergone no precipitation) to determine the tannin content. This method can be applied to tannin in solution (aqueous), and will be applied to grape marc extracts to determine water extractable tannin.

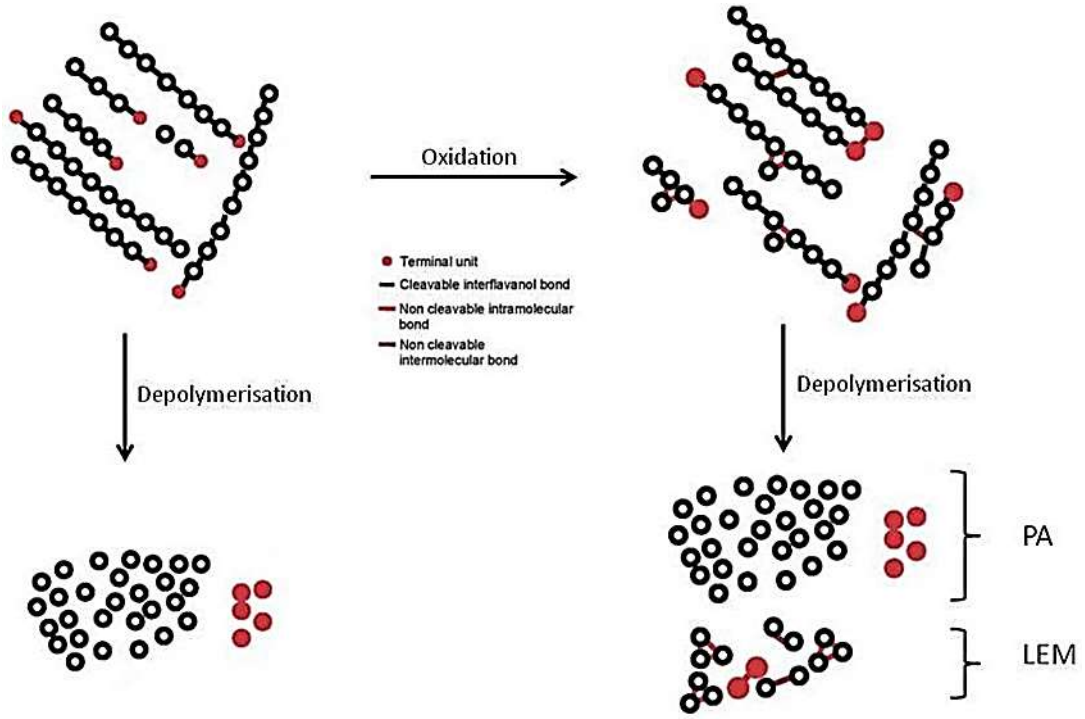
Phloroglucinolysis is a tool by which tannin composition is determined through depolymerisation and subsequent analysis of the individual subunits present (Fig. 4). The depolymerised extension subunits are 'trapped' using phloroglucinol (subunits 4-7) while the terminal subunits remain unadulterated (1-3). The ratio of these subunits give us compositional information of the tannin in terms of: length of chain, or mean degree of polymerisation (mDP); the stereochemistry of the subunits by comparing catechin based subunits with epicatechin based subunits (cis/trans ratio); extent of hydroxylation in terms of percentage containing tri-hydroxylated subunits (%Tri-OH), and; the extent of gallic acid substitution (%Gall).

Figure 4. Conversion of depolymerised extension subunits to phloroglucinol analogues, to give common grape derived phloroglucinolysis analytes (compounds 1-7).



The depolymerisation of single, perfect tannin chains will result in a complete conversion of tannin to subunits. Tannin becomes oxidised readily and undergoes cross linking both between subunits in the same chain, and between different tannin chains (Fig. 5). The depolymerisation of oxidised tannin results in the individual subunits which can be identified (PA) and the small oxidised fraction which can be analysed but not understood on a compositional level (LEM, late eluting material).

Figure 5. Depolymerisation of tannin (left) and of oxidised tannin (right) into PA subunits or PA and LEM, respectively



The use of phloroglucinolysis provides two pieces of information on tannin concentration and four on composition (as shown in Table 1) which can be exploited in understanding the link between the tannin present in grape marc and responses from in vitro and in vivo systems.

Table 1. Information obtained using phloroglucinolysis and subunits used to determine each tannin variable (numbering from Fig. 4).

Tannin Variable	Description (ratios of subunits used to determine)
Concentration	
PA	Identifiable subunits (sum of **1-7**)
LEM	LEM fraction (epicatechin equivs)
Composition	
mDP	Length of chain (compounds **1-3** vs **4-7**)
cis/trans	Stereochemistry (compounds **1,4** vs **2,3,5-7**)
%Tri-OH	Hydroxylation (**6** vs **1-5,7**)
% Gall	Gallic acid substitution (**3,7**, vs **1,2,4-6**)

1.6 Vintage time period

Wine production is constrained to that time of the year when wine grapes are ripe for harvesting. In turn this means that grape marc is only available as a fresh product from late January or early February to April. White marc is generally available from the warmer climate regions first (late January) with red marc generally available 3-4 weeks later. The vintage period ends with red wines generally finishing in April for the cooler climate regions. For grape marc to be accessed outside of the vintage period, it must be collected during and stored until required. Alternatively, grape marc can be sourced from a processing facility if they have material remaining in their stock piles.

2. Objectives

The objective of this project is to reduce Australia's agricultural methane emissions through:

- Quantifying grape marc compositional variation, including a survey of potential agrichemical residues and any other harmful or toxic chemical compounds, with a specific view to generating information about tannin levels and types in grape marc.
- Conducting in vitro screening of grape marc samples with known tannin variations, in partnership with DPI Victoria through project (B.CCH. 6460), This will determine how tannin variations (level and type/complex) effect methane emissions.
- Based on the findings of the tannin and marc characterisation work and the results of in vitro screening, conduct in vivo assessment of grape marc as a feed additive. This will be completed in partnership with DPI Victoria through project B. CCH. 6460.
- Characterising grape marc tannins and elucidating to what extent they are the active ingredients responsible for reducing ruminant emissions -this could guide improvements across all agricultural feedstocks.

The objectives of this project were fully met.

3. Methodology

3.1 Marc collection and processing

Marc samples were collected during vintages 2012 from around Adelaide, and during vintage 2013 from other Australian regions in small parcels (approximately 500g) and remained in storage at -20°C until used. These samples were initially used in developing analytical methods, understanding processing and compositional changes in marc tannin, and were then used in the agrochemical residue survey.

Marc samples were collected during and post-vintage 2013 in larger volumes than previous sampling (now approximately 5kg), allowing enough material to be both analysed and submitted to in vitro experimentation. Samples were collected from the marc process stages A-D, refer to Fig. 1, and stored at -20 °C prior to analysis.

Fresh marc samples are those collected from a winery, either directly from the press or from the winery marc heap (maximum of a few hours post-pressing). Ensiled marc samples are those left in a heap for approximately 1 week (have undergone a short- term ensilage process) prior to steam distillation. Steam distilled or spent marc samples are those that are post-steam distillation with any additional processing (crimping, drying etc.) as specified.

All samples were freeze-dried and milled to pass a 1.0mm screen prior to analysis, so that they were analysed in the same form in which they were to be submitted to subsequent in vitro fermentation.

For in vivo evaluation grape marc was placed into silage bags (approx. 40kg per bag), sealed, and the sealed bags re-bagged to avoid any ingress of air. Samples that were collected directly prior to use in trials were loaded onto pallets and transported. Those samples that were collected into bags in advance of trials were stored at 4° C until transported.

3.2 Agrochemical residue analysis

Grape marc samples collected from across Australia (122 samples) were selected based on their origin and characteristics and submitted to agrochemical residue analysis. Residue analysis was provided by The Australian Wine Research Institute Commercial Services Laboratory for 64 common grape agrochemicals. Data for wet marc samples (fresh marc moisture content 50-75%) was converted to dry matter content (DM) and compared with known maximum residue limits (MRLs) for grape pomace animal feeds, where known (APVMA, see Appendix 1, Table A1: Agrochemicals analysed for in grape marc with associated maximum residue limits (MRLs) for agrochemical residues in grape pomace animal feed, other animal feed, and grape derived food products, compiled using data from the Australian Pesticides and Veterinary Medicines Authority (APVMA).). Samples were selected based on climatic regions with temperature and rainfall having a large impact of the prevalence of pests, and hence regimes for chemical usage. Sample choice also represented colour of grape and size of winery. Climate regions were separated by Mean January Temperature (cool: MJT < 19°C, warm: MJT 19-21 °C, hot: MJT > 21 °C) and Growing Season Rainfall (wet: GSR > 300 mm, dry: GSR < 300 mm). Winery size was based on annual crush (small: < 1,000 tonnes, medium: 1,000-10,000 tonnes, large: > 10,000 tonnes).

3.3 Tannin Standards

Tannat grapes were manually separated into skin and seed and each extracted with 70% aqueous acetone. Acetone was removed and the remaining aqueous extracts were diluted with methanol and trifluoroacetic acid (TFA), to approximately 45 % MeOH containing 0.01 %v/v TFA. The solution was loaded onto a Toyopearl column and subsequently washed with 1:1 MeOH:H2O containing 0.01 %v/v TFA. The purified tannin was eluted using 2:1 acetone:H2O containing 0.01 % v/v. The acetone was removed under vacuum using a rotary evaporator (water bath at 30 °C to avoid tannin degradation), and the aqueous tannin extracts were lyophilized. The resulting powders (purified skin and seed tannin) were analysed by phloroglucinolysis for condensed tannin content and subunit composition.

3.4 Butanol-HCL tannin assays

A traditional butanol-HCl colourimetric assay was employed in an attempt to align our grape marc tannin data with both what was perceived as the agricultural industry standard, and that used by one of our partner organisations (DEPI Victoria). A quick and convenient method, the butanol-HCl, assay is limited most strongly by choice and suitability of standard, and linearity in calibrations using grape tannins, as well as evidence of incomplete tannin removal from plant fibres.

An improved acetone containing assay provided more reliable results, but is less accessible to the more basic laboratory than the traditional assay. The volatility of the solvent (50% acetone) facilitates the need for pressure withstanding test tubes and still requires the use of a carefully chosen tannin standard. The method was modified slightly from the literature method so that it could be applied to both marc fibres and marc extracts under the same conditions. The assay reagent was designed to account for a final 300 µL addition of 70% acetone to each reaction vessel from an extract. Given the evidence that slight changes in solvent volumes and proportions alter the production of colour, (Grabber, 2013) assay reagent added to marc fibre directly compared with the

same reagent added to an extract would require different calibration curves, which was deemed undesirable.

The butanol-HCl method, both traditional and modified, had to be improved for use with grape fibre to account for the presence of anthocyanins and other pH dependant coloured species, by use of a blank that was acidified immediately prior to colour determination. Both the traditional and acetone containing assays have shown good reproducibility between replicates for tannin standards, marc samples and extracts.

For marc samples with tannin content that can only be determined by the modified butanol-HCl assay (not accessible by phloroglucinolysis), an investigation into the use of post-assay analysis by HPLC is required to determine whether the composition of this tannin can be partially understood by analysing for the breakdown products, the anthocyanidins.

The analysis of grape marc fibres for post-phloroglucinolysis tannin (PPT) was performed in the same manner as described for acetone-butanol-HCl assays above.

3.5 Grape/wine derived tannin analysis

Analyses for measuring tannin content and composition native to the wine industry (phloroglucinolysis and methyl cellulose precipitation assay) are at the cutting edge of tannin analysis and provide a wealth of detail, but are not readily applicable to tannin incorporated in plant material fibres, having been developed to be used in conjunction with aqueous media (wine, water etc.). Furthermore, compositional data obtained by phloroglucinolysis does not achieve full removal of tannin from marc fibres and as such provides the composition for a large proportion of the marc tannin, but not all.

The methyl cellulose precipitation assay was performed on aqueous extracts of marc and quantified as epicatechin equivalences (based on the absorbance of this compound) using extracted and purified grape tannin as a standard.

The literature phloroglucinolysis method (employing a shaking water bath) was modified to a dry heating block with regular agitation (every 5 mins by vortex mixer), which provided for a more complete recovery of marc tannin, and allowed for a higher through-put by increasing sample capacity.

The concentration of tannin in each marc sample is quoted as both the total sum of known subunits (PA, in g/kg of dry matter) and as the LEM fraction as determined in epicatechin equivalences (PA+LEM, g/kg DM). The concentration of monomeric flavan-3-ols was determined by submitting a single marc sample to the same reaction conditions (50 °C, 25 minutes) in the presence of methanol alone. Any detected monomeric flavan-30ols were deducted from those determined in the depolymerised samples.

3.6 Further compositional analysis of grape marc

All analysis on grape marc samples that were used during in vitro fermentation studies were analysed in the same form in which they were submitted to fermentation: freeze-dried and ground to pass a 1mm sieve.

Sub-samples of grape marc used during in vitro fermentation studies were sent to AgResearch (New Zealand) for fats and oils analysis and to Dairy One (Ithaca, New York, USA) for nutritive analysis.

Concentrations of monomeric flavan-3-ols (catechin, epicatechin and epigallocatechin) were determined from blank samples used in phloroglucinolysis tannin analysis.

Organic acid and sugar profiles were determined by HPLC from grape marc extracts. For each grape marc sample 500 mg was extracted with 80% ethanol to determine free acids, centrifuged and extract recovered. The resulting solid was then extracted with 1M hydrochloric acid solution to recover organic acid salts. The compounds analysed for were acetic acid, glycerol, lactic acid, malic acid, succinic acid, tartaric acid, glucose, fructose and citric acid).

3.7 In vitro round 1 - grape marc screening experiment

Twenty grape marc samples that were diverse in tannin concentration and composition, and that were obtained from across the grape marc processing chain were submitted to in vitro evaluation. In vitro batch fermentation experiments were conducted by the School of Land and Environment at Melbourne University using the ANKOM gas production modules. (Basic ANKOM 2015) Dairy cow rumen fluid was collected from rumen fistulated cows at Ellinbank, Victoria. Fermentations were replicated (8 reps, 1 gram of marc per ferment) for each marc sample, with only the average of the replicates reported here. Gas production, final gas composition, final volatile fatty acid (VFA) concentration and final pH were all determined.

3.8 In vitro round 2 – grape marc dose response experiments

From the initial in vitro screening study eight grape marc samples were chosen to be used in further inclusion/dose response studies, using the same ANKOM (Basic ANKOM 2015) batch fermentation system. Two separate experiments were performed, the first an inclusion experiment and the second a supplementation experiment. The fermentation data was collected as per the previous in vitro experiment.

For the inclusion experiment 1g of dry matter was present in each ferment with different levels of grape marc included. All eight grape marc samples were fermented with three different forages (chicory, perennial ryegrass or hay) at 3 inclusion rates (25%, 50% or 75% DM) as well as 100% forage and grape marc, with a total of 1g of dry matter in each ferment.

For the supplementation experiment, 0.5g of forage was present in every fermentation with two different levels of grape marc supplemented, either 0.125g (20% marc) or 0.250g (33% marc). The same three forages and eight grape marcs were used as for the inclusion experiment.

The label given to each fermentation was abbreviated to show the forage (chicory = C, Hay = H or perennial ryegrass = P), the grape marc sample used (1, 7, 8, 10, 12, 18, 19 or 20), and the rate at which it was included (25, 50 or 75% for inclusions and 20 or 33% for supplementations). For example, a 25% inclusion of AWRI 012 into chicory is shown as ‘C12.25’.

3.9 In vitro round 3 tannin or fat

Further experiments to differentiate the anti-methanogenic activity of fat and tannin were performed by The University of Western Australia using sheep rumen fluid. Each treatment was performed in triplicate on 500 mg of substrate, with grape marc inclusions at 30% of the total dry matter (150 mg into each ferment with 350 mg of a concentrate based diet – Milne standard pellet, Milne Feeds, Western Australia).

3.10 Dairy Cow in vivo feeding trial

MLA is committed to investing in top quality scientific research, performed by suitably qualified, experienced and registered researchers and organisations. In experiments that involve livestock, MLA acknowledges that such research needs to be done under the auspices of a recognised Animal Care and Ethics Committee (AEC). The responsibility for obtaining AEC approval lies with the researcher. MLA has in the past not specifically asked for evidence that such AEC approval had indeed been obtained.

White ensiled, crimped grape marc and red steam distilled, crimped grape marc were used in the in vivo feeding trial to represent two types of marc (white and red) and the processing most likely experienced for marc used as an animal feed (ensiled and steam distilled). The two marc parcels were analysed for tannin and for nutritive value using the same procedures as used previously for in vitro marc samples. Thirty-two dairy cows in three groups were fed a control diet (n = 12), or a diet supplemented with white ensiled grape marc or red steam distilled grape marc (n = 10 for each treatment) as shown below (Table 2). The pasture used for this trial was predominantly ryegrass.

Table 2: Animal diets for dairy cow in vivo feeding trial.

Treatment	Control	White marc	Red marc
No. of cows	12	10	10
Feed intake (kg DM/cow/d)			
Pasture	13.67	9.74	9.84
Cold –pressed canola	1.97	1.81	1.81
Corn	2.98	2.83	2.83
White grape marc	0	4.2	0
Red grape marc	0	0	4.48
Total DMI	18.62	18.58	18.96

3.11 Sheep in vivo feeding trial

Two parcels of grape marc were collected and transported to the Dookie campus of The University of Melbourne. One parcel had been steam distilled and crimped before being stored in a grain bag for 5 months before being bagged and transported. The second parcel had been steam distilled during vintage (May 2014) and stored aerobically at the processing facility until required.

The two grape marc parcels were fed at three inclusion rates (10%, 20%, 30%) into a diet of oaten hay, plus a control group (7 sheep per treatment). Methane measurements were taken using a face mask.

3.12 Analysis of grape marc composition and fermentation outcomes

All data sets were compared with methane volume per unit of metabolisable energy (vCH4/ME) on a single variable basis to identify any obvious linear correlations. In each case the x-axis (compositional variable) was normalised using the highest value to give an x-axis scale of 0 to 1 and make correlation slopes comparable. Further statistical analyses were performed by principle component analysis (PCA) and partial least squares regression (PLS) using the Unscrambler X software package.

Multivariate analyses were performed by principle component analysis (PCA) and partial least squares (PLS) regression using the Unscrambler X software package. Abbreviations used for input and outputs are as follows:

Table 3. Abbreviations used for multivariate analysis of in vitro data.

Factor	Abbreviation	Factor	Abbreviation
Fermentation information		Mineral content (%)	
Rate Constant (h^{-1})	Rate	Calcium	%Ca
Max gas production (ml/g DM)	vMax	Phosphorus	%P
CH_4 production (% of total gas)	%CH4	Magnesium	%Mg
CO_2 production (% of total gas)	%CO2	Potassium	%K
CH_4 volume (ml/g DM)	vCH4	Sodium	%Na
Acetic acid (mmol/L)	Ac	Sulfur	%S
Propionic acid (mmol/L)	Pr	Chloride	%Cl
Butyric acid (mmol/L)	Bu		
Total VFA (mmol/L)	VFA	Mineral content (ppm)	
Acetate:propionate ratio	Ac:Pr	Iron	Fe
Final pH	pH	Zinc	Zn
Volume of methane per unit of ME (mL/MJ)	vCH4/ME	Copper	Cu
Mass of forage in ferment (g)	Forage (g)	Manganese	Mn
Mass of grape marc in ferment (g)	GM (g)	Molybdenum	Mo
Nutritive information		Phloroglucinolysis tannin	
Metabolisable energy (MJ/kg)	ME	Flavan-3-ol subunit concentration (g/kg DM)	PA
Crude protein (%)	CP	LEM concentration (g/kg DM)	LEM
Available protein (%)	Av P	Mean degree of polymerisation	mDP
Acid detergent insoluble protein (%)	ADICP	cis/trans ratio	cis/trans
Adjusted protein (%)	Adj P	Trihydroxylation	Tri-OH
Soluble protein (% of CP)	Sol P	Galloylation	Gall
Neutral detergent insoluble protein (%)	NDICP		
Acid detergent fibre (%)	ADF	Other tannin analyses (g/kg DM)	
Neutral detergent fibre (%)	NDF	Porters assay tannin	PAT
Lignin (%)	Lignin	Water extractable tannin	WET
Non-fibre carbohydrate (%)	NFC	Post-phloroglucinolysis tannin	PPT
Starch (%)	Starch		
Ethanol soluble carbohydrate (%)	ESC	Flavan-3-ols (g/kg DM)	
Crude fat (%)	Cfat	Total	Flavs
Ash (%)	ash	Catechin	C
		Epicatechin	EC
Fatty acid content		Epigallocatechin	EGC
Sum of all fatty acids (g/kg DM)	FA		
Palmitic (% of fatty acids)	C16	Acids and sugars (g/kg DM)	
Palmitoleic (% of fatty acids)	C16:1	Acetic acid	Acetic
Stearic (% of fatty acids)	C18	Glycerol	Glyc
Oleic (% of fatty acids)	C18:1	Lactic acid	Lac
Linoleic (% of fatty acids)	C18:2	Malic acid	Mal
Linolenic (% of fatty acids)	C18:3	Succinic acid	Succ
Behenic (% of fatty acids)	C22:0	Tartaric acid	Tart
Lignoceric (% of fatty acids)	C24:0	Glucose	Glu
		Fructose	Fru

4. Results

4.1 Agrochemical Residue study

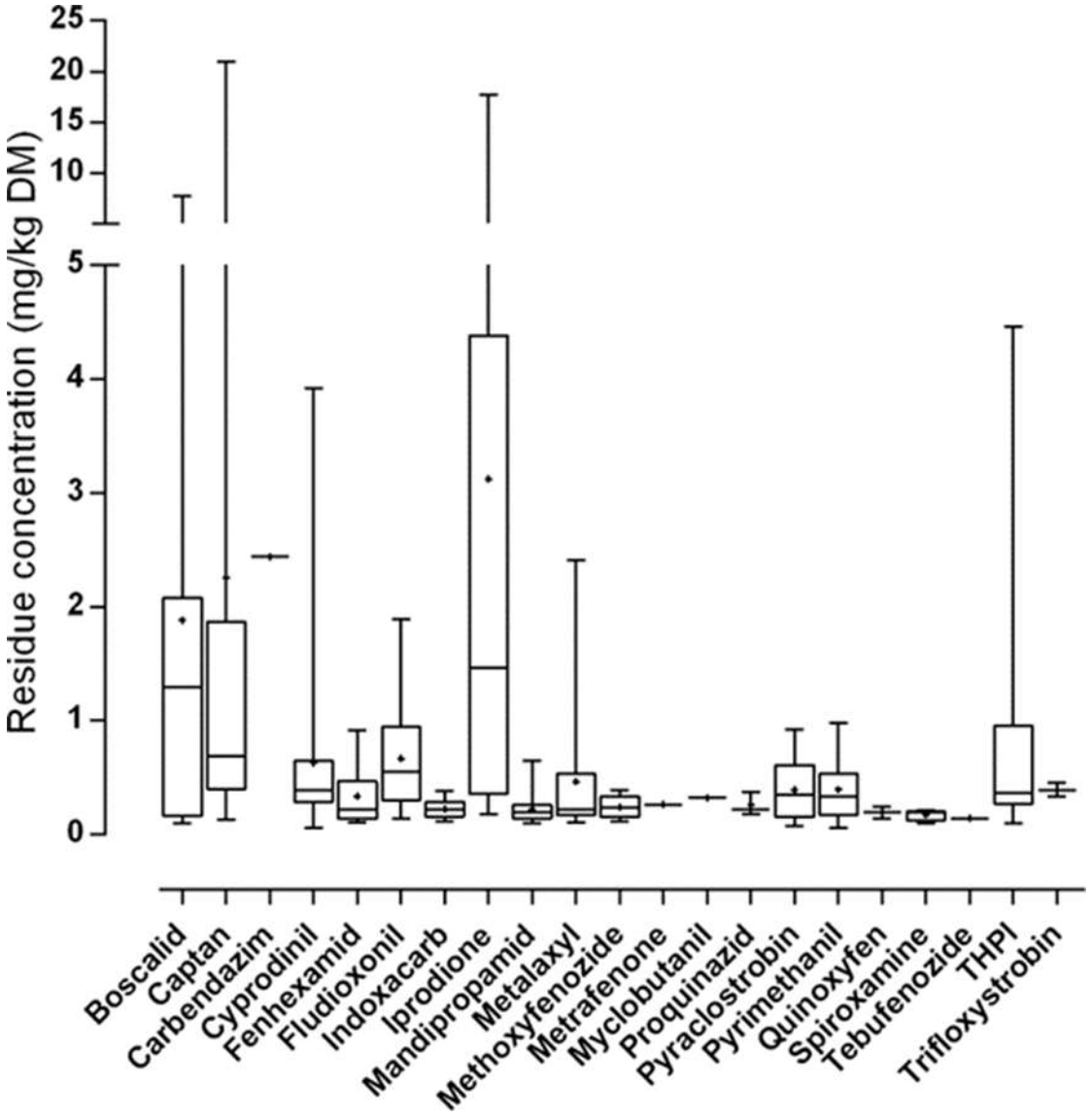
Marc samples collected during the 2013 vintage, and some remaining from the 2012 vintage, were analysed for the presence of 64 agrochemical residues, across climate, colour, processing, and winery size.

