Parameters involved in the enzymatic deconstruction of the Wine Grape cell wall matrix during winemaking

by

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Declaration

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Summary

Commercial enzyme preparations, consisting of pectinases and glucanases, are commonly used in the wine industry to; (1) enhance the extraction of beneficial compounds (e.g. anthocyanins, tannins, aroma etc.) from the grape berry, (2) to facilitate the clarification of the wine before the filtration, and (3) to maximize juice yield for alcoholic fermentation. All three factors are important in improving quality parameters (e.g. body, aroma, ageing potential) and market value for winemakers. The efficacy of any enzymes, including wine enzymes is dependent on their ability to target specific glycan linkages for catalysis, and thereby deconstruct polymeric architecture. However, in the case of grapes, very little information is known on grape berry cell walls, that can assist in a rational enzyme mixture design strategy for customizable applications (e.g. enhanced colour). This is partly due to the complex nature of fruit cell walls, factors such as grape maturity, inter-vineyard variability in ripeness levels at harvest, potential cultivar differences and the crude nature of enzyme mixtures used by the wine industry.

New tools are needed to understand cell wall architecture, and here the use of Carbohydrate Microarray Polymer Profiling (CoMPP) technology, which combines glycan microarrays with sets of characterised monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) for plant cell wall polymer epitopes detection. CoMPP is extensively used with multivariate data analysis methods in this thesis to evaluate the complex nature of the grape cell walls and how enzymes are able to deconstruct them under winemaking conditions.

This study consists of three parts focusing on a Cabernet Sauvignon vineyard:

(1) The validation of cell wall profiling tools combined with fractionation approaches on berry pomace cell walls isolated from wine fermentations. This is to confirm the utility of the approach in the winemaking conditions, and provide a baseline reference dataset for later studies. The study revealed for that grape pomace consists of two main fractions: (i) a

partially methyl-esterified pectin-rich outer layer and (ii) a highly methyl-esterified pectin-rich layer which 'coats' an inner layer of xyloglucan-cellulose rich skin cells.

(2) A study focusing on the inter-vineyard variation of grape berry cell walls collected at harvest using a panel based sampling design. This was combined with how commercial enzyme preparations influenced the potential inter-vineyard variation in harvested grape cell walls that were brought into the winemaking process. Here the study showed that the enzymes performed efficient de-pectination, without de-esterification, and significantly reduced cell wall variability in pomace and wines from treated panels.

(3) In order to study the grape berry cell wall deconstruction process in more detail, the use of pure recombinant enzymes (singly and in combinations), were used in an extensive winemaking study for the first time that we are aware of. The use of cell wall profiling tools revealed: (i) that pectin lyase appears to be a core enzyme in probably many enzyme preparations, because it results in effective de-pectination without de-esterification (ii) other enzyme combinations that combine only endo-polygalacturonase activity with de-esterification appear to only unravel the walls and not degrade effectively. This study, plus the contexts provided by the first two, permitted us to develop a hypothetical model which illustrates the cell wall structures in different tissue layers of the grape berry.

This new grape berry cell wall model apart from extending our scientific knowledge in this area, will now permit hypothesis testing in, fruit development, plant-pathogen interactions, as well as helping designing the tailored enzymes for use in different winemaking scenarios.

OPSOMMING

Kommersiële ensiembereidings, wat bestaan uit pektinases en glukanases, word algemeen gebruik in die wynbedryf om; (1) die ekstraksie van voordelige verbindings(bv. antosianiene, tanniene, aroma ens.) vanuit die druifkorrel te verbeter, (2) om die verheldering van wyn voor filtrasie te fasiliteer, en (3) die sap opbrengs vir alkoholiese fermentasie te verhoog. Al drie faktore is belangrik om kwaliteitseienskappe (bv.volheid, aroma, verouderingspotensiaal)en markwaarde vir wynmakers te verbeter. Die effektiwiteit van enige ensieme, insluitende wynensieme, is afhanklik van hulle vermoë om spesifieke glukaanverbindings te teiken vir katalise, en daardeur die polimeriese argitektuur af te breek. In die geval van druiwe is daar egter baie min inligting beskikbaaroor die druifkorrel selwande wat kan help met die ontwikkelingstrategie vir ‘n rasionele ensiemmengsel vir pasgemaaktegebruike (bv. kleurverbetering). Dit is deels die gevolg van die komplekse samestelling van vrugte selwande, faktore soos druif rypheid, verskille in rypheid binne ‘n spesifieke wingerd tydens parstyd, potensiële kultivar verskille en die ruwe aard van ensiembereidings wat in die wynbedryf gebruik word.