Table 4. Breakdown of marc samples submitted to analysis for agricultural chemical residues.

Attribute	No of Samples
All Samples	122
Marc Type	
White	53
Red	52
Processed	17
Vineyard Climate	
Cool	23
Hot	26
Warm-wet	27
Warm-dry	28
Winery Size	
Large	45
Medium	19
Small	41

Marc samples were divided into climate (n=104) and winery size (n=105) only where the origin could be adequately determined. No processed marc samples were included in analysis of climate and winery size due to an inability to accurately determine the origin. Of the 64 residues analysed for, only 21 were detected across all samples. The average and range of concentrations (in mg/kg of DM) of agrochemical residues detected in these marc samples are show below (Fig. 6).

Figure 6. Concentration of agrochemical residues found in grape marc samples.



* Boxes show median value, 25thand 75th percentile, whiskers to maximum and minimum values. Mean value marked with a cross (+).

The number of individual residues detected in a single marc sample was as high as nine (in one instance), with the majority of samples containing between zero and four residues (82%, Table 5). The occurrence of each residue across the sample range is shown in Table 6.

Table 5: Distribution of the number of residues detected in individual marc samples.

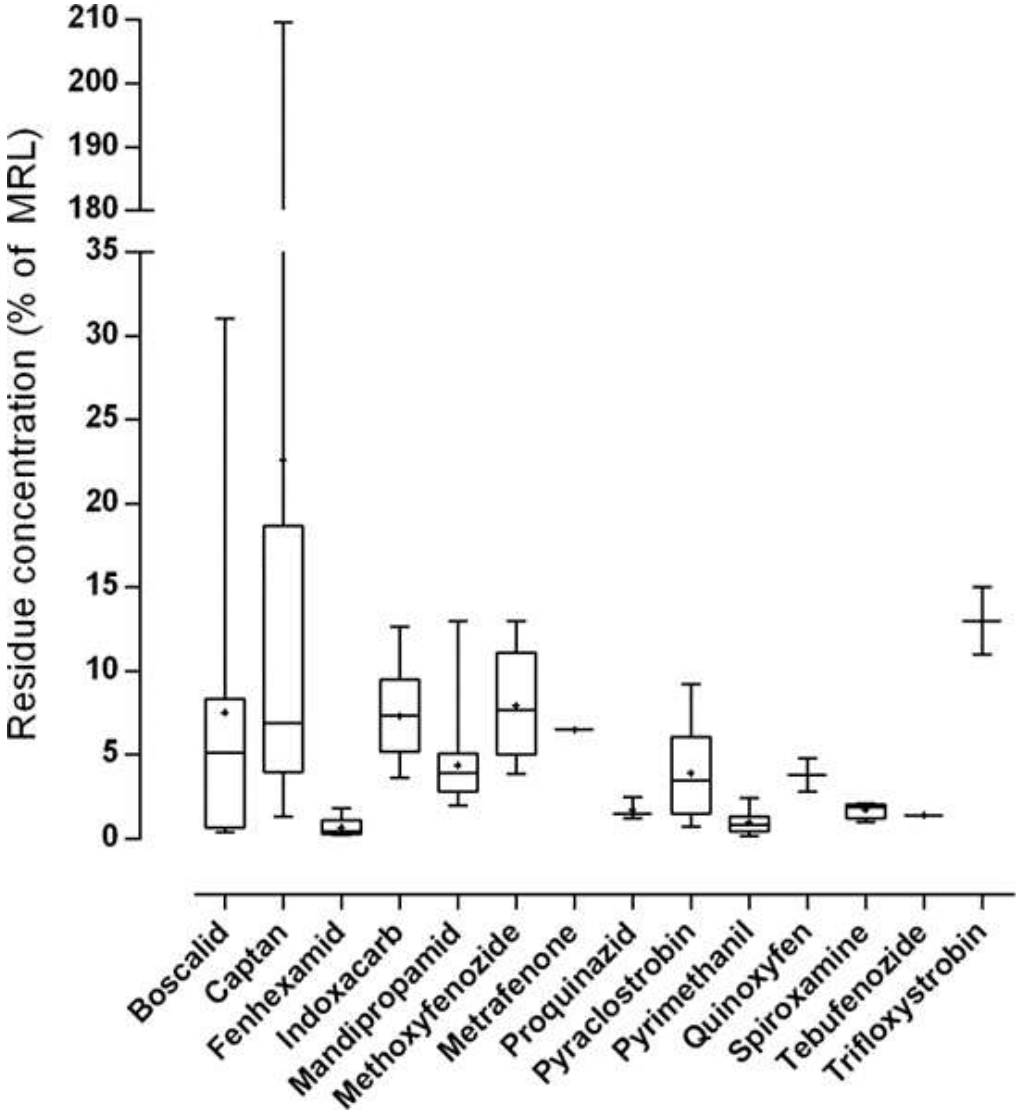
No of residues detected in sample	No of samples	% of samples
0	15	12.3%
1	25	20.5%
2	24	19.7%
3	18	14.8%
4	18	14.8%
5	12	9.8%
6	4	3.3%
7	5	4.1%
8	0	0.0%
9	1	0.8%

Table 6: Occurrence of each of the 21 residues in grape marc samples.

Residue	No of samples detected in	% of samples
Boscalid	7	5.75%
Captan	23	18.9%
Carbendazim	1	0.8%
Cyprodinil	45	36.9%
Fenhexamid	14	11.5%
Fludioxonil	18	14.8%
Indoxacarb	17	13.9%
Iprodione	45	36.9%
Mandipropamid	16	13.1%
Metalaxyl	12	9.8%
Methoxyfenozide	13	10.7%
Metrafenone	2	1.6%
Myclobutanil	1	0.8%
Proquinazid	3	2.5%
Pyraclostobin	39	32.0%
Pyrimethanil	27	22.1%
Quinoxyfen	2	1.6%
Spiroxamine	4	3.3%
Tebufenozide	1	0.8%
THPI	35	28.7%
Trifloxystrobin	2	1.6%

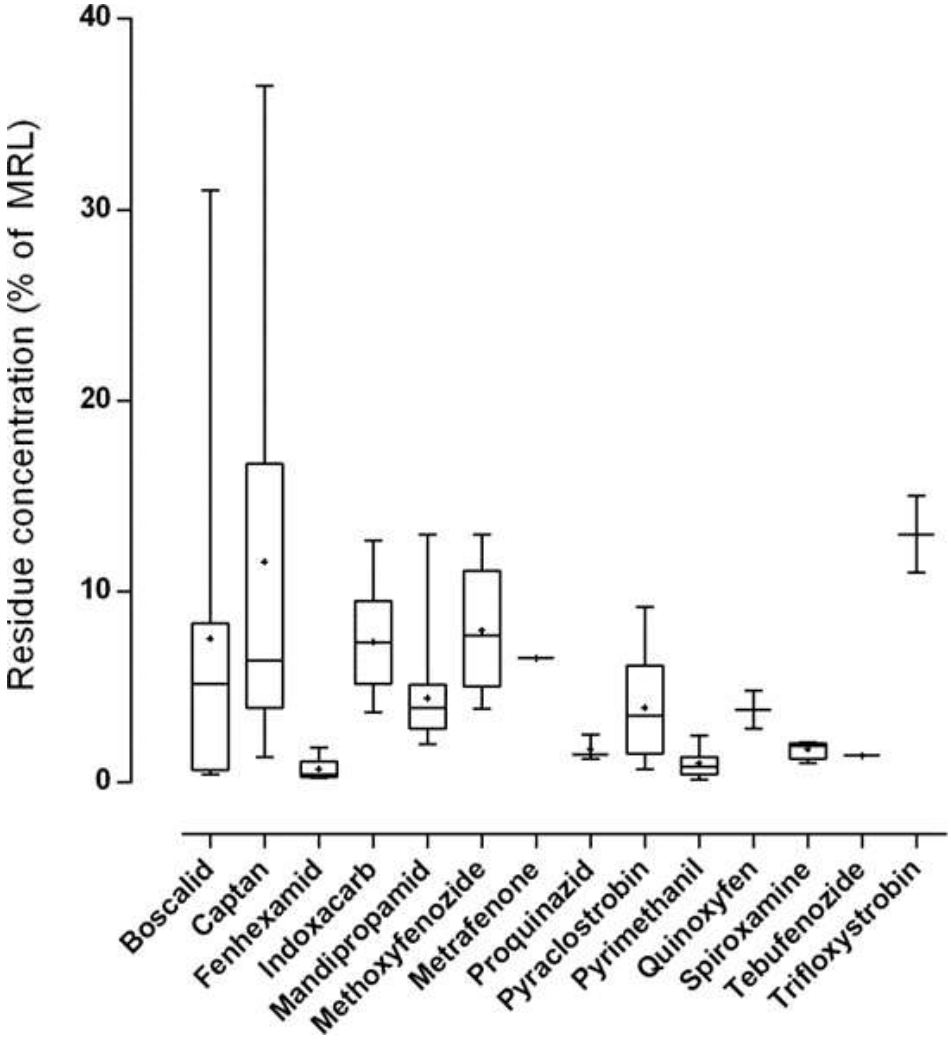
With respect to the maximum residue limits (MRLs), only 14 of the 21 detected residues have determined limits for grape pomace animal feed, as shown in Figure 7. One grape marc sample contained a residue above the stated MRL (captan), and there is a notable concentration of iprodione in a small number of samples, for which there is no existing MRL.

Figure 7: Agrochemical residue content in grape marc, expressed as percentage of allowable limit in grape pomace based animal feed (APVMA data). Boxes show median value, 25th and 75th percentile, whiskers to maximum and minimum values. Mean value marked with a cross (+).



The survey of agrochemical residues found one sample with a residue above a known MRL (209.5% of captan MRL, see Figure 7), and another sample with a reasonably high content of the same compound (67.5% of captan MRL). With these two instances removed, the content of compounds with known MRLs in grape pomace animal feed can be more clearly seen (Figure 8). Outside of the two removed points, there is no apparent issue with any of the residues detected with respect to MRLs.

Figure 8: Agrochemical residue content in grape marc, expressed as percentage of allowable limit in grape pomace based animal feed (APVMA data), with two high concentration samples removed. Boxes show median value, 25th and 75th percentile, whiskers to maximum and minimum values. Mean value marked with a cross (+).



However, there may be some concern for compounds containing iprodione, a compound that does not have an established MRL in grape pomace animal feed. Tabulated below, are the eight marc samples that had total combined agrochemical contents above 10 mg/kg. Of these, iprodione is a major factor in six, being found above 10 mg/kg in four instances (Table 7).

Table 7: Attributes of marc samples with high overall content of residues.

Sample Total (mg/Kg DM)	Climate	Colour	No of residues detected	Highest Residue (mg/kg DM)	2nd Highest Residue (mg/kg DM)
27.8	Warm-wet	Red	6	Captan (20.95)	THPI (4.46)
23.23	Warm-dry	White	7	Iprodione (12.45)	Boscalid (7.76)
18.44	Warm-dry	Red	3	Iprodione (17.69)	Pyrimethanil (0.051)
15.98	Hot	Red	5	Iprodione (13.83)	Boscalid (1.29)
14.34	Hot	Red	4	Iprodione (13.64)	Cyprodinil (0.30)
13.27	Warm-wet	Red	5	Captan (6.75)	Iprodione(4.62)
12.34	Warm-dry	Red	5	Iprodione (8.47)	Boscalid (2.08)
10.73	Cold	White	9	Cyprodinil (3.92)	Fludioxonil (1.89)

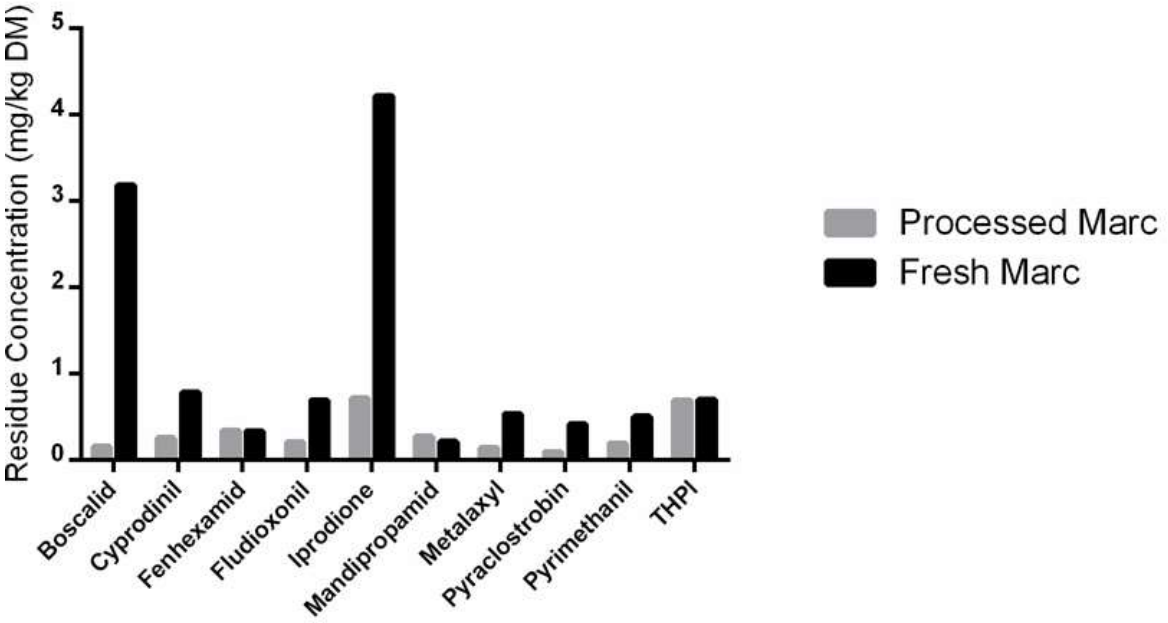
While there is no MRL for this exact purpose, there are established MRLs for iprodione in other animal feeds: alfalfa forage, 20 mg/kg DM; canola, 1 mg/kg DM; citrus, 15 mg/kg DM; peanut forage, 20 mg/kg DM, and; soya bean forage, 5mg/kg DM. Currently, it cannot be said whether these concentrations of iprodione in grape marc will pose a problem, but they do warrant further investigation.

The average iprodione content in fresh marc samples is 2.80 mg/kg for white marc and 4.79 mg/kg for red marc. Processed marc, however, has an average iprodione content of 0.71 mg/kg with a maximum concentration of 2.83 mg/kg. This trend continues for processed marc, whether it is simply ensiled or steam distilled. Table 8 shows the content of agrochemical residues that have established MRLs in processed marc samples. Only five residues can be seen, and all well below MRLs. The apparent reduction in residue concentration with processing is highlighted in Figure 9: Comparison of average residue content in fresh marc samples (n=105) and processed marc samples (n=17), which compares the average content of the 10 residues common to fresh and processed samples. The majority show a vast reduction in processed samples against fresh ones.

Table 8: MRL data for the 17 processed samples.

	Boscalid	Fenhexamid	Mandipropamid	Pyraclostrobin	Pyrimethanil
MRL (mg/kg DM)	25	50	5	10	40
Occurrence	3	2	2	2	9
Average (% of MRL)	0.60	0.68	5.47	1.00	0.47
Maximum (% of MRL)	0.77	0.77	5.80	1.10	1.00

Figure 9: Comparison of average residue content in fresh marc samples (n=105) and processed marc samples (n=17).



As well as reductions in specific residues, the average residue content in processed samples is lower than in fresh samples (Table 9). There were fewer residues detected across the processed samples than in fresh, and the average concentration of the residues observed is much lower.

Table 9: Summary of agrochemical residues by colour and processing.

Colour /Process	Number of different residues detected	Average no. of residues per sample	Average residue concentration (mg/kg)
White marc	18	2.81	0.83
Red Marc	17	2.35	1.45
Processed marc	10	3.24	0.41

We expected that climate would be the biggest factor in determining residue usage, and hence prevalence (see Table 10). Much as in the above table, those climates with the greater number of agrochemicals per sample have lower average concentrations. So as the climate becomes cooler, there is a decrease in residue concentration, but also a greater range of chemicals used in the cool and warm-wet climates than in hot and warm-dry. This could be a result of a greater number of pests that can thrive in colder climates, so a larger range has to be applied. However, as shown in Table 7 (above), samples with high overall residue concentrations were observed from all climates, and hence no generalisations should be made regarding the presence of residues and the climate that the grapes were grown in.

Table 10: Summary of agrochemical residues by climate

Climate	Number of different residues detected	Average no. of residues per sample	Average residue concentration (mg/kg)
Hot	14	2.46	1.23
Warm-dry	13	2.31	1.19
Warm-wet	14	2.89	0.91
Cool	16	2.70	0.62

Similarly to above, no solid conclusions can be made regarding agrochemical content and winery size (results not shown), with no obvious differences in content or number of residues across small medium and large wineries.

4.2 In vitro round 1 – grape marc screening experiment

The grape marc screening experiment was performed in two separate batches due to a maximum capacity in the fermentation set-up of around 120 samples. The nine processed samples made up the first batch and the eleven fresh samples made up the second. Each grape marc was fermented in 8 replicates, with some key fermentation outcomes shown in Table 11.

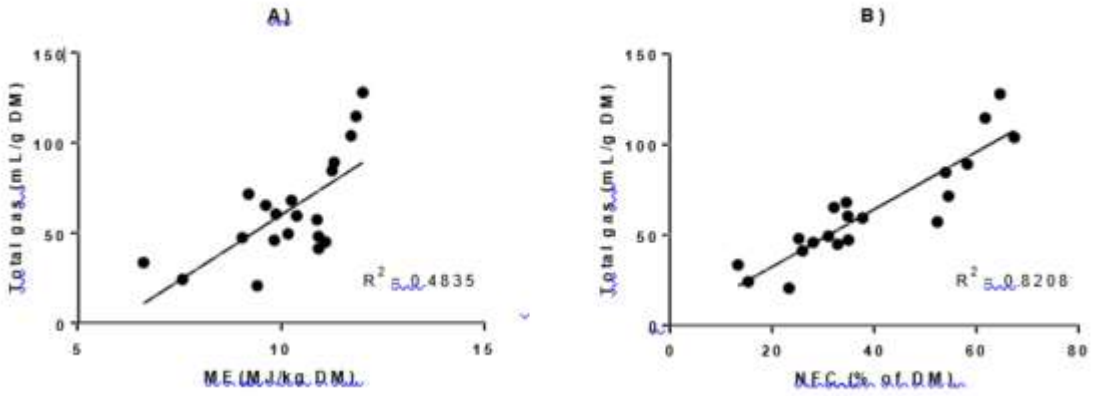
Table 11: In vitro fermentation outcomes (average of replicates).

	Sample	ME (MJ/kg)	Max Gas (mL/g DM)	%CH_4	vCH_4/ ME	Ac:Pr
AWRI 001	Steam distilled, dried	5.61	33.6	35.84	1.85	2.30
AWRI 002	White Ensiled	10.92	48.0	31.89	1.40	2.71
AWRI 003	White Crimped	10.92	41.3	34.54	1.31	3.25
AWRI 004	White Spent	10.38	59.4	29.29	1.68	3.23
AWRI 005	Red/White Ensiled	9.83	45.9	34.75	1.63	3.86
AWRI 006	Red/White Spent	10.25	68.0	28.17	1.88	3.54
AWRI 007	Red Ensiled	10.17	49.4	37.72	1.82	4.19
AWRI 008	Red Crimped	11.09	44.9	37.05	1.47	4.35
AWRI 009	Red Spent	9.62	65.3	29.46	2.02	3.80
AWRI 010	Riesling Marc	11.25	84,5	21.35	1.63	1.54
AWRI 011	Chardonnay Marc	11.84	114.7	21.83	2.21	1.42
AWRI 012	Sauv. Blanc Marc	11.30	89.2	22.67	1.79	1.53
AWRI 013	White Skin	12.01	127.9	23.04	2.56	1.62
AWRI 014	White Seed	9.41	20.7	32.30	0.73	2.64
AWRI 015	Pinot Noir Marc	10.88	57.3	19.55	1.04	1.72
AWRI 016	Shiraz Marc	9.87	60.4	31.16	1.90	5.03
AWRI 017	Cab. Sauv Marc	9.04	37.3	33.89	1.78	4.38
AWRI018	Red Skin	11.72	103.9	23.06	2.05	1.65
AWRI 019	Red Seed	7.57	24.2	53.63	1.70	3.59
AWRI 020	Red Stalk	9.20	71.6	13.60	1.07	1.59

The gas production appears to be heavily determined by the type of grape marc. The skin only samples yielding among the highest gas volume per gram of dry matter, along with the fresh white samples. The processed samples follow a similar trend with the steam distilled/spent samples yielding more gas than the ensiled or ensiled and crimped.The trend of fermentation extent, or maximum gas production, is not well correlated with metabolisable energy (Figure 10A) but instead with the amount of readily fermentable sugars or carbohydrates (Figure 10B).