Nuwe metodes word benodig om die selwandargitektuur te verstaan, en hier is die gebruik van dieomvattende mikrorooster polimeer profielbepalings tegnologie (*Comprehensive Microarray Polymer Profiling*; CoMPP), wat glukaan mikroroosterskombineer met gekarakteriseerde monoklonale teenliggaampies (mAbs) en koolhidraatbindende modules (CBMs) vir plant selwand polimeer epitope, baie waardevol.In hierdie tesis word CoMPP breedvoerig gebruik,tesame met multiveranderlike statistiese data analise metodes, om die komplekse aard van die druifkorrel selwand te evalueeer asook die vermoë van ensieme om dit te dekonstrueer onder wynmaak toestande.

Die studie bestaan uit drie dele wat fokus op 'nCabernet Sauvignon wingerd:

(1) Die bevestigingvan die selwand profielbepalingsmetodes tesame met fraksioneringstegnieke opselwande afkomstig vangepersde druiwedoppetydens die fermentasieproses. Hiermee is die nuttigheid van die benadering onder wynmaaktoestandebevestig, en ‘n basislyn verwysingsdatastel geskep vir verderestudies. Hierdie studie het gewys dat gepersde druiwedoppe bestaan uit twee hoof fraksies: (i) ‘n gedeeltelik metiel-veresterde pektienryke buitenste laag en (ii) ‘n hoogs metiel-veresterde pektienryke laag wat ‘n binneste laag van xiloglukaan- en selluloosryke dopselle bedek.

(2) ‘n Studie wat fokus op die binne-wingerd variasie van druifkorrel selwandsamestelling,wat bepaal is op driuwe wat versamel is tydens oestyd volgens ‘n paneelgebaseerde steekproefontwerp. Dit is gekombineer met 'n ondersoek na die invloed van kommersiële ensiembereidingsop die moontlikebinne-wingerd variasie tydens wynbereiding. Die studie het gewys dat die ensieme effektiewe depektienase uitgevoer het, sonder de-esterifikasie, en die variasie in selwande van gepersde druiwedoppe en wyn, vanaf behandelde panele, is aansienklik minder gemaak.

(3) Gesuiwerde rekombinante ensieme (alleenof in kombinasie) is, sover bekend,vir die eerste keergebruik in ‘n breedvoerigewynmaakstudie om sodoende die druifkorrelselwand dekonstruksie proses in meer detail te bestudeer. Die selwand profielbepalings metodes het gewys: (i) dat pektien liase skynbaar ‘n kernensiem in baie ensiembereidings is, omdat dit effektiewe depektinase veroorsaak sonderde-esterifikasie (ii) ander ensiemkombinasies, bestaande uit endo-poligalakturonase en de-esterifikasie aktiwiteite, kan skynbaar slegs die selwande ontrafel, maar nie effektiewe degradasie veroorsaak nie. Hierdie studie, asook die konteks wat deur die eerste twee verskaf is, laat ons toe om ‘n hipotetiese model te ontwikkel wat die selwandstrukture in die verskillende weefsellae van die druifkorrel aandui.

Hierdie nuwe druifkorrel selwandmodel verbreed ons wetenskaplike kennis in die veld en maak hipotesetoetsing moontlik ten opsigte van vrugontwikkeling, plant-patogeen interaksies, en help met die ontwikkeling van ontwerpersensieme vir gebruik onder verskillende wynmaak toestande.

This dissertation is dedicated to my parents and my wife

Biographical sketch

Yu Gao was born in Luoyang city, He'nan Province, China on 14 May 1984. He matriculated in 2003 at No. 11 High School of Zhengzhou, China and enrolled for a BSc (Biotechnology) in 2004 at University of Western Cape, South Africa. His BSc (Biotechnology) was completed in 2008 and he received his Bsc (Honours) degree in Biotechnology in 2010. He enrolled for a MSc (Biotechnology) degree in Institute for Microbial Biotechnology and Metagenomics (IMBM), Department of Biotechnology, University of Western Cape, South Africa in 2010 and this degree was obtained in 2012 with Cum Laude. His PhD was started from 2013 in the Institute for Wine Biotechnology (IWBT), Department of Viticulture and Oenology, Faculty of Agriculture Sciences, Stellenbosch University, South Africa.

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Preface

This dissertation is presented as a compilation of six chapters. Chapter 2 is prepared for publication to Trends in Food Science and Technology, Chapter 3 was accepted for publication in the Carbohydrate Polymers and Chapter 4 will be submitted to Journal of Agricultural and Food Chemistry. Chapter 5 will be submitted to Carbohydrate polymers.