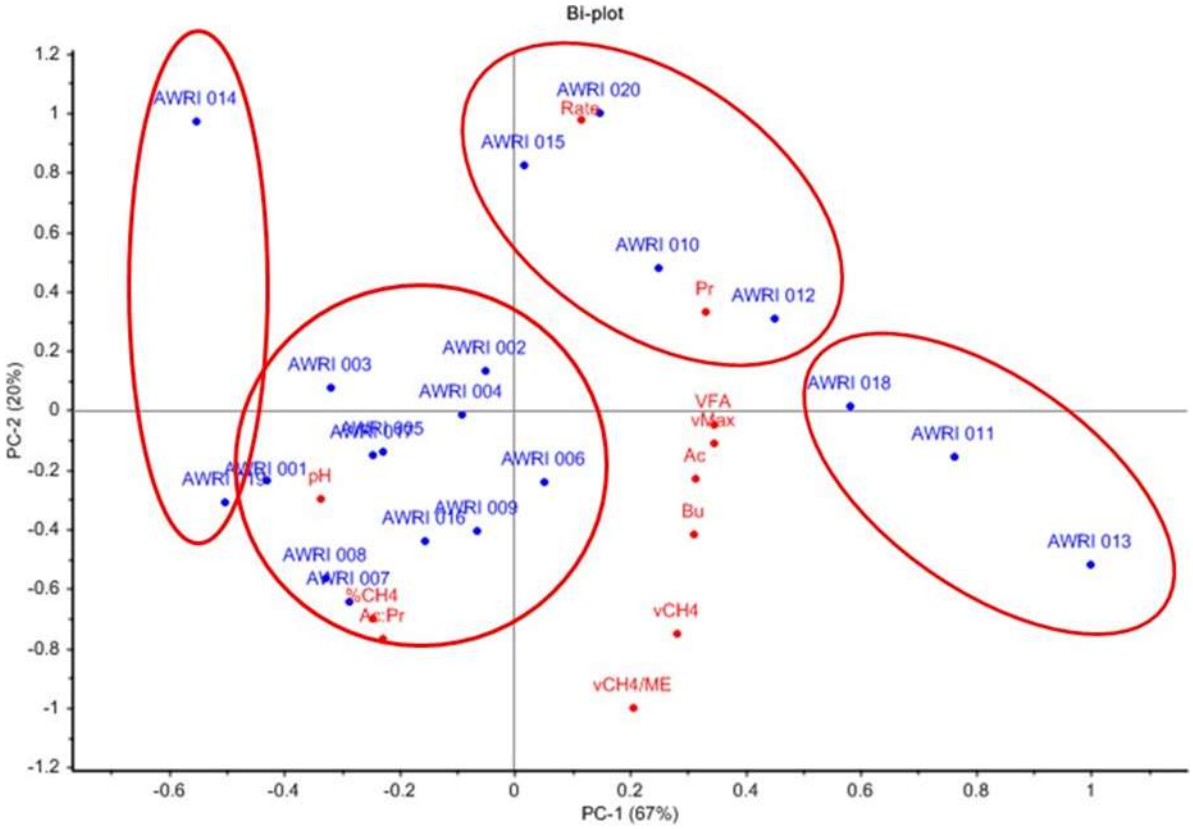
For the fresh grape marc samples, this can be related directly to the type of marc. Fresh white grape marc has greater amounts of simple and readily fermentable carbohydrate (glucose, fructose and pectin) than red marc, a result of red wine fermentation occurring in contact with grape solids and removal of those simple sugars from the red marc. The skin only samples are expected to contain a higher proportion of sugar as these are all non- extracted samples and the seed only samples contain very little simple sugar.

Figure 10: Comparison of total gas production with grape marc composition: A) Metabolisable energy; B) non-fibre carbohydrate



Importantly, there is variation in the fermentation of different grape marc samples, confirming that there is a need to understand differences between grape marc samples when applying it as an animal feed. To understand the differences in fermentation performance between the marc samples the fermentation output data from each of the 20 sets of fermentations was analysed by principle component analysis (PCA, Figure 11).

Figure 11: PCA bi-plot of fermentation outcomes for all twenty grape marc samples, showing clustering as identified in grape marc composition PCA.



The main cluster of samples observed in the PCA plot for sample composition contains the same set of samples that cluster in the PCA plot for fermentation outcome with the exception of a slight shift of AWRI 019 towards the general cluster of samples. This result suggests that we expect similar fermentation outcomes from these processed marc samples. Also, those that do not cluster based on fermentation profile are those with unique compositional characteristics (tannin, fat/oil, sugar etc.).

The majority of grape marc fermentation outcomes are clustered in the bottom left hand quadrant of the PCA bi-plot, which is removed from loadings for gas production (vMax), total VFA and fermentation rate, suggesting that grape marc is largely a low energy substrate. Those that are removed from that main cluster are varied in their correlations with gas compositions ratios, total gas productions and vCH_4/ME.

From in vitro screening of twenty grape marc samples a simple initial comparison of each individual compositional variable was plotted against the methane volume per unit of metabolisable energy (vCH_4/ME) in a xy plot and the strength of the linear correlation as well as the slope of the correlation was determined and tabulated (Table 12).

Table 12: Correlations between single grape marc compositional variables and methane volume per unit of metabolisable energy (vCH_4/ME). Listings in red correlate with ME (R2 > 0.35).

Marc Component	R^2	Normalised Slope
Tannin		
PA	0.259	-0.497
LEM	0.071	-0.249
mDP	0.256	0.447
cis/trans	0.383	0.531
%Tri-OH	0.037	0.185
%Gall	0.129	-0.335
PAT	0.219	-0.514
WET	0.500	-0.579
PPT	0.026	0.117
Minerals		
%Ca	0.342	-0.506
%P	0.032	-0.151
%Mg	0.558	-0.691
%K	0.123	0.315
%Na	0.052	-0.252
FE	0.032	0.192
Zn	0.014	-0.086
Cu	0.280	0.499
Mn	0.053	-0.220
Mo	0.012	-0.116
%S	0.041	0.213
%Cl	0.012	-0.111
Organic Acid/Sugar Profile		
Acetic	0.015	-0.111
Glyc	0.006	0.044
Lac	0.012	-0.068
Mal	0.022	0.133
Succ	0.021	0.082
Tart	0.220	0.446
Glu	0.103	0.201
Fru	0.101	0.205

Marc Component	R^2	Normalised Slope
Fats/Oils		
FA	0.113	-0.321
C16	0.151	0.311
C16:1	0.020	0.084
C18	0.040	0.193
C18:1	0.037	-0.163
C18:2	0.056	-0.190
C18:3	0.018	0.122
C22:0	0.000	-0.013
C24:0	0.140	0.301
Unsat	0.081	-0.221
Polyunsat	0.077	-0.206
Nutritive Information		
ME	0.045	0.192
CP	0.035	0.194
AvP	0.068	0.312
ADICP	0.004	-0.049
AdjP	0.068	0.312
SolP	0.056	-0.221
NDICP	0.018	0.116
ADF	0.092	-0.275
NDF	0.138	-0.299
Lignin	0.083	-0.236
NFC	0.109	0.250
Starch	0.023	-0.156
ESC	0.068	0.166
Cfat	0.124	-0.295
ash	0.106	0.274
Monomeric flavan-3-ols		
Flava	0.474	-0.662
C	0.523	-0.574
EC	0.372	-0.628
EGC	0.319	-0.441

On the basis of single component comparisons, there are a number of compositional variables that are correlated with reductions in methane per unit of ME to some extent. Factors of interest, such as tannin and fat, are correlated with lower methane yields, while other factors of interest, such as tartaric acid and glycerol, do not relate to methane reductions at this level of analysis. All of the compositional factors were also aligned with methane percentage (Table 13).

Table 13: Correlations between single grape marc compositional variables and percentage of methane gas produced. Listings in red correlate with ME (R2 > 0.35).

Marc Component	R^2	Normalised Slope
Tannin		
PA	0.071	-0.246
LEM	0.028	0.148
mDP	0.153	-0.322
cis:trans	0.135	-0.295
%Tri-OH	0.408	-0.576
%Gall	0.703	0.731
PAT	0.068	-0.270
WET	0.070	-0.203
PPT	0.060	0.168
Minerals		
%Ca	0.223	0.385
%P	0.459	0.539
%Mg	0.040	0.175
%K	0.257	-0.429
%Na	0.215	-0.483
FE	0.017	0.133
Zn	0.349	0.403
Cu	0.003	-0.049
Mn	0.164	0.364
Mo	0.083	0.283
%S	0.230	0.475
%Cl	0.377	-0.581
Organic Acid/Sugar Profile		
Acetic	0.075	0.234
Glyc	0.271	0.293
Lac	0.031	0.103
Mal	0.023	0.128
Succ	0.167	0.219
Tart	0.005	0.062
Glu	0.513	-0.423
Fru	0.516	-0.437

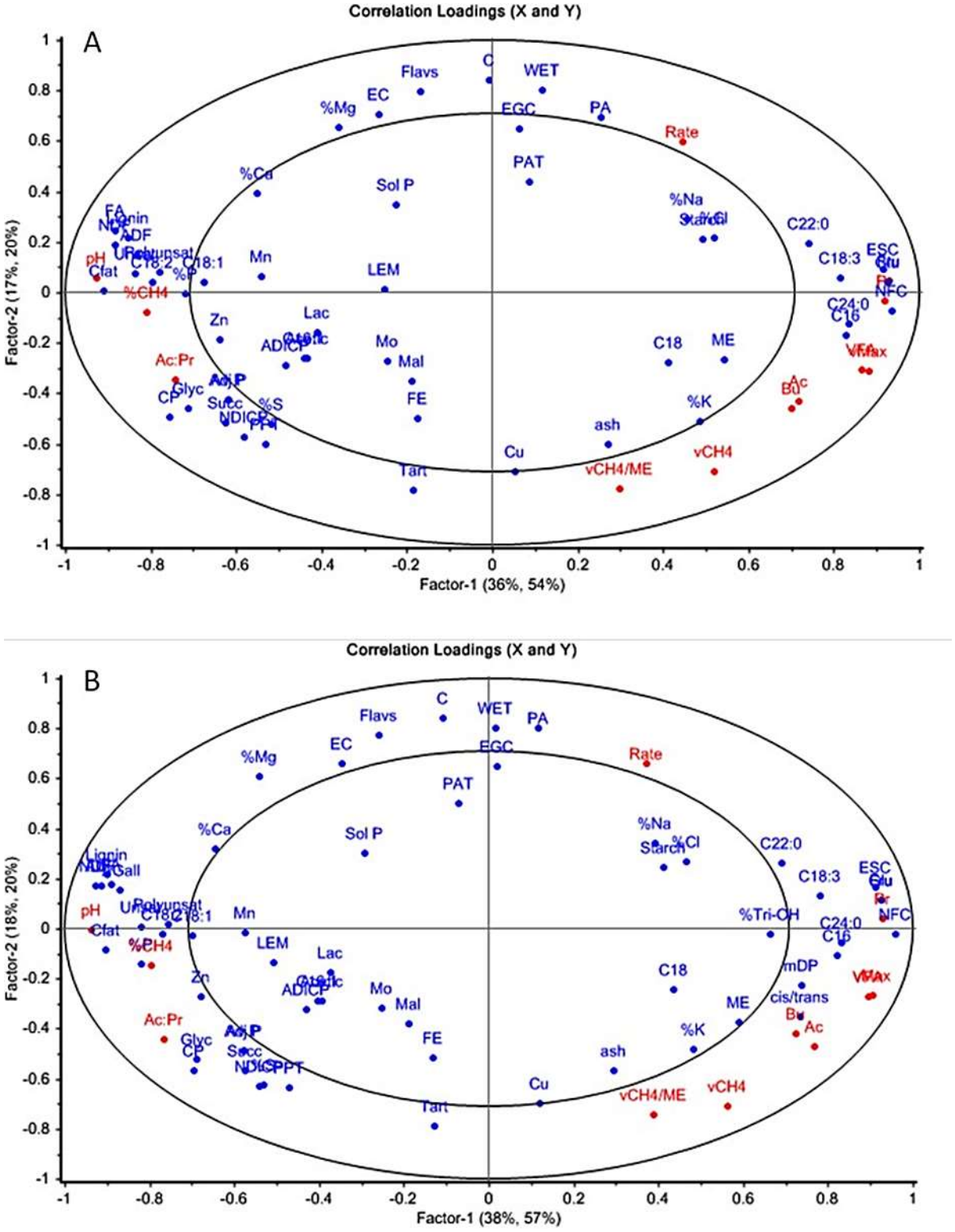
Marc Component	R^2	Normalised Slope
Fats/Oils		
FA	0.740	0.775
C16	0.303	-0.415
C16:1	0.242	0.280
C18	0.142	-0.342
C18:1	0.242	0.392
C18:2	0.315	0.422
C18:3	0.369	-0.519
C22:0	0.328	-0.522
C24:0	0.284	-0.403
Unsat	0.339	0.427
Polyunsat	0.285	0.374
Nutritive Information		
ME	0.300	-0.466
CP	0.412	0.624
Av.P	0.376	0.691
ADICP	0.069	0.207
Adi.P	0.376	0.691
Sol.P	0.035	0.165
NDICP	0.123	0.284
ADF	0.471	0.587
NDF	0.614	0.595
Lignin	0.577	0.586
NFC	0.664	-0.584
Starch	0.255	-0.486
ESC	0.541	-0.441
Cfat	0.521	0.570
ash	0.103	-0.254
Monomeric flavan-3-ols		
Flavs	0.016	-0.113
C	0.087	-0.220
EC	0.000	0.001
EGC	0.098	-0.230

Very few marc components were correlated with reductions in methane percentage, although many tannin variables can be related to reductions in methane. Fatty acid content (FA) shows a correlation with increases in methane percentage, as do all of the organic acids.

To further clarify the relationship between fermentation outputs and compositional factors, data was further analysed using partial least squares regression. This method aims to take the multiple pieces of compositional information and build a data model that expresses marc composition in a single dimension that can be plotted against fermentation outputs.

As mentioned previously, this analysis had to be performed in two separate ways to account for the lack of tannin compositional data for one of the samples. In the first instance, all compositional variables were combined for the 19 samples for which they were available (AWRI 001 excluded), and secondly using those that were available for all 20 samples (excluding 6 phloroglucinolysis inputs). As with principle component analysis, the impact that each variable has on the data model can be observed in the loadings plots (Figure 12), with those contributing little to the model placed within the inner ellipse.

Figure 12: A) PLS loadings plot for all 20 marc samples with tannin composition excluded; B) PLS loadings plot for 19 marc samples with tannin composition included and sample AWRI 001 excluded. Compositional variables shown in blue, fermentation output variables in red.



From the above loadings plots, it can be observed that for each of the models, there are a number of variables that are considered to be of limited significance, and revised models were produced with those eliminated (Table 14).

Table 14: Compositional variables removed from the revised PLS models.

Variables removed from the models			
All samples model		**19 samples model**	
%Ca	Fe	% Cl	LEM
%Cl	Lac	%Na	Mal
%Na	LEM	Acetic	Mn
Acetic	Mal	ADIP	Mo
ADCIP	Mn	Ash	PAT
Ash	Mo	C16:1	Sol P
C16:1	PAT	C18	Starch
C18:1	SolP	EGC	Tri-OH
C18:1	Starch	Fe	
EGC	Zn	Lac	

These new revised models were developed using fewer compositional factors. Table 15 shows the correlations between the revised models and each of the pieces of output data, with factors that are of importance to this study well correlated.

Table 15: Correlations between compositional models and fermentation output measure using revised PLS regression models. Both revised models contain five factors.

Model: PLS all samples revised		**Model: PLS 19 samples revised**	
Ferment measure	**R^2**	**Ferment measure**	**R^2**
Rate	0.628	Rate	0.754
vMax	0.935	vMax	0.926
$\%CH_4$	0.881	$\%CH_4$	0.965
vCH4	0.893	vCH_4	0.894
Ac	0.835	Ac	0.857
Pr	0.958	Pr	0.962
Bu	0.777	Bu	0.745
VFA	0.912	VFA	0.918
pH	0.970	pH	0.973
Ac:Pr	0.885	Ac:Pr	0.927
vCH_4/ME	0.820	vCH_4/ME	0.809

In each case, the PLS regression analysis allows us to look at each of the output variables individually and explore to what extent each of the inputs is contributing to the model. Those with regression coefficients greater than ±0.1 are considered to contribute a significant extent, while those greater than ±0.05 have also been highlighted. Those variables with a higher magnitude regression coefficient (positive or negative) have more influence on the data model, and hence on the output being compared with.

In terms of maximum gas production (essentially an indication of energy) it is not surprising that sugars and carbohydrate fractions are the major contributing factors (Table 16). However, the fact that tannin composition variables have been identified is surprising. There is evidence that tannin reduces digestibility, so a negative correlation with tannin concentration might be expected, as is seen with water extractable tannin in the model excluding tannin chemistry. Other tannin variables have not been identified, and is most likely due to these being 'swamped' by the fibre and carbohydrate variables which are having a much greater effect on gas production than does tannin.

Table 16: Regression coefficients for important x-variables in correlation with maximum gas production.

Model: PLS all samples revised		Model: PLS 19 samples revised	
x-variable	**Regression coefficient**	**x-variable**	**Regression coefficient**
%Mg	-0.101	%Ca	-0.087
NDF	-0.099	%Mg	-0.075
ADF	-0.086	NDF	-0.067
WET	-0.075	ADF	-0.060
Lignin	-0.072	%Gall	-0.059
FA	-0.068	Lignin	-0.052
NFC	0.086	WET	-0.052
ESC	0.088	PA	-0.051
Fru	0.091	cis/trans	0.053
Glu	0.091	NFC	0.057
ME	0.098	ESC	0.059
		Fru	0.061
		Glu	0.051
		ME	0.072

Table 17 shows those variables with regression coefficients greater than ±0.05 when correlated with vCH4/ME. In both models PA and water extractable tannin (WET) negatively correlated with vCH4/ME, suggesting these facilitate a reduction in methane production. Compositionally tannin should have low mDP and low cis/trans ratio, which also came out in the simple, single variable correlations shown earlier (Table 12).

Table 17: Regression coefficients for important x-variables in correlation with methane volume per unit of metabolisable energy.

Model: PLS all samples revised		Model: PLS 19 samples revised	
x-variable	**Regression coefficient**	**x-variable**	**Regression coefficient**
C	-0.186	C	-0.159
WET	-0.165	WET	-0.131
%Mg	-0.153	Flavs	-0.126
Flavs	-0.144	%Mg	-0.114
PA	-0.114	PA	-0.102
EC	-0.103	EC	-0.094
PPT	-0.078	%Ca	-0.068
Cu	0.067	PPT	-0.038
C16	0.071	Cu	0.051
Glu	0.071	CP	0.056
Fru	0.072	C16	0.059
CP	0.072	mDP	0.068
C24:0	0.090	C24:0	0.072
AvP	0.092	AvP	0.079
AdjP	0.092	AdjP	0.079
		cis/trans	0.098

When volume of gas is disregarded, and grape marc components compared simply to the percentage of methane produced, highly bound tannin (PPT) is highlighted as correlating to a lower methane percentage (Table 18), as is water extractable tannin. Unlike the results above, in this case tartaric acid does impact the data model, and so does the fatty acid content (FA) but in the opposite direction as previously expected.

Table 18: Regression coefficients for important x-variables in correlation with percentage of methane gas production.

Model: PLS all samples revised		Model: PLS 19 samples revised	
x-variable	**Regression coefficient**	**x-variable**	**Regression coefficient**
C	-0.141	C	-0.163
%K	-0.136	PPT	-0.148
PPT	-0.122	%K	-0.128
ME	-0.113	Flavs	-0.123
NFC	-0.092	ME	-0.110
Flavs	-0.082	WET	-0.101
CP	0.076	EC	-0.083
NDF	0.102	NFC	-0.080
AvP	0.113	Tart	-0.075
Lignin	0.113	NDF	0.093
%P	0.117	Lignin	0.094
FA	0.129	%P	0.119
	0.149	Zn	0.121
		FA	0.142
		%Gall	0.182

Ultimately, for any one variable to be affecting fermentation outcome considerably the regression coefficients would be higher. However, in a complex mixture with many variables, it can be seen that there are a range of factors that are contributing to some extent in altering methane production, or fermentation in general; some more than others.

Ideally, for tannin to be considered as a key anti-methanogenic property, the model which includes this data should be considered, but it is also beneficial to observe what variables are emerging once tannin chemistry has been removed as a contributing factor. These statistical analyses do in fact suggest that tannin concentration and chemistry are contributing factors in methane production, but not to such an extent that they are the sole driving variable.

4.3 In vitro round 2 – grape marc dose response experiments

From the twenty grape marc samples used for the in vitro screening study, eight were selected based on their methane outputs or on unique variation in tannin chemistry (Table 19). The key compositional characteristics for these eight marc samples can be extracted from the tables containing data for all 20 in vitro samples.

Table 19: Eight grape marc samples used for in vitro dose response study.

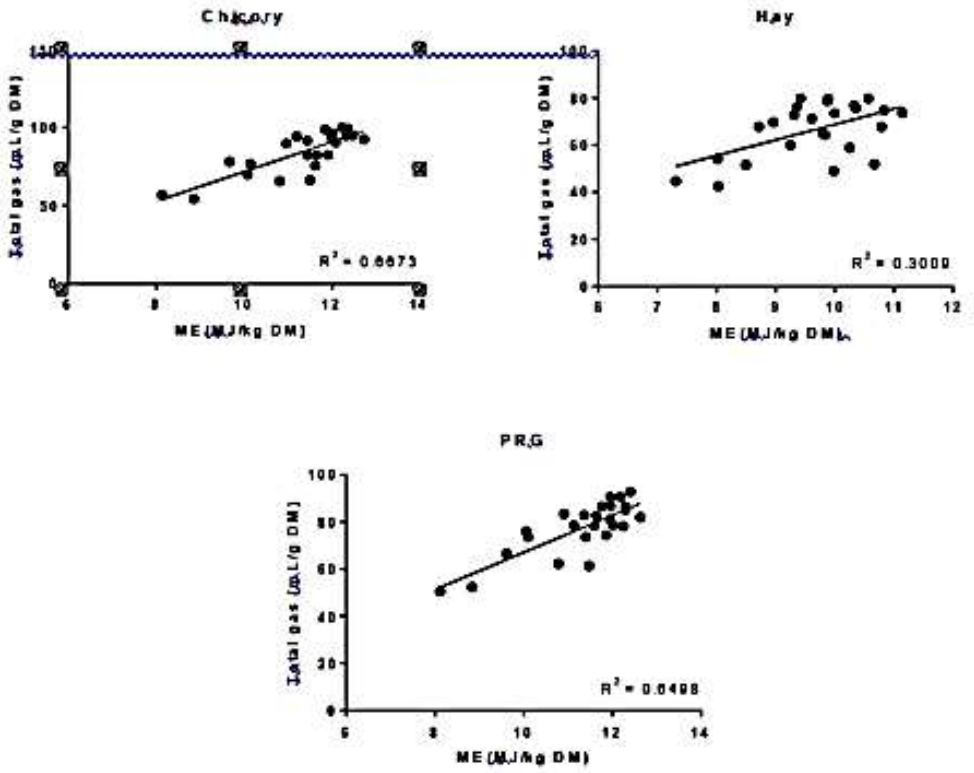
Sample code	Description
UWRI 001	Steam distilled, dried
UWRI 007	Red Ensiled
UWRI 008	Red Crimped
UWRI 010	Riesling marc
AWRI 012	Sauv. Blanc Marc
AWRI 019	Red Skin
AWRI 019	Red Seed
AWRI 020	Red Stalk

The response to grape marc dosing was investigated in two ways using separate experiments. The first method was an inclusion study, whereby 1g of dry matter was fermented at all times, but different levels of grape marc was included into this 1g at the expense of the base forage. The second experiment involved supplementing a constant 0.5g of forage with two different amounts of grape marc, resulting in fermentations with different amounts of total dry matter, but a constant mass of base forage.

4.3.1 Inclusion experiment

From the metabolisable energy content of the base forages, those of the grape marc samples, and the percentage inclusion, the total metabolisable energy in each ferment was calculated. The metabolisable energy was compared to the gas production to determine the extent of fermentation as a function of the calculated energy (Figure 13).

Figure 13: Comparison of total gas production with metabolisable energy of the substrates, by forage type.



For both chicory and perennial ryegrass (PRG) the gas produced relates to the energy in the feed to some extent, however for the hay-based ferments this is not the case. For the hay ferments, there appears to be more happening than purely an energy based fermentation. To compare measures of fermentation performance, the gas produced in all ferments has been aligned with total VFA production (Figure 14). The good linear relationship shows that either are good, and equal, measures of overall fermentation performance.

Figure 14: Comparison of total gas production and volatile fatty acid production in all grape marc dose response fermentations. Forage only in blue, 25% inclusions in red, 50% inclusions in green, 75% inclusions in black.

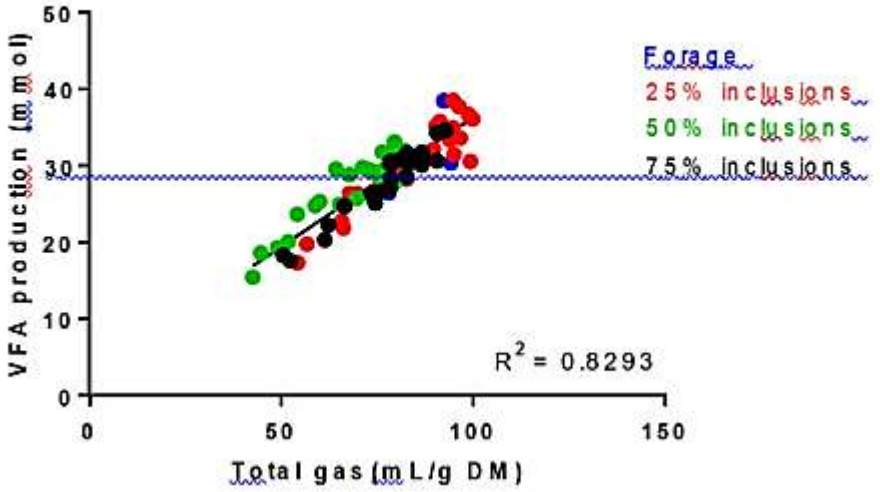
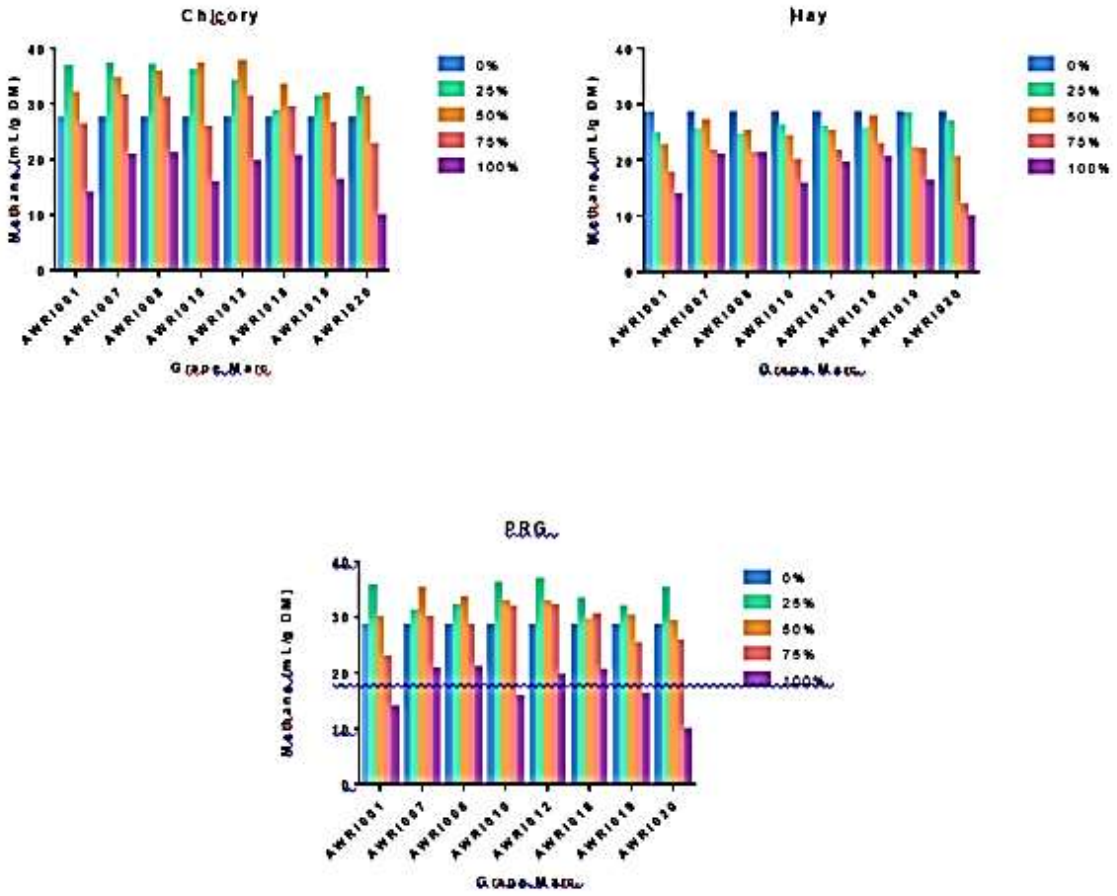


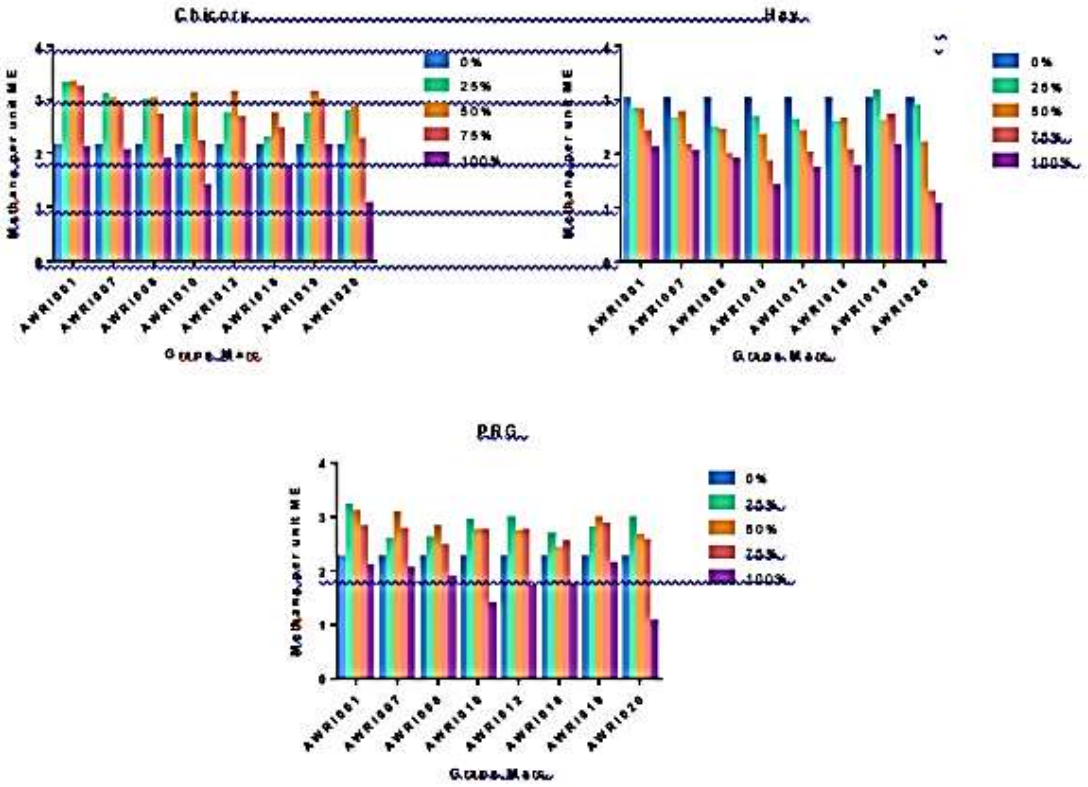
Figure 15 displays the methane volume per unit of dry matter for each of the grape marc inclusions broken down by base forage. The trend across chicory and perennial ryegrass is for an increase in methane volume with a 25% inclusion of grape marc and again for a 50% inclusion. Largely, the 75% inclusion produces less methane that the 25 and 50%, and in some instances is lower than the forage only control. The trends in the hay fermentations differ from those in the other two forages, but also in nutritional content (low ME, low crude protein). For most grape marc samples used there is a reduction in methane with a 25% inclusion of marc, and a much greater reduction with a 50% inclusion.

Figure 15: Methane volume per gram of dry matter for grape marc inclusions (0, 25, 50, 75, 100%) for each of the forages (chicory, hay, perennial ryegrass).



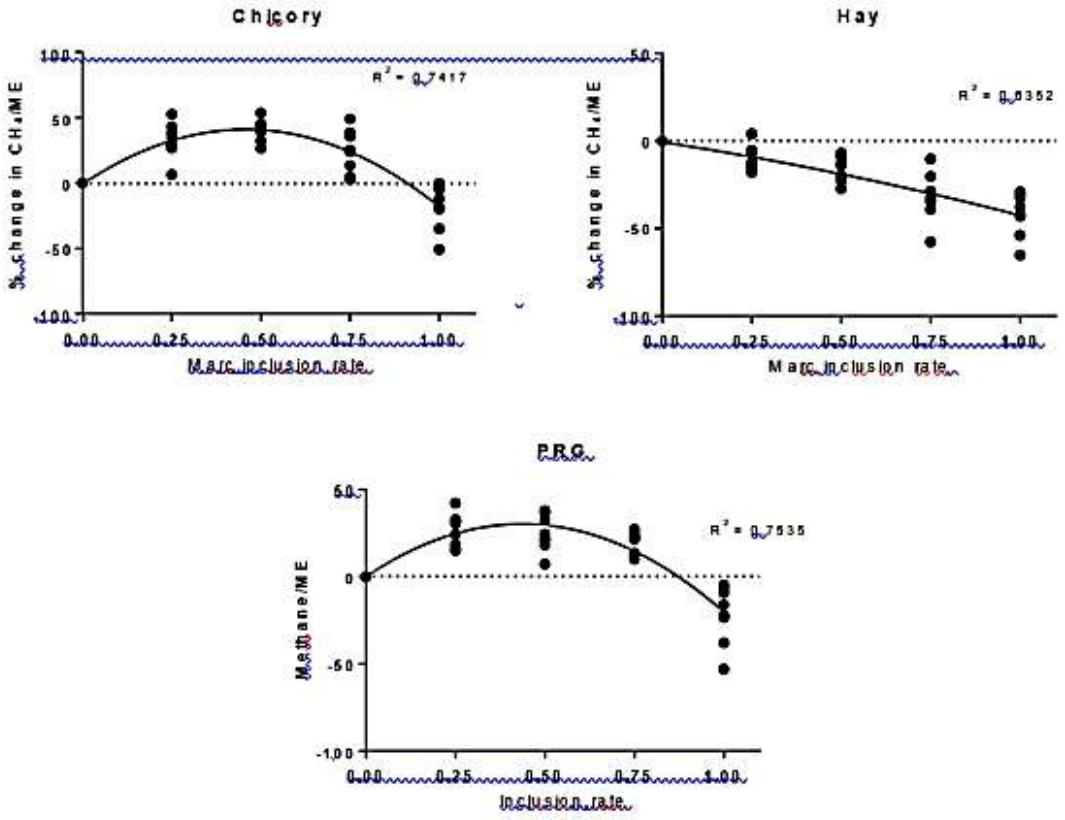
When you express the methane production as a volume of methane per unit of ME in the ferment (Figure 16), the similarities between chicory and perennial ryegrass continue, and largely still show an increase in methane with an inclusion of 25% grape marc. All the grape marc samples used have a lower ME than these two forages, so inclusion of marc is also a reduction in ME. As such, a similar methane volume in a system with a lower ME will be observed as an increase in methane per unit of ME.

Figure 16: Methane volume per unit of metabolisable energy for grape marc inclusions (0, 25, 50, 75, 100%) for each of the forages (chicory, hay, perennial ryegrass).



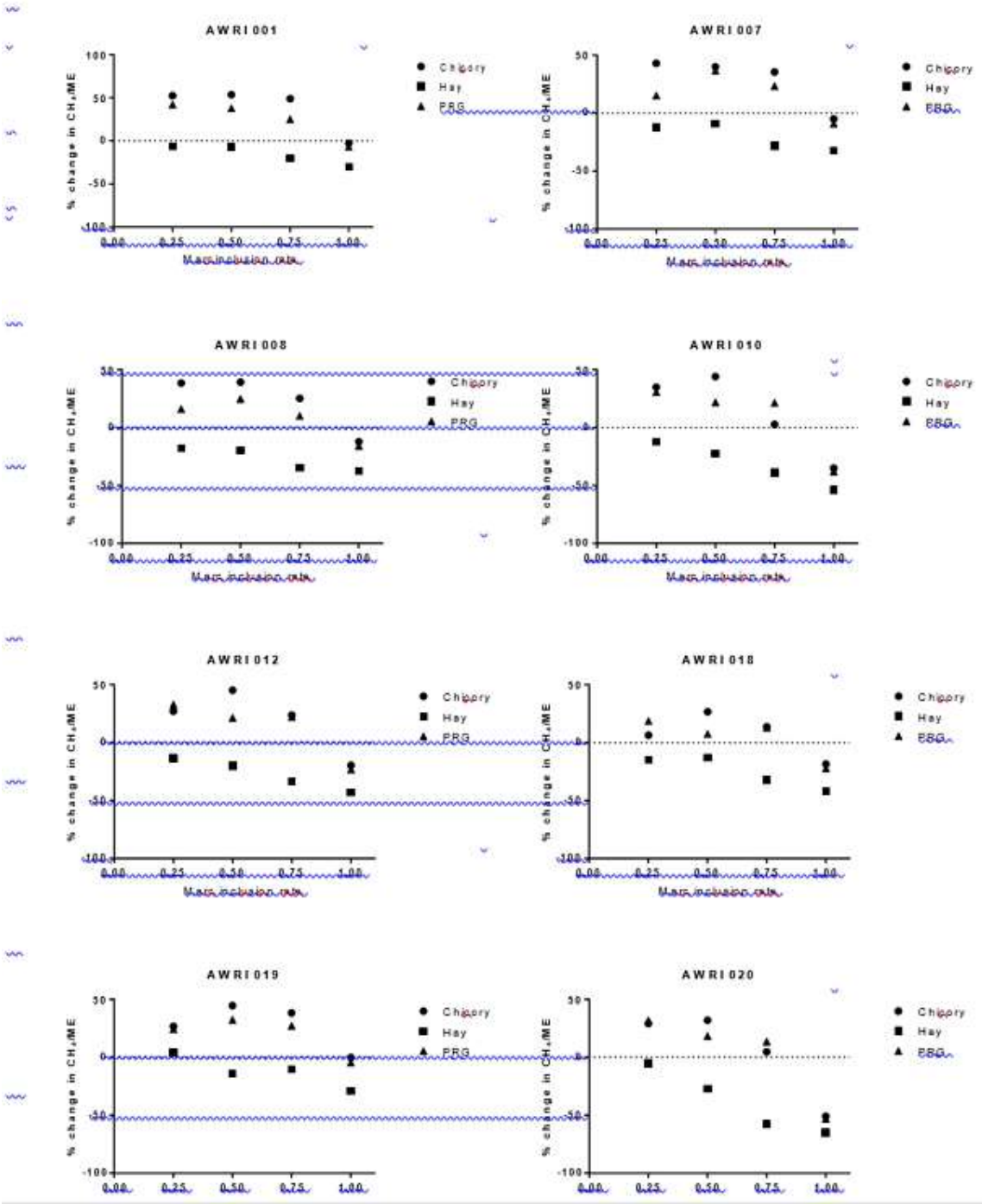
The hay fermentations show a reduction in methane for a 25% inclusion across the board, which continues for a 50% inclusion and again for the 75% inclusions. The hay has an ME of approximate 9.4 MJ/kg DM, so for three of the grape marcs (AWRI 001, 019 and 020) a reduction in ME results from inclusion, but for the remainder there is an increase in ME in the fermentations.

Figure 17: Percentage change in methane per unit of metabolisable energy by inclusion of grape marc into chicory, hay or perennial ryegrass.



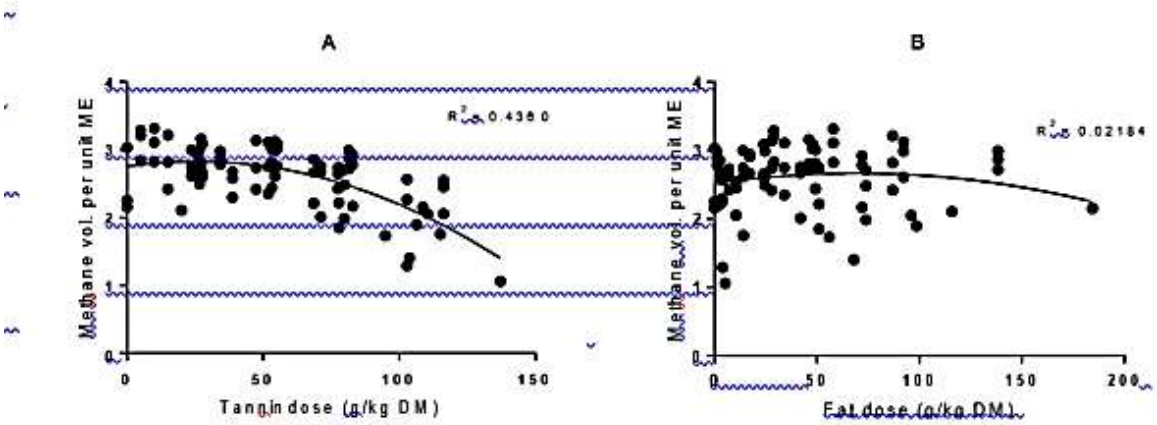
When the inclusion levels are grouped for each of the forages (as seen in Fig. 17) it can be seen more clearly that for both chicory and perennial ryegrass, a 25% and 50% inclusion of grape marc gives an increase in methane per unit of ME, while inclusions with hay give a reduction for all 25% inclusions. However, for chicory and perennial ryegrass the grape marcs that give a similar methane output to that of the base forage are not the same samples. The same dataset is shown for inclusions broken down by marc sample, as opposed to each forage, in Fig. 18.

Figure 18: Percentage change in methane per unit of metabolisable energy for grape marc inclusions, broken down by grape marc variety.



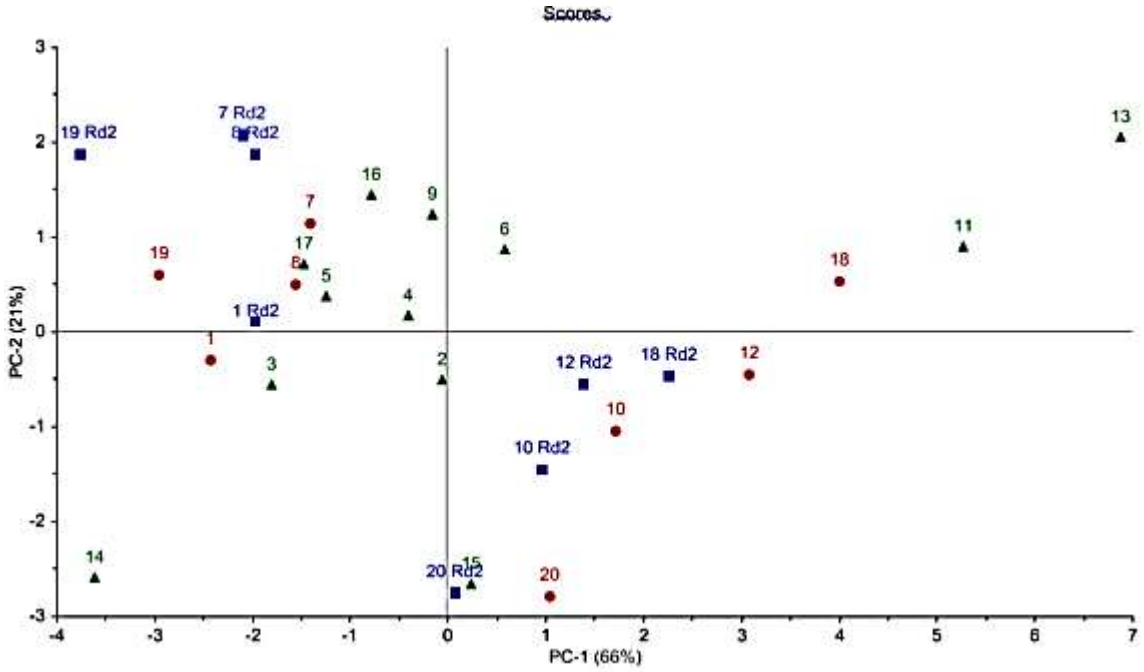
Disregarding the hay fermentations, which largely show reductions in vCH4/ME, the inclusion of AWRI 018 into chicory shows the smallest increase. This marc, along with AWRI 007 and 008, appear to provide the smallest increases of vCH4/ME when included into perennial ryegrass. For all fermentations, the vCH4/ME was plotted against the total tannin dose and the total fatty acid dose (Fig. 19A and Fig. 19B, respectively). The regressions fitted to the data do not show high correlation, but the trend is for reductions when a higher tannin dose is used and for little change with increasing fatty acid dosing.

Figure 19: Methane volume per unit of metabolisable energy against A) total tannin dose, and B) total fatty acid dose.



The pure grape marc fermentations were compared with those observed in the initial in vitro screening experiments (using 20 grape marc samples) via principle component analysis of the fermentation outputs, to confirm that they held true to the base data from which they were selected (Fig. 20).

Figure 20: Principle component analysis of grape marc fermentations from multiple in vitro experiments. Samples from the first round labelled 1-20 (green and red), samples from the second round indicated by 'Rd2' label (blue). Samples from round 1 that were used subsequently in round 2 are shown in red.