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Abbreviation

AG: arabinogalactan

AGP: Arabinogalactan protein

AIR: alcohol insoluble residue

CoMPP: comprehensive microarray polymer profiling

CBM: carbohydrate binding module

CDTA: cyclohexanediamine-tetra-acetic acid

EPG: endo-polygalacturonase

FT-IR: fourier transform – infrared spectroscopy

GC: gas chromatography

HG: homogalacturonan

mAb: monoclonal antibody

OPLS: Orthogonal projections to latent structures

PCA: principal component analysis

PL: Pectin lyase

PME: pectin methyl esterase

RG: rhamnogalacturonan

Chapter 1

General introduction and project aims

1.1 General Introduction

Our knowledge of the grape berry cell wall is still surprisingly limited (Moore *et al*, 2014a); this is particularly remarkable given the advances made in other agronomically important fruits such as tomato and apples etc. (Ruiz-May *et al*, 2013, Ng, *et al*, 2015, Palmer *et al*, 2015, Minoia *et al*, 2016). Given the importance of grapevine as the most planted fruit crop worldwide (OIV, http://www.oiv.int/oiv/info/enpublicationsstatistiques), there is clearly motivation to remedy this deficiency in scientific insight into grape cell walls, especially since expanded and relatively recently developed cell wall profiling technologies have become available (Hervé *et al*, 2011, Sørensen and Willats, 2011).

The first period of major study of grape cell wall substances was the late 80's and early 90's with work performed by Luc Saulnier then at INRA (Montpellier) in France (Saulnier and Thibault, 1987a) (personal discussion and communication with Dr Luc Saulnier at INRA Nantes-Angers, France). The studies involved characterization of grapes pectic substances (Saulnier and Thibault, 1987b, Saulnier *et al*, 1988); and isolation and classical carbohydrate chemical characterization of an arabinogalactan protein (Saulnier and Brillouet, 1989, Saulnier *et al*, 1992). The second major period of study was in the late 90's and early 2000's in Australia mainly (but also partly in France) (personal communication John Moore; with Dr Ian Sims (Victoria University of Wellington, New Zealand) and Prof Tony Bacic (University of Melbourne, Australia). A number of studies were performed on the viticultural side which included mainly understanding berry development and ripening at the cell wall polysaccharide, microscopical and molecular level (Nunan *et al*, 1997, 1998, 2001, Barnavon *et al*, 2000, 2001, Davies and Robinson, 2000, Yakushiji *et al*, 2001,).

A shift occurred in the early 2000's to focus on plant cell wall polysaccharides mainly in the context of oenological relevance (Vernhet *et al*, 1996) and important with major studies emerging from France in collaboration with the Complex Carbohydrate Research Center (CCRC, Georgia, USA) (e.g. Vidal *et al*, 2001, Doco *et al*, 2003a). This was also the period in plant cell wall community where major advances in understanding the complex structure of

rhamnogalacturonan II (RGII) were being made in plant biochemical contexts (O'Neill *et al*, 1996, Ishii *et al*, 1999) and measuring its content and structural studies in fruit juices and vegetables (Doco *et al*, 1997); and presence in red wine (Vidal *et al*, 2000a). This focus on wine polysaccharides was driven by understanding their content and potential properties during winemaking (Vidal *et al*, 2000b, Riou *et al*, 2002, Doco *et al*, 2003b) and in the wine (Doco *et al*, 2000, Vidal *et al*, 2004). Since that period a major focus of research has been measuring polysaccharides in wine following various practices (Ayestarán *et al*, 2004) Approaches have included FT-IR spectroscopy (Coimbra *et al*, 2002, 2005) but more often than not the technique employed involves some sort of enrichment and gas chromatography based analysis of grape-derived polysaccharide monomers produced after acid hydrolysis (Arnous and Meyer, 2009, Ducasse *et al*, 2010, Guadalupe *et al*, 2012). Recent work has attempted to bridge-the-gap in our understanding of the changes in cell wall composition and its relationship to wine polyphenol contents/composition during and after winemaking (Bindon *et al*, 2010, 2012, Bindon and Kennedy, 2011, Ruiz-Garcia *et al*, 2014).

A major challenge regarding plant cell wall studies in general are not only their architectural complexity linked with heterogeneous polymer compositions, but also the complex nature of their cross-links (covalent and non-covalent)(Jarvis, 2011), and also cell wall variability among various plant species, organs and tissues (Somerville *et al*, 2004, Fangel *et al*, 2012). Hence, the current idea of grape berry cell wall structure is still inferred from a number of hypothetical cell wall models from other plants (e.g. Arabidopsis Somerville *et al*, 2004). However, these generalisations are likely from reality of a grape berry which contains tissue layers (e,g, seeds, mucilage, pulp and skins) (Coombe, 1973, Gomez *et al*, 1994, Nunan *et al*, 1998), but have been questioned from some recent studies (Moore *et al*, 2014a, Zietsman *et al,* 2015a).