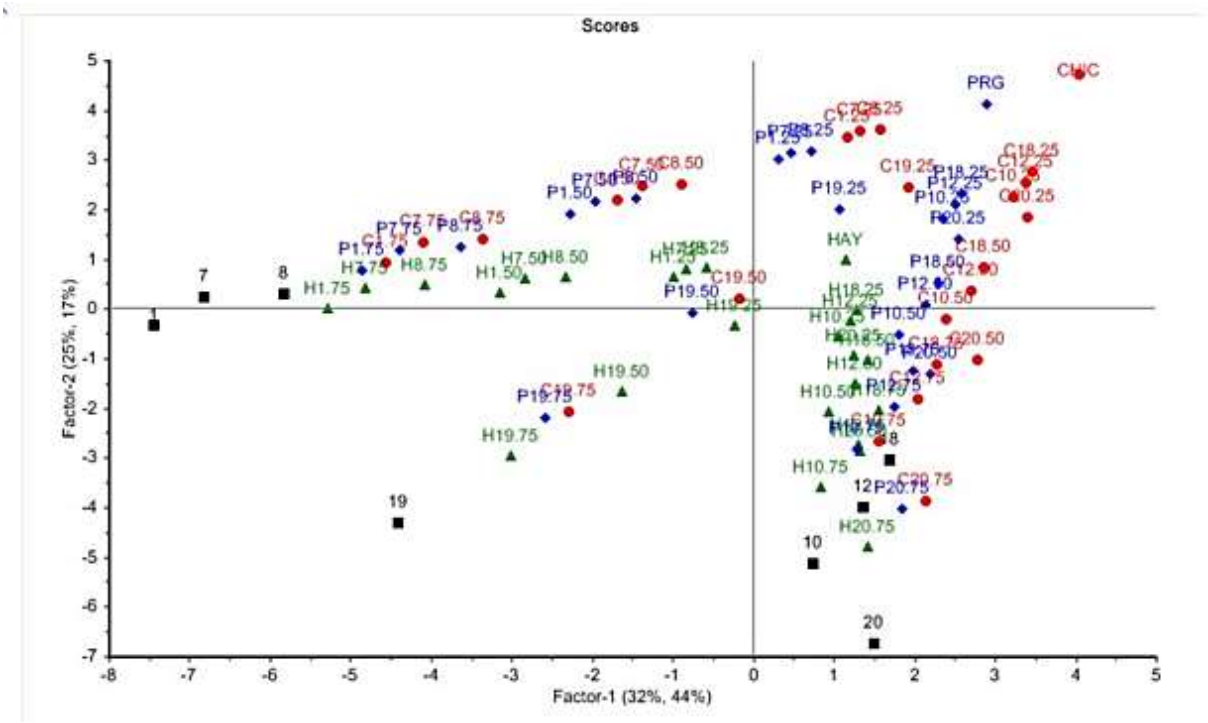


The grape marc fermentations from this experiment (blue points) are within the general cluster of fermentations, although they are slightly removed from the same grape marc samples from the previous experiment (red points). This is most likely due to the difference in the rumen fluid, caused

by the timing of the experiments with the first round of in vitro experiments being performed in June/July and the second round in January when the energy and protein requirement of the animals would be much higher.

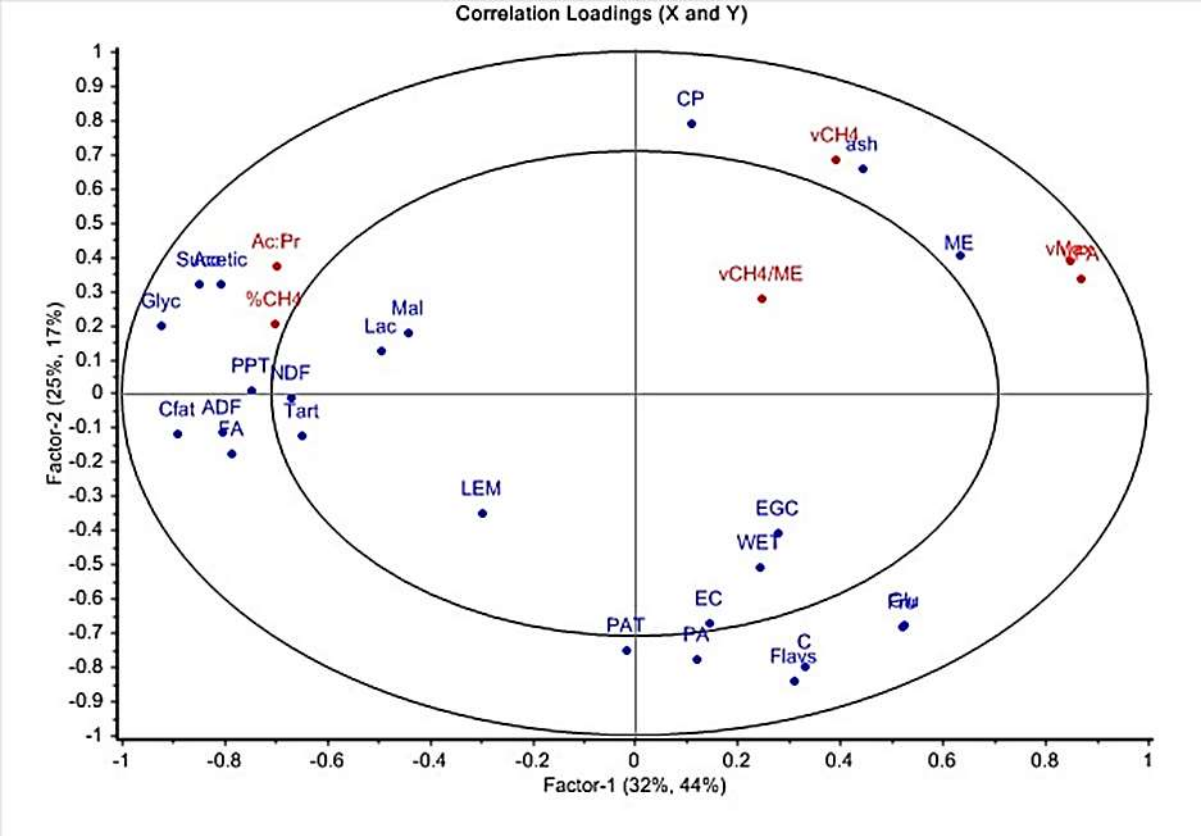
The in vitro fermentation experiment outcomes were analysed by multivariate statistics to better understand the differences between the fermentations and the factors influencing fermentation changes. As one of the samples (AWRI 001) could not be analysed for tannin composition by phloroglucinolysis, the analysis was performed in two separate ways. The first method used all treatments and eliminated the tannin composition data, the second method used all the analytical outputs with those treatments containing AWRI 001 being removed. Only the PLS scores plot for the first method can be seen below (Fig. 21), although by using the second method the outcomes were nearly identical.

Figure 21: Partial least squares regression scores plot for all fermentation samples. Chicory based fermentations shown in red, hay in green, perennial ryegrass in blue, pure marc fermentations in black.



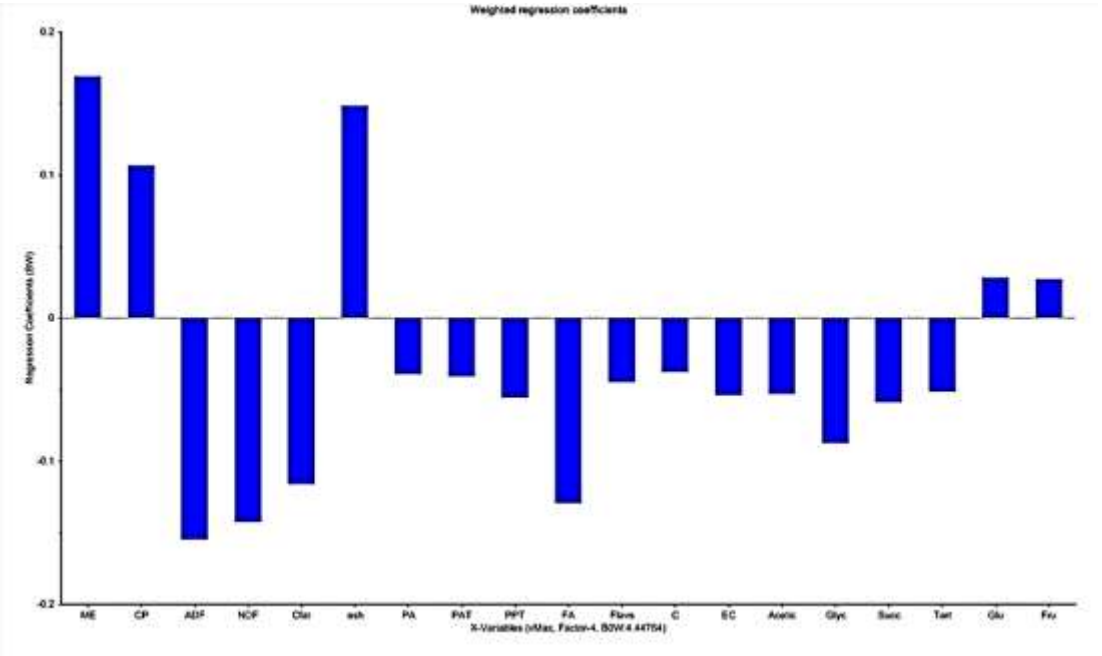
This scores plot shows an origin in the top right corner, where the pure chicory and perennial ryegrass fermentations are, with an increase in grape marc inclusion moving towards the bottom left quadrant. This appears to be occurring in three main clusters or vectors which include samples AWRI 001, 007 and 008 in one cluster, AWRI 019 alone, and AWRI 010, 012, 018 and 020 in the third. The loadings plot for this PLS analysis (Fig. 22) shows that the top right corner of the plot contains most of the fermentation outputs including total gas production, methane volume and methane per unit of ME (red points). The two main input clusters (in blue) are represented by fat content on the right hand side and tannin content at the bottom of the plot. This aligns with samples AWRI 001, 007 and 008 being lower in tannin and higher in fat content, AWRI 010, 012, 018 and 020 being higher in tannin content and AWRI 019 having high tannin and fat content.

Figure 22: Partial least squares regression loadings plot for all fermentation samples.Input variables in blue, outputs in red.



The loadings plot (Fig. 22) also shows many of the fermentation outputs in a large cluster, which means that with inclusions of grape marc the fermentation moves away total gas production and total VFA content, but towards a higher methane percentage. As such, it can be assumed that inclusions of grape marc are inhibiting fermentation in general. When the percentage of marc and forage are included into the PLS regressions (not shown) these two factors dominate and appear in the top right and bottom left quandrants (when moving from pure forage to pure marc ferments). It appears that the one of the biggest drivers in changing fermentation outcomes is simply the amount of grape marc that is included. For this PLS regression model the weighted regressions coefficients for both maximum gas volume (Fig. 23) and percentage methane (Fig. 24) are shown below.

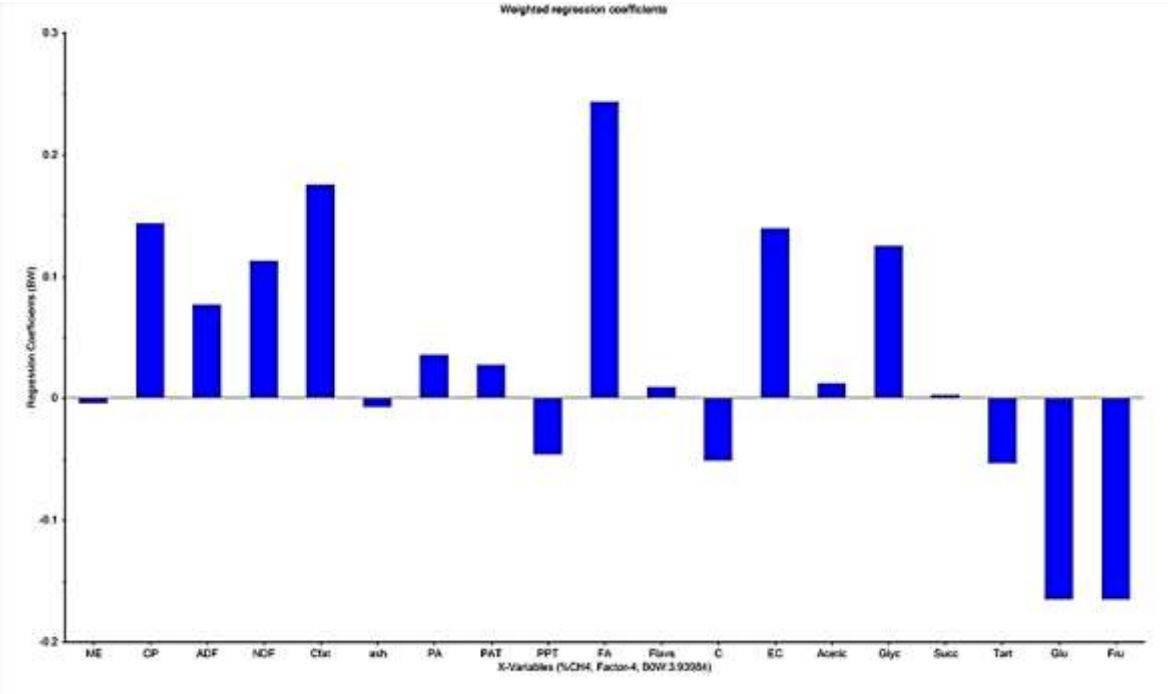
Figure 23: Weighted x-variable regression coefficients for maximum gas volume from PLS model.



Overall fermentation as represented by maximum gas volume is affected negatively by the percentage of ADF and NDF, as well as considerably by the fat content (Cfat and FA). Many other components also affect gas production negatively, but none as significantly as fibre and fat.

The percentage of methane is aligned with fat content (Cfat and FA) but in the opposite direction as expected, with increases in fat correlated with increases in the methane percentage. So not only does the fat content relate to inhibiting fermentation, but also in increasing the proportion of methane produced.

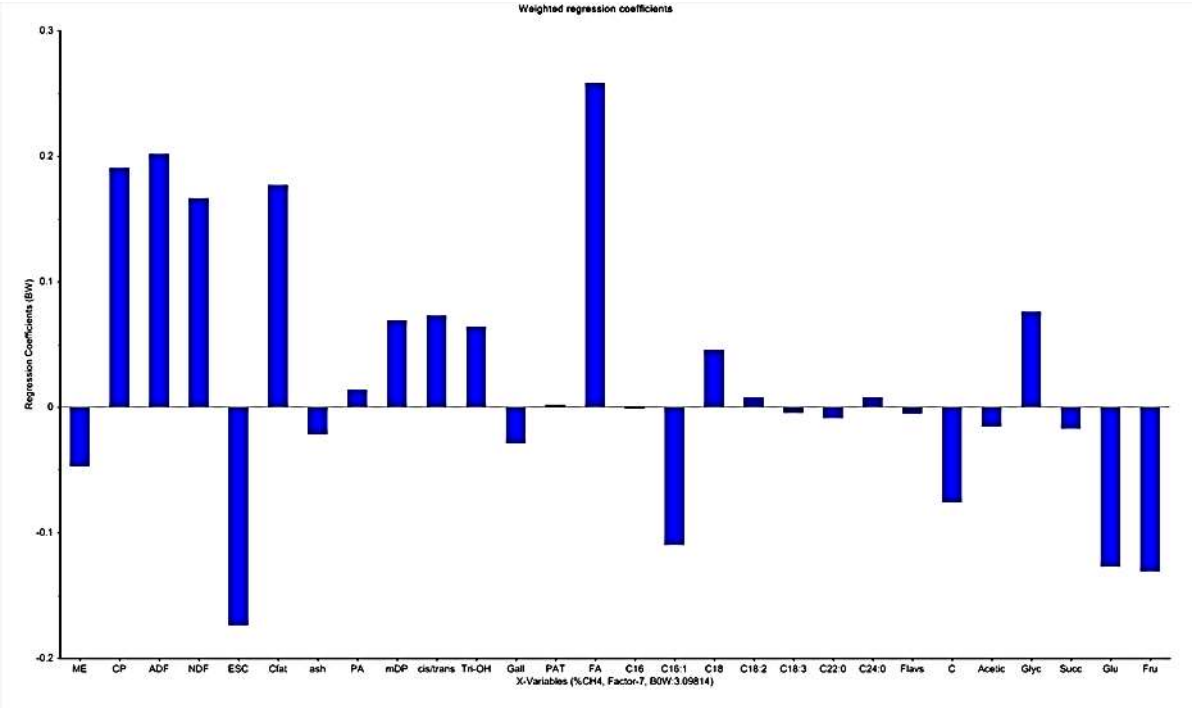
Figure 24: Weighted x-variable regression coefficients for percentage of methane from PLS model.



If some of the overriding factors are removed from the model (marc percentage, forage percentage, ME, ADF, NDF) to better understand the role of tannin and fat, the same clustering towards fat or tannin dominated samples are still observed. If only tannin and fat inputs are modelled with only methane and total gas outputs, the resulting regression model is not adequate for showing variation between samples (produces a poor model).

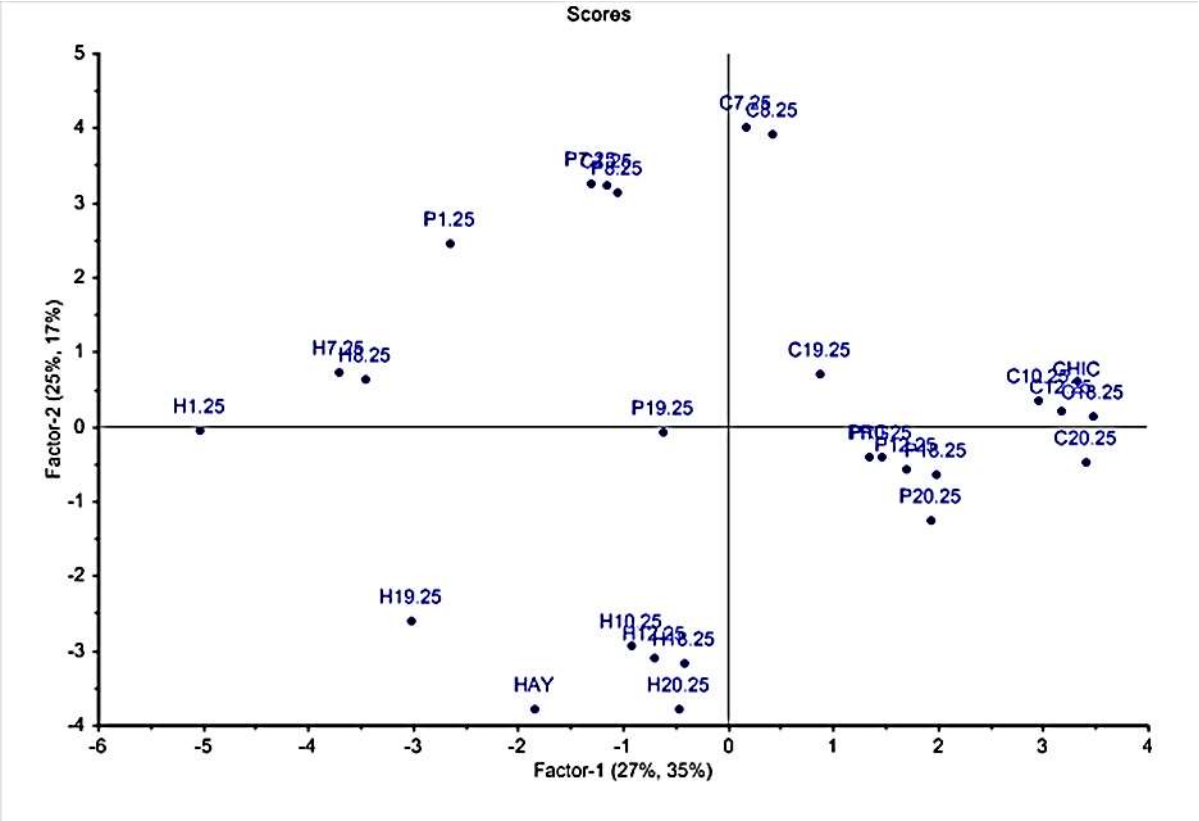
While fat appears to be quite influential in the above model, tannin concentration does contribute to altering fermentation outcomes to a small extent. In the data model that contains tannin composition, these also contribute to altering methane production (Fig. 25).

Figure 25: Weighted regression coefficients for percentage of methane gas using data model including tannin compositional variables.



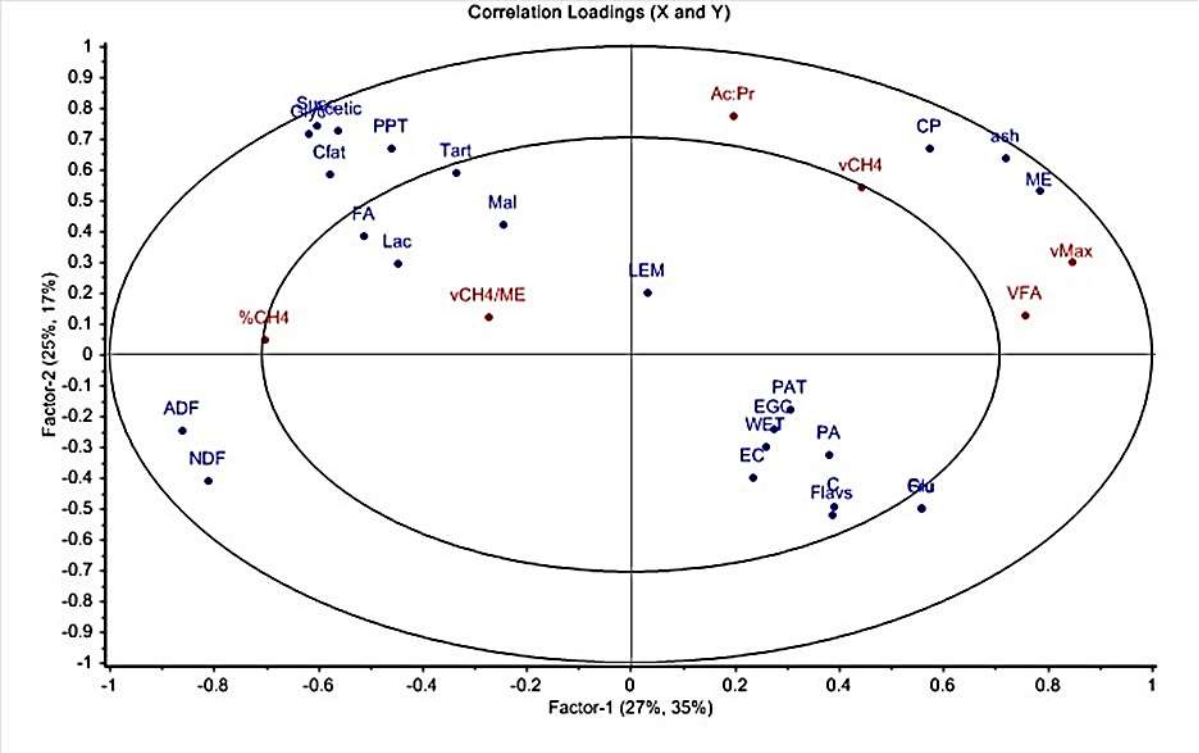
To further deconvolute the in vitro data, the samples that include grape marc inclusion percentages that are unlikely to be applied in a real-life feeding regime (50, 75 and 100% marc) were removed leaving only the pure forage samples and 25% inclusions. The PLS scores plot (Fig. 26) shows a clustering of hay based treatments away from chicory and perennial ryegrass, which was also evident in the basic methane production graphs presented earlier (Fig. 15 and Fig.16).