In order to expand our understanding of grape cell wall architectural changes in the context of winemaking we need to apply new tools to the problem. The recently developed high throughput glycan microarray (CoMPP) provides direct and insight view of cell wall on the polymer level, give the opportunity to study the structure and the polymer associations

in detail (Moller *et al*, 2007). This method has been combined with other classical cell wall analysis methods and validated on the research of grapevine related studies, such as grape leaves, berry skins and wine (Moore *et al*, 2014a, 2014b, Zietsman *et al*, 2015a, 2015b). Moore *et al.* (2014a) used high-throughput profiling approach to analyse grapevine leaves, providing a baseline analyse combining various approaches such as GC-MS, ATR-FT-MIR spectroscopy, MS fingerprinting and CoMPP to show the utility of such an approach in a screening analyses (e.g. for disease symptoms). Such an approach was then applied in a comparative study (Moore *et al,* 2014b) of ripening stages in wine grapes (cv. Cabernet Sauvignon) and table grapes (cv. Crimson Seedless) using cell wall profiling tools and multivariate tools; revealed potential cell wall biomarkers, requiring further study. The first application of the cell wall profiling approach in a winemaking context was performed by Zietsman *et al*. (2015a) looking at the effect of maceration enzymes on grape skins (cv. Pinotage) using a novel OPLS multivariate approach. A further study by Zietsman *et al*. (2015b) excluded the complexity of the wine matrix by investigating the actions of enzymes on isolated skin cell wall material (cv. Pinotage) in buffered conditions leading to the concept of cell wall 'unravelling' by enzymes (i.e. incomplete degradation). Thus these prior studies have provided us a platform which allows us to study further, in more depth, on the compositional structure of grape berry cell walls and their potential polymer associations, and their impact and influence in wine fermentations.

1.2 Specific aims and objectives

The special aims of this study, using Cabernet Sauvignon as our cultivar of choice, are divided into three major step-wise progressions: (1) firstly to perform a baseline analysis of grape berry cell walls undergoing standard red winemaking to generate a foundation of information on composition and architectural components that vary and are invariant. (2) to investigate the intra-vineyard grape cell wall variability and its impact on commercial enzyme treatments; here two studies are incorporated into one; (i) grape cell wall variability and (ii) commercial enzyme effects, both in a vineyard and then winemaking context. (3) To use recombinant enzymes, generously supplied by Novozymes, to determine the effect of single

enzyme treatments with combinatorial applications in order to sequentially deconstruct the grape berry cell walls during winemaking. All three special aims involve the use of cutting-edge high-throughput cell wall profiling approaches (Moller *et al*, 2007, Moore *et al*, 2014a, 2014b, Zietsman *et al*, 2015a, 2015b). The expanded versions of the three special aims are provided as follows:

1.2.1 Validation of using high-throughput cell wall profiling tools and fractionation approaches to understand the grape berry cell wall deconstruction process more clearly during a standard red wine fermentation.

Traditionally, plant cell wall information is inferred indirectly from analysis using classical approaches at the monosaccharide level (such as Gas chromatography, York *et al*, 1985) and has revealed that grape berry cell walls undergo compositional changes during development (Vicens *et al*, 2009). In this study, harvested grape berries (cv. Cabernet Sauvignon) will taken through a standard red wine fermentation. The pressed pomace at different stages of fermentation will be processed and analysed using a combined cell wall profiling approach (Moore *et al*, 2014b), to investigate the degradation process of berry cell wall throughout the winemaking, for establishing a baseline reference dataset for further study. The chemical and enzymatical fractionation methods will also be used on the isolated berry cell wall material to provide additional information on the content and composition. In addition to pomace, the wine polysaccharides will also be isolated and analysed for tracking the composition and distribution of degraded berry cell wall polymers.

1.2.2 Studying the intra-vineyard variation of berry cell wall and its contribution to the winemaking in the context of the application of commercial enzymes.

Previous research work on berry parameters (e.g. sugars, acids, phenolics, etc) found that the intra-vineyard variation are present due to the microclimate effect (Bramley, 2005, Paggy and Cheng, 2010), the berries in the same vineyard, vine and bunch do not necessarily sit at same development stages. Standard viticultural practice often ignores these variation factors,

but rather performs a pooled harvest and fermentation. However, the content variability of these beneficial compounds (e.g. pigments, tannins, aromatics etc.), as well as their differential extractabilities due to the specific development stage may be cell wall related and hence could contribute to the inconsistency of final wine quality. However, there is no study has been done on berry cell wall analysis correlated to the intra-vineyard variation, and if the cell walls of the berries from the vineyard will undergo the similar degradation processes when pooled and harvested in separate panels. Furthermore, the question of how this variation is impacted by the addition of the commercial enzyme preparations, which are widely used in current wine industry, in a winemaking scenario is largely unknown. The vineyard will be dissected into adjacent panels, and the berries from each panel will be harvested and fermented individually, with or without the commercial maceration enzymes. Cell wall information will be generated by analyzing the fresh berries and pressed pomace using the validated cell wall profiling approach, in order to determine the degree and nature of the variability in grape berry cell wall composition, which is presumably present within the vineyard. Furthermore how this cell wall variability influences the maceration steps in winemaking, as well as the effect of the addition commercial enzyme preparations, will be followed.

1.2.3 The use of a combination of recombinant cell wall degrading enzyme to sequentially deconstruct grape berry cell wall structure during winemaking.

As a semi-purified product extracted from microorganisms (e.g. fungi), commercial enzyme preparations have been used in wine industry for decades for enhancing maceration and clarification processes (Romero-Cascales *et al*, 2012). However, the assessment of the efficiency of these enzymes is still exclusively based on the measurement of indirect parameters (i.e. juice yield, color, etc). The direct study on the berry cell wall is difficult because of two main reasons, (i) unknown composition and purity of enzyme preparations, and the reliance on (ii) and classical cell wall methods (e.g. GC-MS monosaccharide profiling approaches) with insufficient attention paid to newer techniques investigating the polymer level (e.g. using mAbs and CBMs to study polymers directly by virtue of their glycan epitopes).

The use of recombinant cell wall degrading enzymes can be a first step to elucidate cell wall architecture more deeply using a number of designer “enzyme cocktails”. In order to look at the degradation by the enzyme(s) on polymer epitope level, the relatively new glycan microarray (CoMPP) technology (Moller *et al*, 2007) is a useful approach to determine the actions of these recombinant enzymes in winemaking conditions. Thus, by combining this technique with well characterised recombinant enzymes, we can investigate the degradation of berry cell walls under the actions of different enzyme(s), following wine fermentation. Cell wall profiling tools will be applied to the pressed pomace to generate information on the degree and nature of cell wall degradation. Then the multivariate data analysis tools will be used to analyses these datasets to assess the impact of single and combinations of recombinant enzymes in comparison to commercial enzymes. This should provide much more insight into the nature of the enzymatic deconstruction of grape berry cell wall during winemaking then is known thus far.

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Chapter 2

Literature review

Parameters involved in the enzymatic deconstruction of the wine grape cell wall matrix during winemaking

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2.1 Winemaking: a short summary

Grapes are well known as one of the most valuable and widely planted fruit-tree crops worldwide (OIV, http://www.oiv.int/oiv/info/enpublicationsstatistiques). Generally, grapevine use can be divided into two main categories, table grape cultivars (for eating e.g. Crimson Seedless) and wine grape cultivars (for winemaking e.g. Cabernet Sauvignon) (Creasy and Creasy, 2009). Human-mediated domestication of grapevines for fermented beverage production has yielded the European grapevine *Vitis vinifera* cv. which are considered to produce the best diversity of quality wine styles. However, the global wine industry still faces numerous challenges; in the area of wine production these can be related to optimizing the yield per hectare (per cultivar) for bulk wine and spirits while still maintaining consistent and good overall quality (a very subjective term) (Moore and Divol, 2011), while still expressing the "typicity" of the different cultivars used (e.g. Cabernet Sauvignon) (Robinson, 2006), as well as facing the influence from global climate change (Hannah *et al*, 2013). Wine fermentation can be broadly divided into the production processes involved for making red and white wines which involves both distinct and similar treatments in the cellar (see Fig. 2.1 for a summary diagram, adapted from Pretorius (2000). Unlike white wine which is mainly fermented from clarified must (juice) obtained from destemming and crushing the white grapes; red wines are fermented on skins (i.e. the cap) to enhance the extractability of a number of beneficial compounds related to colour, mouthfeel and flavour (e.g. anthocyanins, tannins and aromas) obtained through breakdown of the pomace (skin and pulp tissues), in order to improve various important oenological parameters (e.g. body, aroma, ageing potential). However, the efficiency of this step is not always consistently guaranteed and is often highly variable, due to the complex nature of grape berry tissues and the grape lots obtained from the harvests (e.g. vintage etc.) (Ortega-Regules *et al*, 2008).

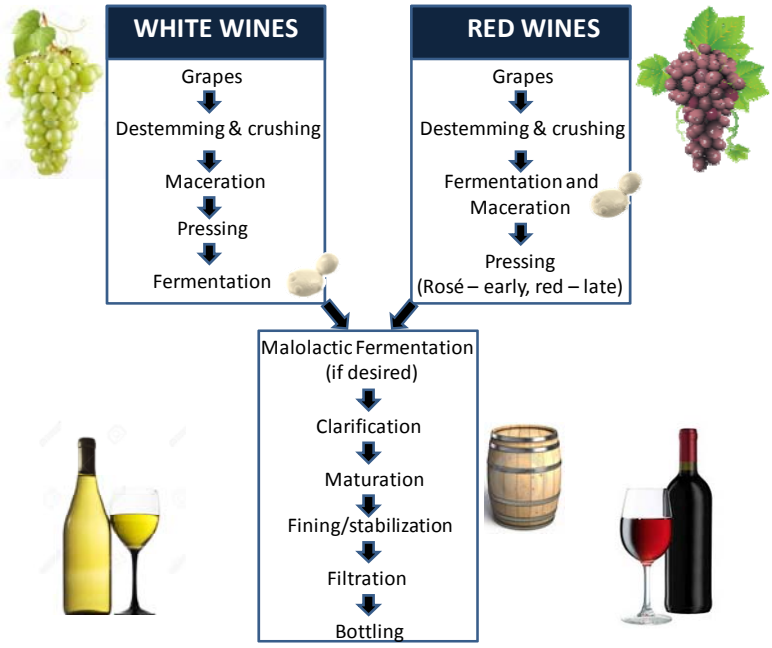

Figure 2.1 The standard winemaking process of white and red wines (adapted from Pretorius, 2000).