Figure 26: Partial least squares regression scores plot for pure forage samples and 25% grape marc inclusions.



From the loadings plot (Fig. 27) the distribution of samples from the bottom right quadrant to the top left within a base forage set show a movement from high tannin samples to high fat samples (based on sample inputs – blue points). The hay samples are removed from the other forages based on ME and crude protein (for inputs) and also due to the resulting inferior fermentation outcomes (VFA and total gas volume).

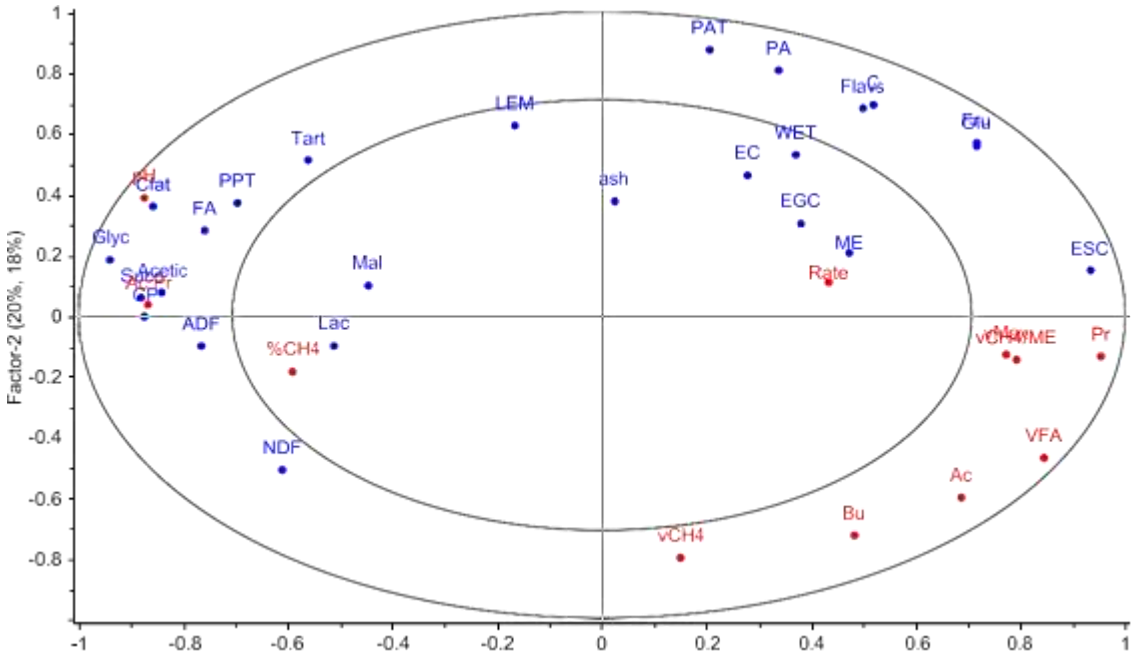
Figure 27: Partial least squares regression loadings plot for pure forage samples and 25% grape marc inclusions. Input variables in blue, outputs in red.



The high tannin samples are more closely correlated with greater fermentation (higher VFA and gas production) than those of high fat, and also those of higher fat are also more closely correlated with higher methane percentage (although only minor correlations are observed).

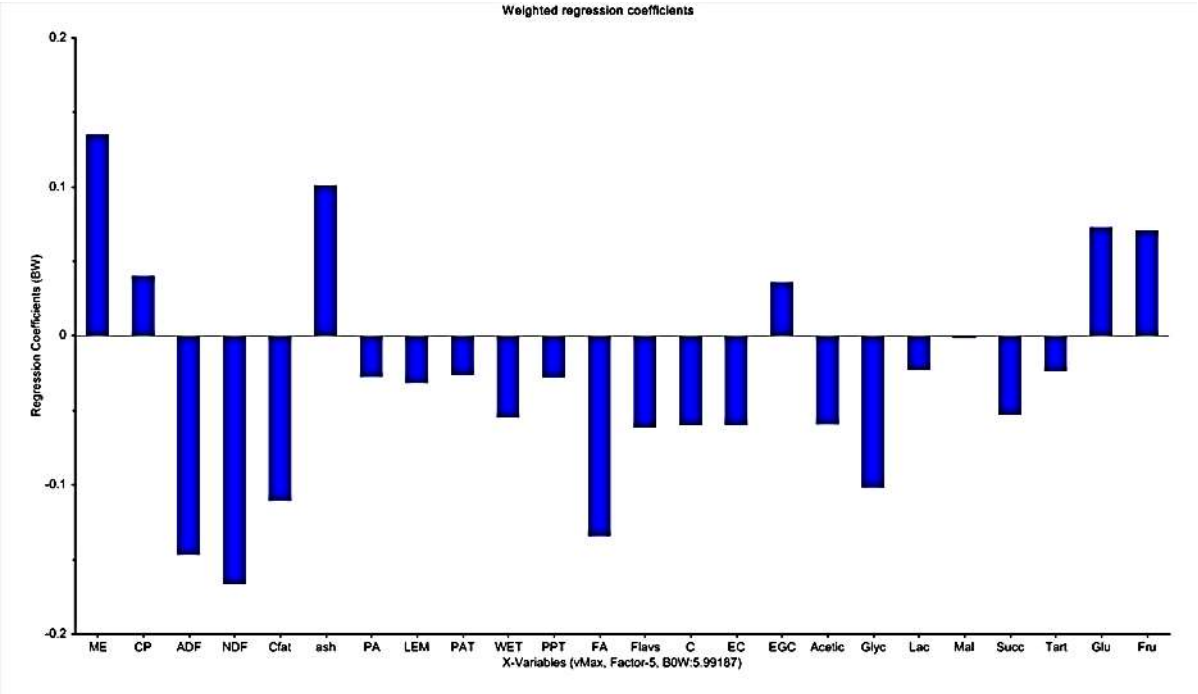
The data for the in vitro fermentations were divided based on forage and analysed separately. Again the chicory and perennial ryegrass samples show similar profiles to each other and resemble that described for all samples combined (Fig. 21 and Fig. 22). All of important fermentation outputs are clustered, and by moving away from a high methane percentage, the fermentation potential (VFA and gas production) is also reduced. Again, the exception is with the hay samples. The loadings plot for the hay samples can be seen below (Fig. 28) and one of the significant characteristics is that the output for methane percentage is not so highly correlated with those total fermentation measures, suggesting it is possible to affect methane production in hay fermentations without inhibiting total fermentation.

Figure 28: Partial least squares regression loadings plot for hay containing fermentations. Input variables in blue, outputs in red.



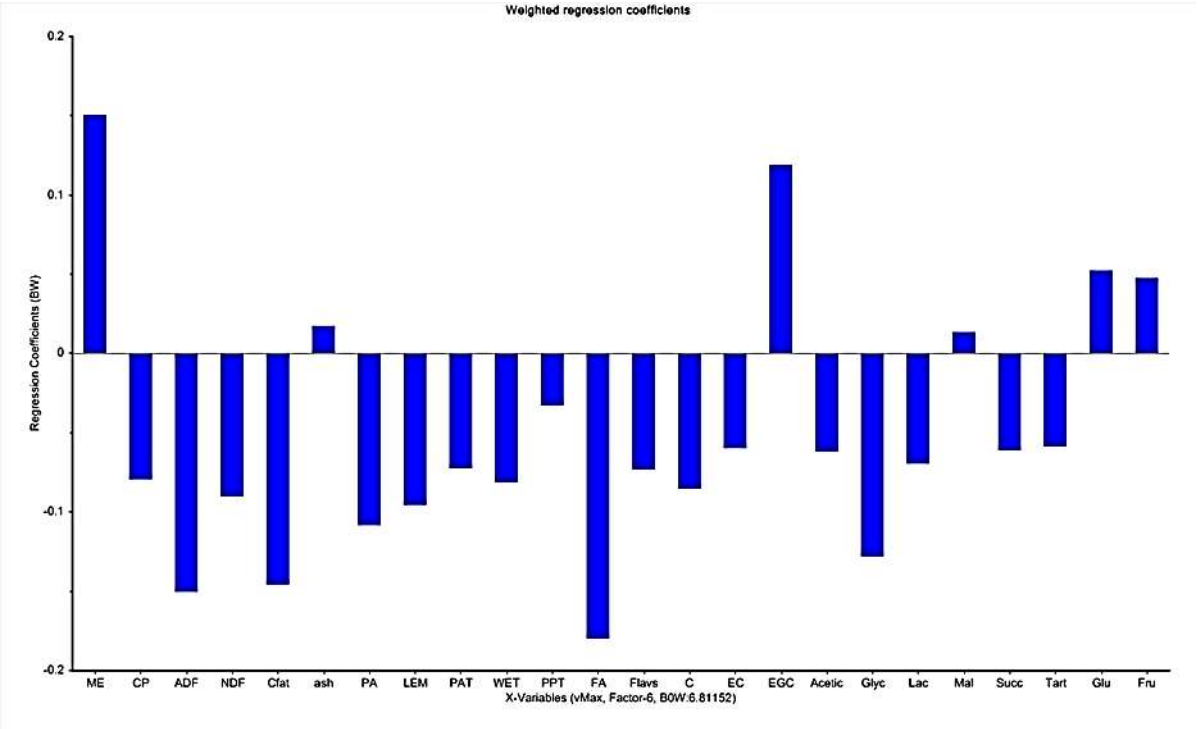
Furthermore, when the regression coefficients are examined for the chicory and perennial ryegrass fermentations, it can be seen that the maximum gas production is affected rather heavily by ME and fibre (ADF, NDF), as well as the fat content (Fig. 29).

Figure 29: Weighted regression coefficients for maximum gas volume in chicory and perennial ryegrass fermentations.



When the same fermentation output is examined for only hay containing fermentations, the gas production is not so obviously dominated by ME and fibre, but there are a number of other contributing factors. This suggests that for these fermentations, it is not just a question of energy relating to fermentation performance but that other compositional factors can play a role.

Figure 30: Weighted regression coefficients for maximum gas volume in hay fermentations.



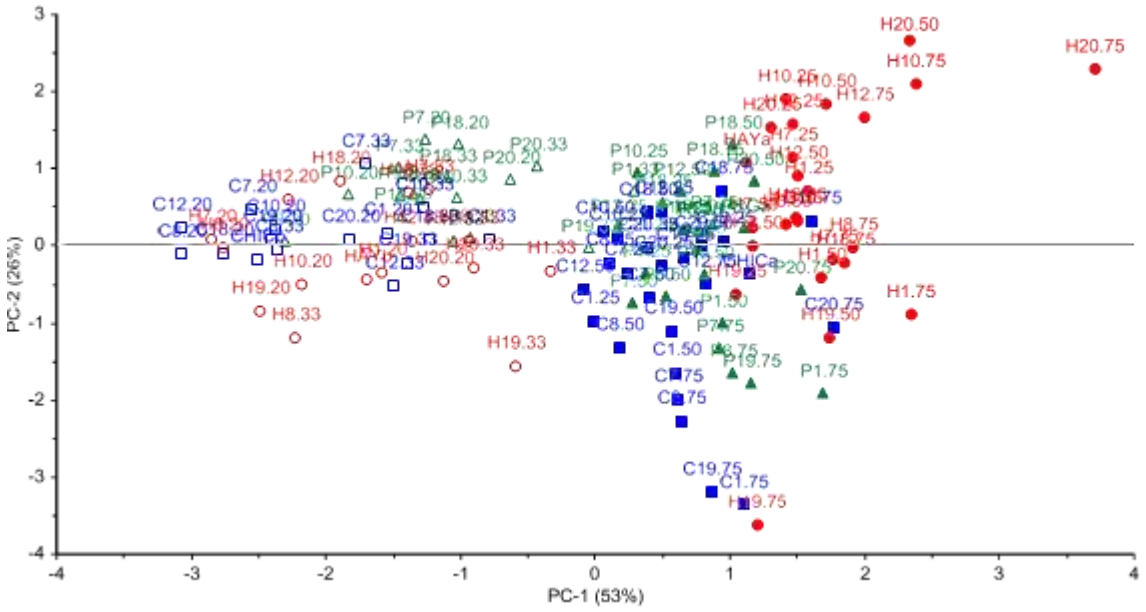
Using the raw output data for the in vitro fermentation experiments combined with the knowledge that 25% inclusions are more important to a real-life scenario and that higher rate inclusions will tend towards a poorer fermentation, the eight inclusions at that level can easily be compared for each base forage. For each, one or two grape marc varieties can be observed that show slight reductions in methane percentage without having a great impact on total gas or VFA production. For chicory, a 25% inclusion AWRI 018 or 020 shows minor benefits, for hay the inclusion of AWRI 010, 012 or 018, and for perennial ryegrass the inclusion of AWRI 018.

With respect to the potential active ingredients (tannin and fat), these four samples represent the high tannin and low fat samples (AWR 018 and 020) and two samples that have moderate tannin content and the lower fat contents of the 'whole' marc samples (i.e. not separated into skin, seed or stalk - AWRI 010 and 012). This aligns with the PLS analysis of the data which suggests that tannin has a small effect on reducing methane, but that fat is more closely aligned with reducing fermentation as a whole.

4.3.2 Supplementation experiment

Grape marc was supplemented into 0.5g of forage at rates of 0.125g and 0.250g representing a final marc addition of 20% and 33% of the total dry matter in the ferment. The fermentation outcomes of the supplementation experiment were compared to those of the inclusion experiment using principle component analysis (Fig. 31).

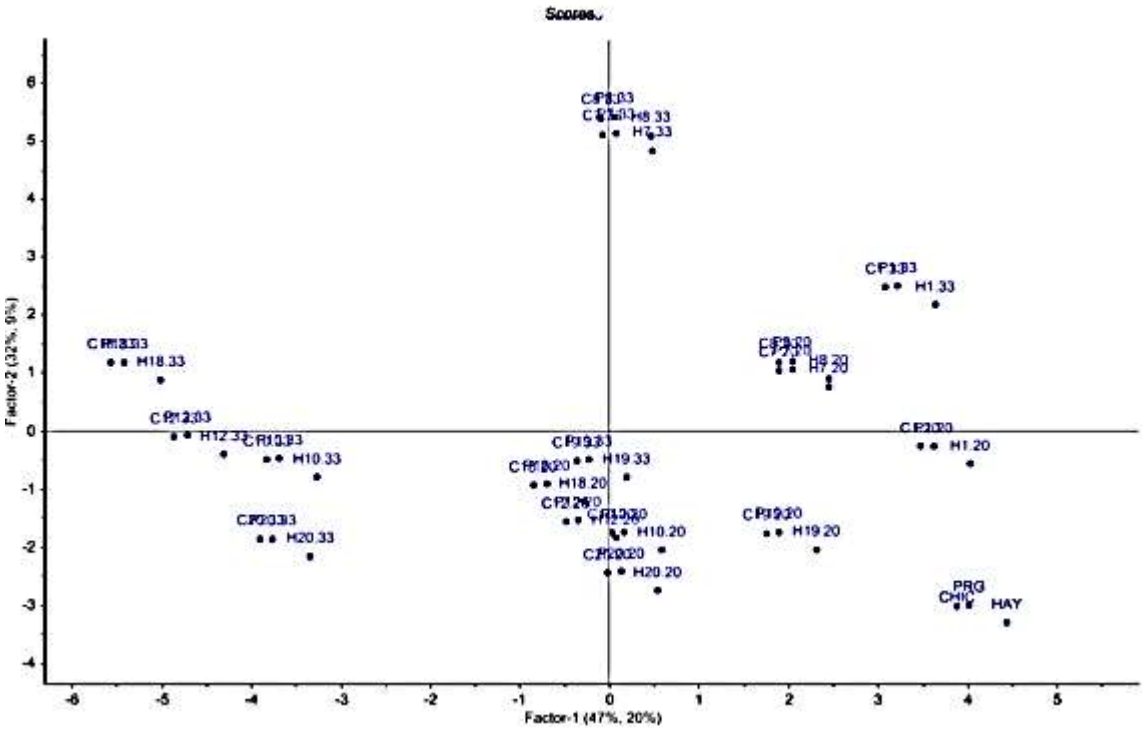
Figure 31: PCA scores plot of fermentation outcome showing both inclusions experiment (solid shapes) and supplementation experiment (hollow shapes).



The fermentation data from each of the experiments cluster well, with very little overlap. The supplementation experiment produced much more gas than the initial inclusion ferments, and also produced a higher percentage of methane. This means that the data sets are unable to be combined as they differ too much in outcome.

As the supplementation ferments contained varying amounts of dry matter, the experiment can be assessed in two distinctly different ways. Firstly, the data can be converted to a ‘per gram’ basis and all samples considered on a level playing field, but making it resemble the inclusion experiments. Secondly, the data can be expressed on a ‘per ferment’ basis and the response of adding grape marc into a diet can be investigated, as seen in Fig. 32.

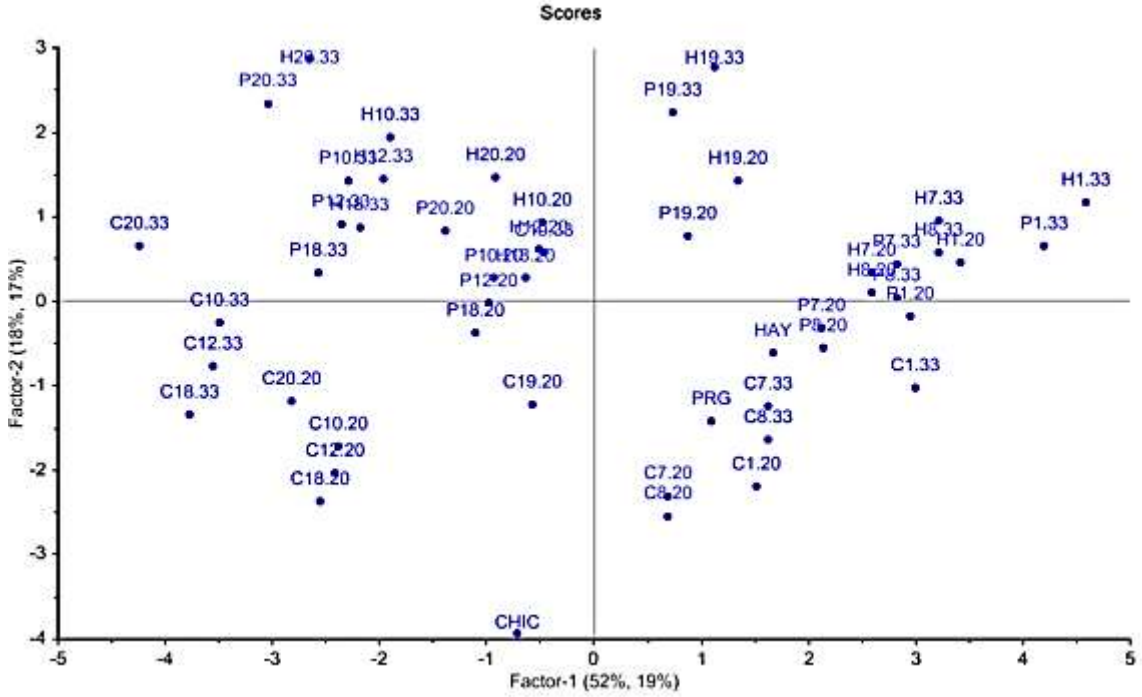
Figure 32: PLS scores plot for supplementation experiment on a per fermentation basis.



The analysis of the data on a 'per ferment' basis is completely dominated by metabolisable energy, which is not unexpected. The base forages are shown at the bottom right of the PLS scores plot and move towards the top left where ME is situated on the loadings plot. However, the clustering is similar to that seen earlier in the inclusion study, whereby AWRI 001. 007 and 008 are together, AWRI 019 is separate, and AWRI 010, 012, 018 and 020 cluster. These clustering are largely due to the presence of tannin and sugars in one cluster, and NDF, ADF and fat in the other, while AWRI 019 is situated half way between the two.

When the fermentation is considered on a 'per gram' basis, the domination of metabolisable energy is no longer seen (Fig. 33). The spread from forage to high marc inclusion rates is not the dominant object taking up both PC1 and PC2. Instead, the base forages are centred at the bottom of chevron-like spread of samples. Again, the same clustering as observed before can be observed.

Figure 33: PLS scores plot for supplementation experiment on a per gram basis.



So while the fermentation outcomes from both dose response studies cannot be combined, the same trend is observed for both experiments. In the above scores plot, percentage methane is on the positive end of PC1 (right hand side), and as such, the samples that are on the left of the plot tend towards lower methane production (AWRI 010, 012, 018 and 020).

When the original fermentation data is considered, the most beneficial supplementations for each forage can be found. Again, the assumption is that 20% addition of grape marc is more realistic and applicable than 33%. Additionally, in only three of the 24 combinations does a 33% inclusion result in a higher gas production per gram.

For perennial ryegrass, the addition of both AWRI 018 and 020 result in comparable fermentations (vMax) and percentage of methane. For chicory based fermentations, the addition of AWRI 010 or 020 results in better fermentation and lower percentages of methane. For hay, every marc sample except for AWRI 007 gives both improved methane percentages and maximum gas production.

4.4 In vitro round 3 –tannin or fat

The previous two in vitro experiments have highlighted that both tannin and fat have been most consistently aligned with alterations to fermentation performance and methane production. However, there are still a number of questions that are remaining. Firstly, the extent to which these two factors (tannin and fat concentration) have affected fermentation individually is not clear as a result of these experiments. Also, the role that changing tannin composition independently of tannin concentration, and the differences between bound and extractable tannin, are not yet clear. As such, the overarching aims of the final in vitro experiments are to: separate the anti-methanogenic activity of tannin from fat; understand role of water soluble tannin versus bound tannin, and; better understand role of changing tannin composition in methanogenesis

However, there are a number of practical limitations that need to be considered when trying to understand the effect of tannin and fat separately. Firstly, there is no practical method for eliminating grape seed oil from marc without heavily affecting overall composition. If grape marc is defatted by extractive methods, many other compounds would also be removed. Conversely, there is no ideal way to add grape seed oil in a way that is representative of the natural state. Any grape seed oil addition is expected to float on top of the fermenting rumen fluid rather than being incorporated into the grape marc. Lastly, there is no way to add 'bound' tannin like that found in grape marc.

With these limitations in mind, four experiments exploiting the natural variations in grape marc will answer the remaining question using the existing twenty grape marc samples as these are already well characterised.

4.4.1 Investigating the role of tannin 'types'

Grape marc possesses small amounts of extractable tannin, and a large amount of bound tannin, whether loosely or highly bound. The difference between bound tannin, the form that is commonly found in grape marc, and extractable tannin, which is expected to be more accessible, will provide evidence as to which is more important when considering methane abatement.

In this experiment a highly available steam distilled grape marc has been used (AWRI 006), which is representative of a marc pile at a processing facility which is going to be largely of unknown origin or variety. The addition of extractable tannin and the inclusion of polyvinylpolypyrrolidone (PVPP) into ferments will uncover the role of these tannin types in methanogenesis (Table 20).

Table 20: Treatments for investigating tannin types and methaogenesis.

Treatment	Diet	Grape marc	Other addition
UWA 1.1	350 mg sheep pellet	150 mg AWRI 006	-
UWA 1.2	350 mg sheep pellet	150 mg AWRI 006	320 mg PVPP
UWA 1.3	350 mg sheep pellet	150 mg AWRI 006	10 mg purified grape tannin
UWA 1.4	350 mg sheep pellet	150 mg AWRI 006	10 mg purified grape tannin + 320 mg PVPP

Table 21: Fermentation results for grape marc of differing tannin type.

Treatment	Gas volume (mL/g DM)	% CH_4	CH_4 volume (mL/g DM)
Control	290.8	13.6	39.6
UWA 1.1	264.4	12.5	33.0
UWA 1.2	288.8	13.5	38.8
UWA 1.3	267.4	11.9	32.4
UWA 1.4	269.6	12.6	34.7
P	0.0214	0.0034	0.0142

AWRI 006 has a tannin concentration of 78.4 g/kg DM (PA), so a 150 mg addition will provide approximately 12 mg of loosely bound tannin into the ferment. The change in fermentation from the control to UAW 1.1 shows a reduction in overall fermentation, but also a reduction in methane percentage (Table 21). When PVPP is also included (UWA 1.2), the fermentation performance recovers and the methane production increases. Loosely bound tannin does have an effect on extent of fermentation but also on methane.

When an additional 10 mg of commercial tannin is added, of which 57% is identifiable tannin subunits, as determined by phloroglucinolysis, this is equivalent to approximately 18 mg of tannin in the ferment, 6 mg of which is extractable. The fermentation performance does not drop in treatment UWA 1.3, when compared with UWA 1.1, although the methane percentage is further reduced. The addition of PVPP in the ferment only recovers the fermentation to that observed in UWA 1.1.

While extractable tannin appears more potent at reducing methane production while not affecting overall fermentation performance, the role of PVPP with both types of tannin is unclear. Potentially, the extractable tannin in UWA 1.4 binds all the PVPP and none remains for binding with the loosely bound tannin.

4.4.2 Investigate the role of altering tannin chemistry

In previous in vitro experiments, the differences in tannin chemistry have been investigated using distinctly different grape marc samples and while the tannin composition has been different, so have other factors, like tannin concentration, fat and metabolisable energy. In this experiment, two grape marc samples were chosen for their differing tannin composition, but similar tannin concentration, fat content and metabolisable energy (Table 22).

The practical limitation of not being able to add tannin to a grape marc in a way that is representative of its natural form requires a different experimental approach. A blend of two grape marc samples that have the right relative compositions allow for this to be investigated without having to adulterate a single sample. A sample of red marc skin (AWRI 018) and grape stalk (AWRI 020) were mixed in four step to give gradual changes in tannin composition (Table 22).

Table 22: Treatments for investigating tannin composition on methanogenesis.

Treatment	Diet	Red Skin	Stalk
UWA 2.1	350 mg sheep pellet	150 mg AWRI 018	-
UWA 2.2	350 mg sheep pellet	100 mg AWRI 018	50 mg AWRI 020
UWA 2.3	350 mg sheep pellet	50 mg AWRI 018	100 mg AWRI 020
UWA 2.4	350 mg sheep pellet		150 mg AWRI 020

Both of these samples contain similar concentrations of tannin, similar metabolisable energy, are both very low in fatty acid and possess the same fatty acid profile. The blending of these two will provide similar tannin content and ME across all ferments, very low fat but with large difference in tannin composition, specifically mDP, cis/trans and %Gall (Table 23).

Table 23: Grape marc composition and changes in key attributes of tannin chemistry across the ferments

		Phloroglucinolysis				Fatty acid	ME
	PA (g/kg)	mDP	cis/trans	%Tri-OH	%Gall	(g/kg)	(MJ/kg)
AWRI 018 - Red skin	120.74	32.55	26.00	24.7%	3.9%	14.01	11.72
AWRI 020 - Stalk	114.79	9.98	9.22	20.0%	6.1%	5.23	9.20
UWA 2.1	36.22	32.55	26.00	24.7%	3.9%	4.20	3.51
UWA 2.2	35.63	25.02	20.41	23.1%	4.6%	3.32	3.26
UWA 2.3	35.03	17.50	14.81	21.6%	5.4%	2.45	3.01
UWA 2.4	34.44	9.98	9.22	20.0%	6.1%	1.57	2.76
Percentage change	95%	31%	35%	81%	156%	37%	79%

Table 24: Fermentation results for grape marc of differing tannin composition

Treatment	Gas volume (mL/g DM)	% CH_4	CH_4 volume (mL/g DM)
Control	290.8	13.6	39.6
UWA 2.1	295.9	12.9	38.2
UWA 2.2	296.6	12.7	37.9
UWA 2.3	272	11.8	32.4
UWA 2.4	288.3	12.2	35.4
P	<0.0067	0.0049	0.0069

4.4.3 Investigate the role of tannin and fat

Separating the affect of tannin and fat on methanogenesis has been achieved in two separate experiments. The first experiment involves removing tannin by way of PVPP from both low fat, high tannin marc and from a high fat, high tannin marc. These alterations will highlight the differences between ferments that are affected by the presence or absence of tannin alone, or by tannin and fat, against fat alone. The second experiment will be a blend of two grape marc samples across a gradient that contain similar fat contents and compositions, but very different tannin concentrations.

4.4.4 Investigate the role of tannin and fat, experiment one

The grape marc samples chosen for this experiment (AWRI 014 and AWRI 020) have similar contents of tannin (PA = 126 and 114 g/kg, PA+LEM = 148 and 132 g/kg) and similar ME values (9.41 and 9.20 MJ/kg), but differ in fat content significantly (152 vs 5 g/kg). The inhibition of tannin activity by PVPP across the first two treatments will highlight the role that tannin content is playing, while the second two treatments will provide ferments that are acted on by tannin and fat, or just fat (Table 25).

Table 25: Treatments for investigating the role of tannin and fat in methanogenesis

Treatment	Diet	Grape marc	Other addition
UWA 3.1	350 mg sheep pellet	150 mg AWRI 020	-
UWA 3.2	350 mg sheep pellet	150 mg AWRI 020	320 mg PVPP
UWA 3.3	350 mg sheep pellet	150 mg AWRI 014	-
UWA 3.4	350 mg sheep pellet	150 mg AWRI 014	320 mg PVPP

The use of AWRI 020 (UWA 3.1) shows a similar fermentation extent to the control, but a much reduced methane percentage (Table 26). When PVPP is also included (UWA 3.2), there appears to be no change to the methane levels. As such, the methane reduction may not be purely due to tannin.

When AWRI 014 is used (UWA 3.3), the fermentation performance is again hindered, which is consistent with earlier results that fat retards fermentation. The addition of PVPP does not improve fermentation performance, but does increase the methane percentage closer to that of the control, further suggesting that tannin is reducing methane while fat is inhibiting fermentation.

Table 26: Fermentation results for grape marc of differing fat concentrations

Treatment	Gas volume (mL/g DM)	% CH_4	CH_4 volume (mL/g DM)
Control	290.8	13.6	39.6
UWA 3.1	288.3	12.2	35.4
UWA 3.2	295.2	12.3	36.4
UWA 3.3	266.5	11.8	31.4
UWA 3.4	271.5	12.7	34.5
P	<0.0001	0.0226	<0.0001

4.4.5 Investigate the role of tannin and fat, experiment two

In a similar experiment to previous, where two grape marc samples were blended, AWRI 001 and AWRI 014, with similar fat contents and identical fatty acid profiles were combined to exploit their vastly different tannin concentrations.

Table 27: Treatments for investigating the role of tannin concentration in methanogenesis.

Treatment	Diet	Dried marc	White Seed
UWA 4.1	350 mg sheep pellet	150 mg AWRI 001	
UWA 4.2	350 mg sheep pellet	100 mg AWRI 001	50 mg AWRI 014
UWA 4.3	350 mg sheep pellet	50 mg AWRI 001	100 mg AWRI 014
UWA 4.4	350 mg sheep pellet	-	150 mg AWRI 014

AWRI 001 has a low tannin concentration but possess a high fat content, while AWRI 014 has high tannin and high fat contents (Table 28). The blend from pure AWRI 001 to pure AWRI 014 provides a small increase in both fat content and metabolisable energy, but also provides a significant tannin concentration gradient.

Table 28: Grape marc composition and changes in tannin composition across ferments.

	Tannin (g/kg DM)	Fatty Acids (g/kg)	ME (MJ/kg)
AWRI –Dried marc	20.5	115.86	6.61
AWRI- White seed	125.13	152.06	9.41
UWA 4.1	6.02	34.76	1.98
UWA 4.2	16.62	38.38	2.26
UWA 4.3	27.23	42.00	2.54
UWA 4.4	37.84	45.62	2.82
Percentage change	629%	131%	142%

As we move from low tannin, high fat marc (UWA 4.1) and towards a high tannin, high fat (UWA 4.4) there is a trend towards greater reductions in methane, while fermentation performance remains relatively steady (Table 29). Much like the previous experiment, there is evidence for the slight anti-methanogenic property of fat, comparing UWA 4.1 with the control, although fermentation is retarded. The introduction of tannin provides further reductions in methane percentage without inhibiting the extent of fermentation.

Table 29: Fermentation results for grape marc of differing tannin concentrations.

Treatment	Gas volume (mL/g DM)	% CH_4	CH_4 volume (mL/g DM)
Control	290.8	13.6	39.6
UWA 4.1	268.1	12.7	34.2
UWA 4.2	269.3	12.2	32.9
UWA 4.3	270.1	12.3	33.3
UWA 4.4	266.5	11.8	31.4
P	0.0007	0.008	<0.0001

4.5 Dairy Cow in vivo feeding experiment

The two marc parcels delivered to DEPI for inclusion in the in vivo feeding trial were sampled directly before being transported (approximately 7 days before trial commencement). These were analysed for tannin using phloroglucinolysis (for loosely bound tannin and composition), methyl cellulose precipitation assay (for water extractable tannin) and by an acetone containing porters assay on the whole marc and on the fibres remaining after phloroglucinolysis (highly bound tannin). The results from the suite of analyses can be seen below (Table 30).

Table 30: Tannin analyses of dairy in vivo grape marc samples.

Tannin analysis	Ensiled white marc	Steam distilled red marc
Phlororglucinolysis concentration (g/kg DM)		
PA	60.31	6.86
LEM	22.61	7.68
Phlororglucinolysis concentration (molar ratios) mDP	9.62	12.38
Cis/trans	9.32	10.10
%Tri-OH	7.3%	20.1%
% Gall	12.9%	8.8%
Tannin by other analyses (g/kg DM) Post-phloroglucinolysis tannin	16.5	20.1
Porter's assay tannin	115.9	48.7
Water extractable tannin	1.01	0.92

The ensiled white grape marc had been collected in February 2013 and stored anaerobically at 4 degrees until September. The red steam distilled grape marc was collected from the processing facility in September 2013 and was remaining from vintage of that year. It was expected that extended storage in aerobic conditions would result in diminished tannin content, which proved to be the case as can be seen in Table 30.

These two grape marc samples were sent to Dairy One for nutritive analysis (Table 31). The white marc possesses a higher ME, which can be attributed to the higher NFC result, and the lower indigestible fraction (lignin).

Table 31: Key nutritional information for dairy in vivo grape marc parcels.

	White marc	Red marc
	9.2	8.2
(MJ/kg)		
Content (% of DM)		
Crude Protein	13.3	14.2
ADF	42.7	51.8
NDF	48.2	55.6
Lignin	27.4	38.0
Crude Fat	12.8	15.5
ESC	3.1	2.1
NFC	19.5	7.4
TDN	58.0	52.0

When collecting the ensiled white marc for this trial a portion was frozen immediately and was included in the original in vitro screening experiments (AWRI 003). After 7 months of storage, the tannin content of this marc was largely unchanged indicating that preservation of tannin is possible (Table 32). However, the ensiling process appears to have a negative effect on the NFC and crude fat. The reduction in these fractions in the ensiled marc results in a relative increase in other fractions, such as crude protein, ADF, NDF and lignin. The loss in NFC and increases in lignin and ADF contribute to a lower ME value in the ensiled marc.

Table 32: Phloroglucinolysis and nutritive data for white crimped grape marc when collected and after 7 months of ensiling.

Tannin analysis	**White marc when collected AWRI 003**	**White marc, 7 months ensiled – in vivo**
Phlororglucinolysis concentration (g/kg DM)		
PA	59.41	60.31
LEM	21.77	22.51
Phlororglucinolysis concentration (molar rat.)		
mDP	9.87	9.62
Cis/trans	10.02	9.32
%Tri-OH	7.7%	7.3%
% Gall	10.3%	12.9%
ME (MJ/kg)	10.92	9.2
Content (% of DM)		
Crude Protein	12.9	13.3
ADF	36.9	42.7
NDF	44.4	48.2
Lignin	21.6	27.4
Crude Fat	16.6	12.8
ESC	3.5	3.1
NFC	26.0	19.5
TDN	68.0	58.0

The diets used in the feeding trial are described in the methods section (above), and the outcomes as determined by DEPI are shown in Table 33. The absolute methane production (g/cow/day) was reduced in both treatments of grape marc, although the milk yield was also affected. On a DMI basis, there is a significant difference between the control and the grape marc diets, but when methane per unit of production (g/kg milk) is considered, there is no significant reduction in methane production.

Table 33: Outputs from in vivo experiment.

Variate	Control	White Marc	Red Marc	SED	P-Value
n	11	10	10		
Total DMI (kg/cow per d)	18.6	18,8	18,9	0.36	0.662
Methane (g/cow per d)	380	333	326	12.9	<0.001
Milk (kg/cow/d)	29.1	25.7	26.6		
Methane (g/kg/milk)	13.4	13	12.6		
Methane yield (g/kg DMI)	20.4	17.8	17.3	0.68	<0.001
CoV of CH_4 yield	6.6	9.8	8.5		

It appears that the biggest factor contributing to the changes in gas production are in fact the reduced energy in the diets that include grape marc. The control diet includes mostly high quality pasture that provides the high energy and protein that is required during early lactation. The diets that contain grape marc show reduced milk yields, while dry matter intake remains relatively constant. The methane yield per unit of dry matter intake is reduced, but the productivity is also reduced.

The two marc parcels used in this experiment are distinctly different in tannin composition, and also reasonably varied in fat content (Table 31). If the changes in tannin concentration were having an effect on methane, then the methane production from the two treatments should differ. However, there remains the possibility that the increased tannin in the white marc is reducing methane to a similar extent that the increased fat content in the red marc is having an effect.

Regardless of the minor contribution of tannin or fat they may be present, the results are overshadowed by the reduction in energy and protein and hence why the milk yield is lower in the grape marc diets.

4.6 Sheep in vivo feeding trial

The two marc parcels collected for the sheep in vivo trial (a crimped and ensiled marc and a steam distilled marc) were analysed for tannin content and nutritive properties.

Table 34: Tannin analyses of sheep in vivo grape marc samples.

Tannin analysis	Ensiled marc	Steam distilled marc
Phlororglucinolysis concentration (g/kg DM)		
PA	9.98	0.00
LEM	10.22	1.85
Phlororglucinolysis concentration (molar ratios)		
mDP	9.45	-
Cis/trans	9.80	-
%Tri-OH	22.8%	33.1
% Gall	8.5%	-
Tannin by other analyses (g/kg DM)		
Post-phloroglucinolysis tannin	38.3	6.6
Porter's assay tannin	47.2	6.7
Water extractable tannin	0.5	-

The tannin analysis of the two grape marc parcels gave expected results (Table 34: Tannin analyses of sheep in vivo grape marc samples., with the grape marc that had been exposed to oxidative conditions for a prolonged period (steam distilled marc) showing very little tannin. The composition of the tannin in this sample could not be analysed due to the low concentration of loosely bound tannin, all of which was identified as LEM. The ensiled grape marc, while having been stored for the same period, was not exposed to as much oxygen and has much higher levels of condensed tannin by phloroglucinolysis and when analysed by other means.

However, the methods of processing and storage had very different effects on nutritional profile than they did on tannin, with the steam distilled marc showing higher nutritive qualities (Table 35). The difference in metabolisable energy between the two marc samples appears to be due to the large difference in non-fibre carbohydrate (NFC), with the ensiled marc showing a much lower content.

Table 35: Key nutritional information for sheep in vivo grape marc parcels.

	Ensiled marc	Steam distilled marc
ME (MJ/kg)	7.2	9.0
Content (% of DM)		
Crude Protein	13.7	13.6
ADF	52.4	44.8
NDF	57.2	46.7
Lignin	36.4	33.1
Crude Fat	12	13.3
ESC	1.6	3
NFC	9.8	18
TDN	47	57

The total digestible nutrient level (TDN) for the ensiled grape marc is lower than any of the grape marc parcels analysed in the project, with the exception of the steam distilled and flash dried grape marc submitted to in vitro screening (AWRI 001, TDN

= 44%). Both the ensiled marc and the steam distilled, dried marc have had their seeds cracked (one from a roller mill and one from the pellet process), and potentially the compounds within the seed have degraded as a result. The possibility exists that the later the seed is cracked and exposed to oxidative conditions, the better it is for the quality of the feed.

The results from the dairy in vivo experiment suggest that the largest contributing factor in altering methanogenesis was the reduced energy content of the grape marc treatments. As such, for this trial, the control diet and the grape marc treatments were designed to be as close to isoenergetic as possible.

Across the four weeks of the feeding trial there was no significant difference between the control and treatment diets for intake and live weight. This suggests that when an isoenergetic diet is used, there doesn't necessarily have to be detrimental effects on animal performance.

However, inclusion of grape marc into the diet, regardless of type or extent of supplementation, did not have a significant effect on methane production due to the variation between animals within treatments. As such, the link between grape marc composition and fermentation outcome cannot be further examined. The key outcome of this trial is that grape marc can be used in animal feeding systems without having negative effects on the energy intake and performance of the animal. It may be that livestock being fed at or just above maintenance is the ideal time to be using grape marc as a feed supplement.

4.7 Discussion

4.7.1 Agricultural residue survey

This survey looked at 122 marc samples from around Australia covering marc colour, processing, climate and winery size, and analysed for 64 different agrochemical residues common to the wine industry. There was a single instance of a residue exceeding a known MRL (captan), and notable concentrations of iprodione in a few instances, although with no known MRL for comparison. The suggestion would be for either an adequate study to determine the MRL for iprodione in grape pomace animal feed, or to base this on a known MRL in another feed.

There are few generalisations that could be made about the residue content by colour, climate, or winery size. It appears impossible to completely rely on any of these factors in helping to determine sources of low residue marc to feed to animals.

The only real trend that was observed was that that exists between fresh and processed samples. The content in processed samples (ensiled or steam distilled) was generally lower, and no processed samples contained residue levels close to an established MRL. However, the number of processed samples is relatively low and may not be a statistically relevant number of samples to be making

4.7.2 Grape marc composition

The tannin survey of grape marc samples has shown that both the concentration of tannin in grape marc and the composition of grape marc is highly varied. The extremes of tannin composition exist in skin and seed only samples, but these are not likely to be relevant to large scale feeding. While the composition in whole marc samples was more consistent, the concentration of tannin falls into a wide range. This large range in tannin does make it hard to put an average value on the level of grape marc tannin.

Because of this, the implementation of a CFI/ERF method for grape marc feeding becomes difficult as it is likely that the concentration of tannin in every marc parcel would need to be assessed before the associated reductions in methane could be determined.

The trend of highly variable composition continues into non-tannin factors, although some of these factors can be related to the nature of the grape marc. Many of the extractable compounds like simple sugars, pectins and organic acids are higher in marc parcels that have not undergone extended extraction, like that experienced by red marc. Additionally the evolution of acetic acid, glycerol, succinic acid and lactic acid appear to be results of storage.

The principle component analysis of grape marc compositional inputs showed that all of the processed grape marc samples were much more closely aligned than were the fresh samples. As such, if compositional consistency is required, than processed grape marc is more likely to provide this.

4.7.3 In vitro assessments of grape marc

The first in vitro experiment, the screening study, showed that there are large differences in the fermentation of different grape marc samples and in the extent of methane production. Some of the changes observed in fermentation performance can be directly related to the nature, or type, of grape marc with samples that had undergone little extraction possessing much higher

concentrations of simple sugars. These additional sugars resulted in greater fermentation (gas and VFA production), and this was also evident in increased ME values being calculated. Those samples with the highest fat concentrations (seed only samples) showed reduced fermentation.

When the experiment was analysed in depth, the concentration of tannin in the samples was correlated with reductions in methane production, while the fat concentration was correlated with reductions in maximum gas production. The correlation of different tannin fractions (WET, PA and PPT) and tannin compositional factors (cis/trans, %Gall and mDP) with fermentation and gas composition show that tannin needs to be considered in more depth than just a single quantitative assay.

When grape marc was used in conjunction with a number of different forages in different ratios, the higher inclusion rates resulted in fermentation inhibition. The volume of methane per gram of dry matter did decrease, but largely because fermentation was retarded. Factors other than methane volume per gram of dry matter needs to be considered when investigating the anti-methanogenic potential of a feed supplement.

In both dose experiments, small inclusions or supplementations (20-25%) can provide improvements to fermentation and methane, but this effect can be overshadowed by energy factors when grape marc is replacing high-energy forage (chicory and perennial ryegrass). This effect was not seen for hay based fermentations, and nearly all grape marcs had positive effects on fermentation performance, methane productions, or both. Across all inclusions, the grape marc samples that provided the most positive results, were those that had higher tannin concentrations, yet low to moderate fat concentrations. This effect may be slightly inflated by the fact that grape marc samples that contain high tannin concentrations are not overly extracted and hence also possess higher simple sugar concentrations.

When individual grape marc compositional factors were investigated the role of tannin and fat was again repeated. Tannin appears to reduce methane production more so than inhibiting fermentation, while fat tends to reduce fermentation performance which gives less methane volume as a result. Again, the correct output metrics need to be analysed to observe real reductions in methane.

Furthermore, the presence of extractable tannin appears to be potent in reducing methane while not effecting fermentation negatively. It is likely that the presence of loosely bound tannin results in some unavailable carbohydrate, and hence why slight reductions in fermentation are observed. Extractable tannin on the other hand, does not have this inherent link with carbohydrate fractions. If extractable tannin is more important, the preservation of tannin in grape marc is key. The amount of extractable tannin is relatively low, and any that is present must be preserved against oxidation to maintain potency.