2.2 The physiology of grapevine: a focus on the grape berry

In general, grape berries can be categorized in three main tissue types (see Fig. 2.2 A for anatomy of a grape berry [1] (Coombe, 1973), which consists of the the skins, pulp and seeds. The chemical compounds and metabolites present within the grape berries also vary, (see Figure 2.2. for the general distribution of compounds in a grape berry [2]), as illustrated by Gomez *et al*. 1994. A waxy layer (cuticle) is present on the outer layers of the skin tissues; as this layer not only protects the fruit from pathogens (e.g. fungi), but also minimises/regulates water loss from the berry (Bargel *et al*, 2006). The microscopical study (Fig. 2.3) performed by Nunan *et al* (1998) show that the cells in skin tissues (i.e. exocarp) are more condensed and tightly packed; these cells accumulate the phenolic compounds during the ripening which contribute positively to wine quality and sensory characteristics. The pulp (i.e. flesh, also known as pericarp) is the main storage parts for sugars (i.e. glucose and fructose) and organic acids, and the cells in pulp tissues expand significantly during and after the veraison stage by volume compared with skin cells which expand according to the net surface area increase (i.e. a surface-to-volume ratio) (Jackson, 2000). The seeds also contain a relatively higher concentration of phenolic compounds (compared with skins and pulp),

however these can contribute to the bitterness of the wine. As berry ripening proceeds though, these compounds become less extractable due to the formation of an outer impermeable layer around the seed during maturation (Cadot *et al*, 2006).

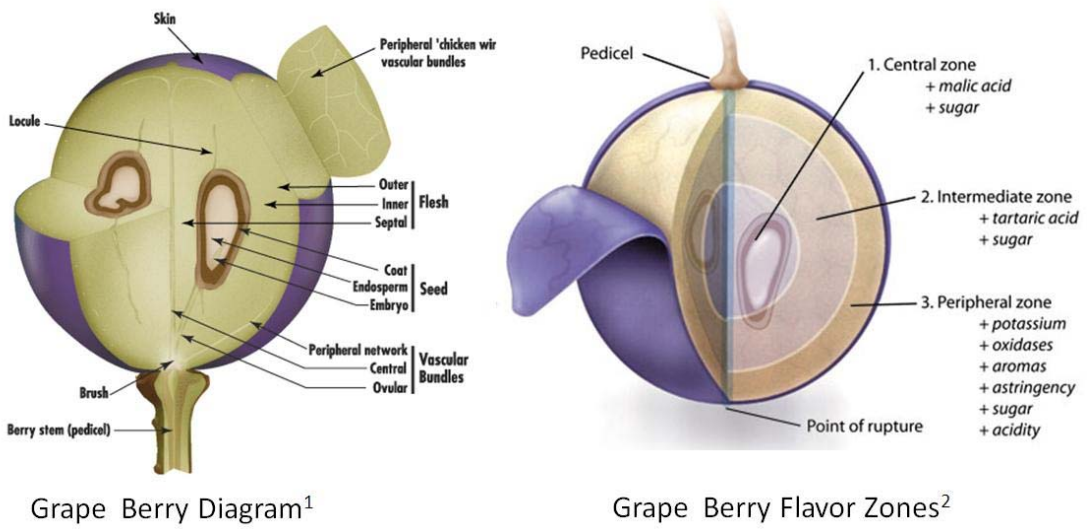

Figure 2.2 The illustrated berry tissue composition (Coombe, 1973), the chemicals and their locations (Gomez *et al*, 1994).

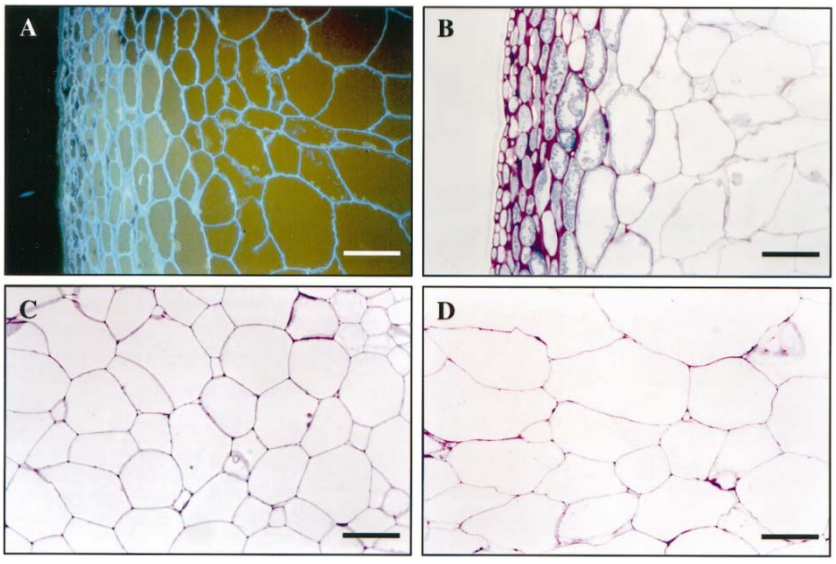

Figure 2.3 Microscopy images of grape berry outermost regions showing the cell difference between tissue types (Nunan *et al*, 1998). **A:** berry at 58 days post-anthesis (pda), stained with calcofluor; **B:** berry at 86 pda, stained with TBO, **A** and **B** show the epidermis at left and mesocarp ar to the right (bars=100 µm). **C** (58 dpa) and **D** (114 dpa) showed the central location at mesocarp, and stained with TBO. (Nunan *et al*, 1998).

2.3 Maceration of grapes during winemaking: advantages and drawbacks

In order to extract qualititave compounds from berries, a crushing step is applied during winemaking. In this process, the pulp cells are relatively easily broken up to release the sugars and organic acids, in the free run juice (must), due to the relatively thinner cell walls of these fully expanded cells (Ortega-Regules *et al*, 2008). In contrast, the cell wall of condensed tightly-packed skin cells is much more resistant to the mechanical disruption, which makes the release of favorable chemical compounds (e.g. polyphenols) quite difficult (Bindon *et al*, 2014). Thus, one of the most important objectives in winemaking is to enhance the effective breakdown of the berry cell wall during the skin contact phase (mainly used in red winemaking; with a few specific, but less commonly applications in white winemaking). It is considered essential in order to maintain (and enhance) the consistency of overall quality and quantity of wine produced.

This important step in winemaking, termed maceration, (Fig. 2.1) is used to soften the skin, and to leach the polyphenol and aroma compounds from skins into the must ((Ribéreau-Gayon *et al*, 2006). In this step, the pomace is usually punched down a few times per day to increase the extraction (other technique may also be applied, e.g. pump over), as the skins naturally float (lower gravity than ethanol) on the top of the must. Sulfur used as an antioxidant and antimicrobial agent, in the must can also contribute the leaching, however, some cultivars are recalcitrant to the release of sufficient amount of favorable compounds into the wine, as the berry cell wall acts like a natural barrier to prevent the cells to be sufficiently broken down (Bindon *et al*, 2014). The berry skin cells contain most of the anthocyanins and tannins, which are not harsh, unlike seed tannins, contributing to wine mouthfeel (Ortega-Regules *et al*, 2008). Thus, to increase the overall quality of final wine, more effective cell wall deconstruction is required. In the past, a number of winemaking techniques waere developed for more effective extraction in maceration step, such as (1) cold maceration which include a short cold fermentation period at 4 to 15 ^{o}C before the standard fermentation, in order to release more favorable compounds; (2) thermovinification which applies elevated temperatures to the fermentation for higher phenolic extraction, as well as the extended maceration in warm temperature, and (3)

physical disruption of the cell structures, such as is performed using electric pulse treatment (Cholet *et al*, 2014). However, although these methods have been shown at times to positively influence the maceration efficiency, the lack-of-consistency is always an issue (Sacchi *et al*, 2005), and some side-effects have also been observed, such as increases in volatile acidity and off-flavors (Koyama *et al,* 2007).

Commercial enzyme preparations, however, have been used for decades in winemaking. These enzymes are crude extracts and are mainly sourced from *Aspergillus niger* (and few other species) grown under specific conditions (very similar to wine fermentation conditions), and they are recognized to have GRAS (generally regarded as safe) status by the OIV (Organisation International de la Vigne et du Vin) (http://www.oiv.int/oiv/info/enspecificationproduit). The main application of wine enzymes is to enhance free run juice, improve clarification and enhance extractability (Ortega-Heras *et al*, 2012). These preparations have been shown to accelerate the maceration and this has been confirmed by analyzing a number of general wine parameters post-treatment (Romero-Cascales *et al*, 2012), showing that valuable compounds in the final wine were increased by adding the enzyme preparations. Cell wall degrading enzymes in the commercial preparations are believed to be able to degrade the cross-links of polysaccharides and polyphenols in berry skin polysaccharide-rich matrices, thereby facilitating the diffusion of the anthocyanins and tannins into the wine during the fermentation (Ortega-Heras *et al*, 2012). This is in order to produce red wines with strong and stable red-purple colours, as well as more body and structure, for ageing potential. White winemaking also employs pectinolytic enzymes in enzyme preparations to facilitate the degradation of the pectin compound content in the pressed juice to reduce the viscosity, in order to facilitate clarification (Sacchi *et al*, 2005). However, as in the case of the other maceration techniques, there are also drawbacks of using maceration enzymes; as a semi-purified product (rules set by OIV, www.oiv.int/oiv/files/CODEX_2012_EN.pd), the enzyme preparations may also contain a number of unwanted activities (Revilla and Gonzalez-SanJose, 1998, Fia *et al*, 2014). Moreover, reliable structural information of the substrate (i.e. grape berry cell walls) which the enzymes presumably target for degradation/maceration is still very limited to date. Without a clearer picture (i.e.

understanding) of the cell wall architecture of grape berries, the ability to develop more efficient enzyme-mediated maceration techniques will be severely curtailed, and this information is therefore of great importance for the wine industry.