When considering tannin chemistry, the presence of other variables makes the identification of key factors difficult. In the initial in vitro experiments, tannin of low mDP and low cis/trans were correlated with lower volumes of methane per unit of ME, while a lower %Gall was aligned with reductions in methane percentage. In the final in vitro experiment, changing tannin chemistry towards smaller (low mDP) and a higher proportion of extractability resulted in a lower methane producing fermentation.

As such, to maximise the benefit of tannin supplementation, smaller tannin with a lower cis/trans ratio should be found. While grape stalk is a relatively low energy feed, the low fat content and high

tannin content, along with low mDP and low cis/trans ratio point to a good supplement for low methane, if the energy loss can be countered. However, there would need to be significant engineering to implement this as it exists as a woody product that would need mulching/pulping before being applied.

4.7.4 In vivo assessments of grape marc

The first in vitro experiment, the screening study, showed that there are large differences in the fermentation of different grape marc samples and in the extent of methane production. Some of the changes observed in fermentation performance can be directly related to the nature, or type, of grape marc with samples that had undergone little extraction possessing much higher concentrations of simple sugars. These additional sugars resulted in greater fermentation (gas and VFA production), and this was also evident in increased ME values being calculated. Those samples with the highest fat concentrations (seed only samples) showed reduced fermentation.

When the experiment was analysed in depth, the concentration of tannin in the samples was correlated with reductions in methane production, while the fat concentration was correlated with reductions in maximum gas production. The correlation of different tannin fractions (WET, PA and PPT) and tannin compositional factors (cis/trans, %Gall and mDP) with fermentation and gas composition show that tannin needs to be considered in more depth than just a single quantitative assay.

When grape marc was used in conjunction with a number of different forages in different ratios, the higher inclusion rates resulted in fermentation inhibition. The volume of methane per gram of dry matter did decrease, but largely because fermentation was retarded. Factors other than methane volume per gram of dry matter needs to be considered when investigating the anti-methanogenic potential of a feed supplement.

In both dose experiments, small inclusions or supplementations (20-25%) can provide improvements to fermentation and methane, but this effect can be overshadowed by energy factors when grape marc is replacing high-energy forage (chicory and perennial ryegrass). This effect was not seen for hay based fermentations, and nearly all grape marcs had positive effects on fermentation performance, methane productions, or both. Across all inclusions, the grape marc samples that provided the most positive results, were those that had higher tannin concentrations, yet low to moderate fat concentrations. This effect may be slightly inflated by the fact that grape marc samples that contain high tannin concentrations are not overly extracted and hence also possess higher simple sugar concentrations.

When individual grape marc compositional factors were investigated the role of tannin and fat was again repeated. Tannin appears to reduce methane production more so than inhibiting fermentation, while fat tends to reduce fermentation performance which gives less methane volume as a result. Again, the correct output metrics need to be analysed to observe real reductions in methane.

Furthermore, the presence of extractable tannin appears to be potent in reducing methane while not effecting fermentation negatively. It is likely that the presence of loosely bound tannin results in some unavailable carbohydrate, and hence why slight reductions in fermentation are observed. Extractable tannin on the other hand, does not have this inherent link with carbohydrate fractions. If extractable tannin is more important, the preservation of tannin in grape marc is key. The amount of extractable tannin is relatively low, and any that is present must be preserved against oxidation to maintain potency.

When considering tannin chemistry, the presence of other variables makes the identification of key factors difficult. In the initial in vitro experiments, tannin of low mDP and low cis/trans were correlated with lower volumes of methane per unit of ME, while a lower %Gall was aligned with reductions in methane percentage. In the final in vitro experiment, changing tannin chemistry towards smaller (low mDP) and a higher proportion of extractability resulted in a lower methane producing fermentation.

As such, to maximise the benefit of tannin supplementation, smaller tannin with a lower cis/trans ratio should be found. While grape stalk is a relatively low energy feed, the low fat content and high tannin content, along with low mDP and low cis/trans ratio point to a good supplement for low methane, if the energy loss can be countered. However, there would need to be significant engineering to implement this as it exists as a woody product that would need mulching/pulping before being applied.

4.7.5 In vivo assessments of grape marc

While the grape marc containing diets delivered the animals the same dry matter intake as the control diet, the energy level of the diets are not expected to be equivalent. The reduction in metabolisable energy in the grape marc diets is expected to result in a loss of productivity, and also the likely reason why the methane production is reduced per unit of dry matter intake. The reductions in milk yield are similar to the reductions in methane and result in the insignificant difference between methane intensity between the control and treatment diets.

As the grape marc parcels are markedly different in tannin content but show similar reductions in methane per animal per day, it can be concluded that the tannin content is not solely responsible for any change in digestion. The crude fat contents are similar, so may be the dominant factor in determining the fermentation outcomes in the rumen, although for this experiment the changes in digestion were not towards greater methane intensity.

The responses of the in vivo grape marc feeding trial suggest that on a dry matter intake basis, supplementation results in reductions in methane that is likely due to a reduction in metabolisable energy in the diet and reduced fermentation in the rumen. On a methane intensity basis (per unit of milk), the supplementation of grape marc showed no significant reductions in methane.

In an initial in vivo feeding trial performed by DEPI27 reductions in methane per milk yield were observed, although these animals are stated as being 203 ± 72.8 days in milk, while in this experiment the cows were fed across September and October in early lactation. Much like the changes in fermentation outcome for the in vitro experiments, these differences may be due to the

requirements of the animals at these times, with a higher energy and protein requirement in early lactation rather than in late lactation.

The in vivo treatments were supplemented at 22.6 and 23.6% of the dry matter intake for the ensiled white and steam distilled red grape marc, respectively. These supplementation rates resemble that of the 25% in vitro inclusion experiments, which for the majority of grape marcs with higher energy forage (chicory and perennial ryegrass) showed no reductions in methane either on a dry matter intake basis or in methane per unit of metabolisable energy.

The second in vivo experiment using sheep was designed to keep the control and treatment diets as close to isoenergetic as possible to overcome the overriding energy differences which appear to have been effecting earlier experiments. The sheep diets were supplemented with two different grape marc parcels at three different inclusion levels (10, 20 and 30%). The variation within treatments meant that no significant variation in methane production could be seen. However, even when grape marc was supplemented into the ration at 30% of the dry matter there is no significant loss in live weight. As such, the supplementation of grape marc does not necessarily come with reductions in animal performance, like that seen during the dairy in vivo trial.

From the inclusion experiments and the dairy in vivo experiments there is much evidence that when grape marc is included into a high energy diet, the reduction in energy results in reduction in fermentation, or reductions in animal productivity. When this is the case, the effect of tannin or fat is extremely hard to identify. When diets are similar in energy to the control, or two grape marc treatments are compared, the effects can be seen. The use of grape marc feeding to achieve reductions in methane should be done during times of low energy requirements, such as the summer-autumn feed gap, during times of drought, or when a feed supplement of equivalent energy is being replaced.

The collection of grape marc for in vivo experiments was achieved by storing marc into 40kg bags. This process was labour intensive and not applicable for large scale storage and transport. An adequate method of storage and transport needs to be investigated before grape marc feeding can become commonplace. Furthermore, when marc was transported for sheep in vivo trials, the presence of mould resulted in a portion of the marc being discarded. The prevention of mould formation in grape marc needs to be investigated to ensure limited waste and a more cost effective feed. In a 40kg silage bag grape marc can be stored anaerobically and appears to be stable in storage for up to a year. The maximum length of storage needs to be established.

5. Conclusion

Grape marc has been thoroughly tested for the presence of agrochemicals, tannins, nutritive profile and other compounds of interest to methanogenesis. The agrochemical survey highlighted iprodione as an area of further research, with high concentrations found in some unprocessed samples. Grape marc tannin was found to vary greatly in concentration and composition across the processing chain with skin only and seed only samples giving rise to the biggest compositional variations.

In vitro experiments highlighted the roles of both fat and tannin in reducing methanogenesis, although fat was also closely related to losses fermentation efficiency. Small tannin was found to be more effective at reducing methane production, with extractable tannin reducing methane without inhibiting fermentation.

Marc parcels that have undergone limited or no extraction will be beneficial due to the presence of small, extractable tannin, but also readily fermentable sugars. However, marc with limited processing needs to be screened for the presence of agrochemical residues.

Grape marc must be applied under the correct conditions, with reductions in total feed energy contributing to productivity losses that overshadow any anti-methanogenic potential. For marc to be used effectively on-farm a number of issues need to be addressed such as preservation of tannin and methods for handling that prevent mould formation.

5.1 Key findings

From the inclusion experiments and the dairy in vivo experiments there is much evidence that when grape marc is included into a high energy diet, the reduction in energy results in reduction in fermentation, or reductions in animal productivity. When this is the case, the effect of tannin or fat is extremely hard to identify. When diets are similar in energy to the control, or two grape marc treatments are compared, the effects can be seen. The use of grape marc feeding to achieve reductions in methane should be done during times of low energy requirements.

5.2 Benefits to industry

Grape marc must be applied under the correct conditions, with reductions in total feed energy contributing to productivity losses that overshadow any anti-methanogenic potential.

6. Future research and recommendations

The agrochemical residue screening highlighted the need to better understand some common wine industry residues and how these affect ruminant animals. The compound of most importance in grape marc is iprodione, which was found in numerous samples, but does not possess a maximum residue limit for grape pomace/marc animal feed.

The supplementation of grape marc into high energy feeds has shown to result in reductions in fermentation performance or animal productivity. When grape marc is used in a lower energy ration, the effect of tannin and fat can be better observed. Additional feeding studies should be undertaken using low energy control diets, during the summer-autumn feed gap, or when animals

are being fed at or just above maintenance. It is likely that the role of grape marc in feeding systems is during one of these scenarios, and that the extent of anti-methanogenic effect would be maximised.

Lastly, the logistics of using grape marc on farm needs to be better understood. Large scale storage and transportation methods, as a well as ways to limit mould formation all while preserving the anti-methanogenic properties of marc. As oxidative conditions are known to be responsible for degradation of tannin, methods for anaerobic long term storage must be the focus of future research, especially when the production of grape marc is limited to such a small window from February to April.

7. References

Alipour, D. and Rouzbehan, Y., 2007. Effects of ensiling grape pomace and addition of polyethylene glycol on in vitro gas production and microbial biomass yield. *Animal Feed Science and Technology*, 137(1-2), pp.138-149.

Aufrere, J., Theodoridou, K., Mueller-Harvey, I., Yu, P. and Andueza, D., 2014. Ruminal dry matter and nitrogen degradation in relation to condensed tannin and protein molecular structures in sainfoin (Onobrychis viciifolia) and lucerne (Medicago sativa). *The Journal of Agricultural Science*, *152*(2), pp.333-345.

Basalan, M., Gungor, T., Owens, F.N. and Yalcinkaya, I., 2011. Nutrient content and in vitro digestibility of Turkish grape pomaces. *Animal Feed Science and Technology*, *169*(3-4), pp.194-198.

Basic ANKOM batch fermentation set-up (https://ankom.com/product/ankom-rfs-gas-production-base-kit.aspx).

Bate-Smith, E.C., 1973. Haemanalysis of tannins: the concept of relative astringency. *Phytochemistry*, 12(4), pp.907-912.

Baydar, N.G. and Akkurt, M., 2001. Oil content and oil quality properties of some grape seeds. *Turkish Journal of Agriculture and Forestry*, 25(3), pp.163-168.

Baydar, N. G., & Akkurt, M. (2001). Oil Content and Oil Quality Properties of Some Grape Seeds.

Becker, P.M., van Wikselaar, P.G., Franssen, M.C., de Vos, R.C., Hall, R.D. and Beekwilder, J., 2014. Evidence for a hydrogen-sink mechanism of (+) catechin-mediated emission reduction of the ruminant greenhouse gas methane. *Metabolomics*, *10*(2), pp.179-189.

Beauchemin, K.A., McGinn, S.M., Martinez, T.F. and McAllister, T.A., 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. *Journal of Animal Science*, *85*(8), pp.1990-1996.

Beauchemin, K.A., McGinn, S.M., Benchaar, C. and Holtshausen, L., 2009. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. *Journal of Dairy Science*, *92*(5), pp.2118-2127.

De Pina, C.G. and Hogg, T.A., 1999. Microbial and chemical changes during the spontaneous ensilage of grape pomace. *Journal of Applied Microbiology*, *86*(5), pp.777-784.

Grabber, J.H., Zeller, W.E. and Mueller-Harvey, I., 2013. Acetone enhances the direct analysis of procyanidin-and prodelphinidin-based condensed tannins in Lotus species by the butanol–HCl–iron assay. *Journal of Agricultural and Food Chemistry*, *61*(11), pp.2669-2678.

Jayanegara, A., Leiber, F. and Kreuzer, M., 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from in vivo and in vitro experiments. *Journal of Animal Physiology and Animal Nutrition,* 96(3), pp.365-375.

Kennedy, J.A. and Jones, G.P., 2001. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *Journal of Agricultural and Food Chemistry*, *49*(4), pp.1740-1746.

Lee, S.Y., Lee, S.M., Cho, Y.B., Kam, D.K., Lee, S.C., Kim, C.H. and Seo, S., 2011. Glycerol as a feed supplement for ruminants: In vitro fermentation characteristics and methane production. *Animal Feed Science and Technology*, *166*, pp.269-274.

Lorenz, M.M., Hayot Carbonero, C., Smith, L. and Udén, P., 2012. In vitro protein degradation of 38 sainfoin accessions and its relationship to tannin content by different assays. *Journal of Agricultural and Food Chemistry*, *60*(20), pp.5071-5075.

Lorenz, M.M., Alkhafadji, L., Stringano, E., Nilsson, S., Mueller-Harvey, I. and Udén, P., 2014. Relationship between condensed tannin structures and their ability to precipitate feed proteins in the rumen. *Journal of the Science of Food and Agriculture*, *94*(5), pp.963-968.

Mercurio, M.D., Dambergs, R.G., Herderich, M.J. and Smith, P.A., 2007. High throughput analysis of red wine and grape phenolics adaptation and validation of methyl cellulose precipitable tannin assay and modified somers color assay to a rapid 96 well plate format. *Journal of Agricultural and Food Chemistry*, *55*(12), pp.4651-4657.

Moate, P.J., Williams, S.R.O., Torok, V.A., Hannah, M.C., Ribaux, B.E., Tavendale, M.H., Eckard, R.J., Jacobs, J.L., Auldist, M.J. and Wales, W.J., 2014. Grape marc reduces methane emissions when fed to dairy cows. *Journal of Dairy Science*, *97*(8), pp.5073-5087.

Molina-Alcaide, E., Moumen, A. and Martín-García, A.I., 2008. By-products from viticulture and the wine industry: potential as sources of nutrients for ruminants. *Journal of the Science of Food and Agriculture*, *88*(4), pp.597-604.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture*, *86*(13), pp.2010-2037.

Porter, L.J., Hrstich, L.N. and Chan, B.G., 1985. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry*, *25*(1), pp.223-230.

Rasmussen, J. and Harrison, A., 2011. The benefits of supplementary fat in feed rations for ruminants with particular focus on reducing levels of methane production. *ISRN Veterinary Science*, *2011*.

Sirohi, S.K., Pandey, P., Goel, N., Mohini, M. and Kundu, S.S., 2012. Effect of tartaric acid addition on rumen fermentation, methane production and digestibility in different diets containing wheat straw in vitro. *Online Journal of Animal and Feed Research*, *2*(3), pp.308-313.

Spanghero, M., Salem, A.Z.M. and Robinson, P.H., 2009. Chemical composition, including secondary metabolites, and rumen fermentability of seeds and pulp of Californian (USA) and Italian grape pomaces. *Animal Feed Science and Technology*, *152*(3-4), pp.243-255.

www.tarac.com.au Tarac process 78% of Australia's grape marc.

8. Appendix

8.1 Acknowledgements

We acknowledge our partner organisations for their expert contributions. Additionally, The University of Melbourne, School of Land and Environment for performing in vitro and sheep in vivo experiments, and the University of Western Australia, School of Animal Biology for performing in vitro experiments.

This work couldn't have happened without the cooperation of Tarac Technologies Ltd, Nuriootpa SA in providing insight into grape marc processing, and access to samples and equipment.

8.2 Maximum residue limit information

Table A1: Agrochemicals analysed for in grape marc with associated maximum residue limits (MRLs) for agrochemical residues in grape pomace animal feed, other animal feed, and grape derived food products, compiled using data from the Australian Pesticides and Veterinary Medicines Authority (APVMA).

			Animal feed		Food	
Residue	Function	Viticultural Purpose	Grape pomace, dry	Other feeds	Grape	Dried grape
Atrazine	Herbicide			0.5-40		
Azinphos methyl (Gulthion, azinphos)	Insecticide	grapevine scale, lightbrown apple moth, grapevine moth, elephant weevil - phased out			2,	
Azoxystrobin	Fungicide	downey mildew, powdery mildew			2	5
Benalaxyl	Fungicide	downey mildew			0.5	
Boscalid	Fungicide	bunch rot	25	1-30	4	15
Buprofezin	Insecticide	mealybugs	5	5	0.3	1
Captan	Fungicide	downey mildew, botrytis cinerea, blackspot, leaf blight	10	10-60	10	15
Carbaryl	Insecticide	cutworms		20-400	5	
Carbendazim	Fungicide			25		
Chlorantraniliprole	Insecticide	grapevine moth, lightbrown apple moth	2	0.5-10	0.3	
Chlorpyrifos (ethyl)	Insecticide	lightbrown apple moth, grapevine moth, grapevine scale		0.5-30	1	
Chlorpyrifos methyl	Insecticide	used for cereal grains				
Clothianidin	Insecticide	mealybug		0.05-2	0.02 (wine grapes), 3 (table grapes)	
Cyprodinil	Fungicide	botrytis cinerea (used with fludioxonil)			2	5
Diazinon	Insecticide	mealybug, locust			2	
Dimethoate	Insecticide	*currently suspended for use*		0.02-30	0.1	
Dimethomorph	Fungicide	downey mildew			2	
Ethion	Animal feed			20	2	
Etoxazole	Insecticide	two spotted mite	2	0.02-2	0.2	0.7
Fenarimol	Fungicide	powdery mildew			0.1	
Fenhexamid	Fungicide	bunch rot	50 (wet)		10	20
Fenitrothion	Insecticide	locust		5-10		
Fenthion	Insecticide	Insects and birds			0.2	
Fludioxonil	Fungicide	botrytis cinerea (used with cyprodinil)		0.01-30	2	
Flusilazole	Fungicide				0.5	
Hexaconazole	Fungicide	powdery mildew			0.05	
Indoxacarb	Insecticide	grasshopper, lightbrown apple moth, grapevine moth, vine weevil, earwig	3	0.02-30	0.5	2
Iprodione	Fungicide	botrytis cinerea		1-20	20	
Malathion (maldison)	Insecticide	vine moth			8	
Mandipropamid	Fungicide	downey mildew	5		0.3	
Metalaxyl	Fungicide	downey mildew		0.05-0.1	1	
Methamidophos	Insecticide	*currently being phased out*		0.5-10		
Methidathion	Insecticide	sugar beetle, elephant weevil, fig longicorn, grapevine moth, grapevine scale, lightbrown apple moth, mealybug, vine weevil			0.5	
Methoxyfenozide	Insecticide	lightbrown apple moth	3	3-50	2	6
Metrafenone	Fungicide	powdery mildew	4		1	
Myclobutanil	Fungicide	powdery mildew			1	
Oxadixyl	Fungicide	downey mildew			2	
Parathion methyl	Insecticide	*currently being phased out*		5-25	0.5	
Penconazole	Fungicide	powdery mildew			0.1	
Procymidone	Fungicide	botrytis cinerea		0.1-5	2 (specifically wine grapes)	
Propiconazole	Fungicide			5-10	1	
Prothiofos	Insecticide	mealybug			2	
Proquinazid	Fungicide	Powdery Mildew	15		0.5	2
Pyraclostrobin	Fungicide	downey mildew, powdery mildew	10	0.05-25	2	5
Pyrimethanil	Fungicide	botrytis cinerea	40	0.3-40	5	
Quinoxyfen	Fungicide	powdery mildew	5		0.5	2
Simazine	Herbicide	broad spectrum		0.1-5		
Spiroxamine	Fungicide	powdery mildew	10	1-10	2	
Tebuconazole	Fungicide	powdery mildew		50	2	
Tebufenozide	Insecticide	lightbrown apple moth	10	10	2	
Tetraconazole	Fungicide	powdery mildew	2		0.5	
THPI (tetrahydrophthalamide)		Breakdown metabolite of captan				
Triadimefon	Fungicide	powdery mildew		10	1	
Triadimenol	Fungicide	powdery mildew		0.5-10	0.5	
Trifloxystrobin	Fungicide	downey mildew, powdery mildew	3	0.02-15	0.5	

8.3 Publications

AWRI eNews article (May 2013), available at http://www.awri.com.au/information_services/enews/2013/05/07/enews-may- 2013/#title10. The AWRI eNews contains short snippets of information in anewsletter style (a few paragraphs per article). This article summarises that projectand is an announcement of the funding acquisition and major project outcomes.

AWRI Technical Review (Issue 204, June 2013) accessible by creating login (http://www.awri.com.au/information_services/technical_review/. This publication provides stakeholders (wine industry levy payers) with technical updates on projects and on key achievements. This article was produced to convey the analytical techniques that we would be applying to the project.

A fact sheet for this project was developed and circulated at the launch of the AWRI's extension and outreach project (available from: http://www.awri.com.au/wp-content/uploads/2014/07/understanding-methane-reducing-tannins.pdf).

The project was highlighted in an AWRI Technical Review article (December 2014). This article detailed the concentrations of extractable compounds in grape marc and some practical considerations for using as an animal feed (accessible with login from: http://www.awri.com.au/tr/Technical_Review_Issue_213.pdf)..

The scope of this project and relevant outcomes were presented at a number of symposia, largely in conjunction with other AWRI projects funded by the Department of Agriculture (some slides relate to other projects).

Using grape marc in livestock enterprises – meeting of Northern Hills FarmersGroup, Nuriootpa, SA, July 18 2014.

SA Wine Industry and Climate Change – Adelaide, SA, July 31 2014.

Crush 2014 grape and wine science symposium – Adelaide, SA, September 25-26, 2014.

The general project objectives and reasons for undertaking this research were discussed in a radio interview with ABC 639 North and West, rural South Australia (live to air Feb 9, 2015). A transcript/podcast has been requested, but has not yet been made available.

To date, 3 manuscripts have been drafted, detailing the outcomes of the project.

- Hixson, J.L., & Smith, P.A.S. Comparison of direct phloroglucinolysis and colorimetric depolymerization assays and their applicability for determining condensed tannin in grape marc. *Journal of Agricultural and Food Chemistry*, IN FINAL PREPARATION.
- Hixson, J.L., Jacobs, J.L., Wilkes, E.N., & Smith, P.A.S. A survey of the variation in grape marc condensed tannin composition and concentration and analysis of key compositional factors. *Journal of Agricultural and Food Chemistry*, IN DRAFT.
- Hixson, J.L., Durmic, Z., Smith, P.A., & Wilkes, E.N. Grape marc bioactive compounds responsible for reducing in vitro methanogenesis. *Journal of Animal Production Science*, IN DRAFT.