2.4 Plant cell wall composition and structure: a summary

Grapevine cells, as is the case of other angiosperms, possess a primary cell wall, composed of cellulose, xyloglucan, pectins and proteins, which is the main type found in berry tissue, excluding the vascular veins and vessels. Figure 2.4 presents the schematic view of the primary plant cell in relation to the apoplast and symplast; and adjacent cells (http://www.ccrc.uga.edu/~mao/intro/ouline.htm, complex carbohydrate research center website). Different parts of the primary cell wall are involved in a number of important processes related to growth, development, functional specialization and biotic and abiotic stressors (Brett and Waldron, 1996). Firstly, its outer layer (termed middle lamella, Fig. 2.4) acts as a barrier to protect cells against inner and outer stress factors, such as cell expansion (i.e. mechanical) and pathogen attacks (i.e. biotic) (Cosgrove, 2005, Verhertbruggen and Knox, 2006); while the inner layer mainly consists of hemicelluloses (e.g. xyloglucan) and cellulose polymers, and is believed to be more rigid but also semi-flexible; acting as the skeletal framework of the cell (McNeil *et al*, 1984). The middle lamella and primary cell wall also control the transport of molecules between the cells, and here the pectin components are strongly believed to control the pore size of the cell wall layers, thus regulating passage of biomolecules (Fleischer *et al*, 1999).

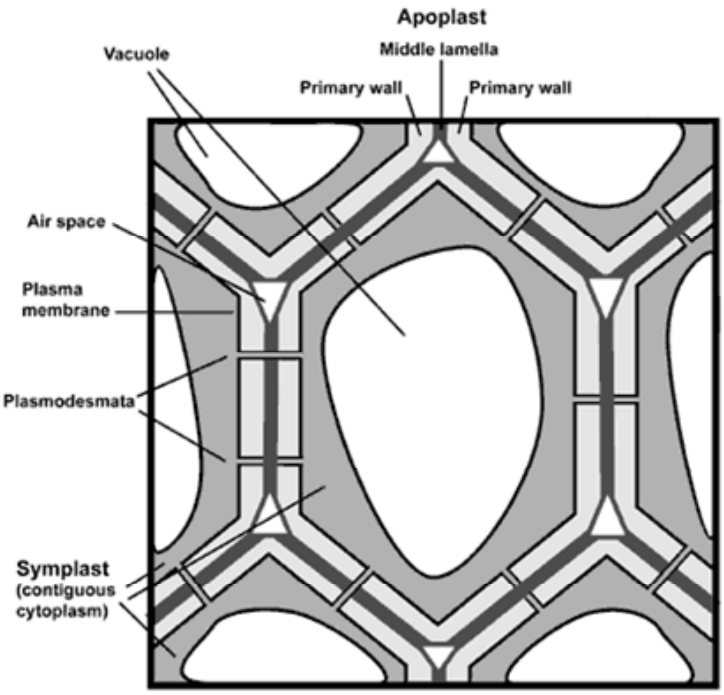

Figure 2.4 The schematic view of plant cell wall surrounds by the primary cell wall. From http://www.ccrc.uga.edu/~mao/intro/ouline.htm, complex carbohydrate research center (CCRC) website.

The plant cell wall is generally recognized to be one of the most abundant and complex biopolymeric structures in the natural world (Carpita & McCann, 2000), due to its diverse heterogeneous composition and complex cross-linking between cell wall polymers *in muro*. Several models have been proposed over the last forty years to develop a unified model for plant cell walls; the first being Keegstra *et al*. (1973); based on biochemical methods of the time. Since then other models have been put forth; for example Carpita and Gibeaut (1993) emphasizing the differences between monocotyledons and dicotyledons in their relative leaf cell wall compositions and general architecture. It is for example more common in dicotyledonous plants such as grapevines to have equal proportions of cellulose, hemicellulose (e.g. xyloglucans) and pectins in their primary cell walls. This is different from monocotyledonous plants where large amounts of secondary cell walls are present (e.g. xlyan-rich layers); thereby shifting this proportion based on dry weight sugar compositions as expounded in Carpita and Gibeaut (1993). The most popular model to date is the tethered-network model (a simplified version taken from the Markus Pauly group website is provided in Fig. 2.5) which shows the cellulose microfibrils 'tethered' by xyloglucan strands

through hydrogen bonding; this skeletal framework is then embedded in pectins, proteins (not shown) and then other neutral polymers such as xylans.

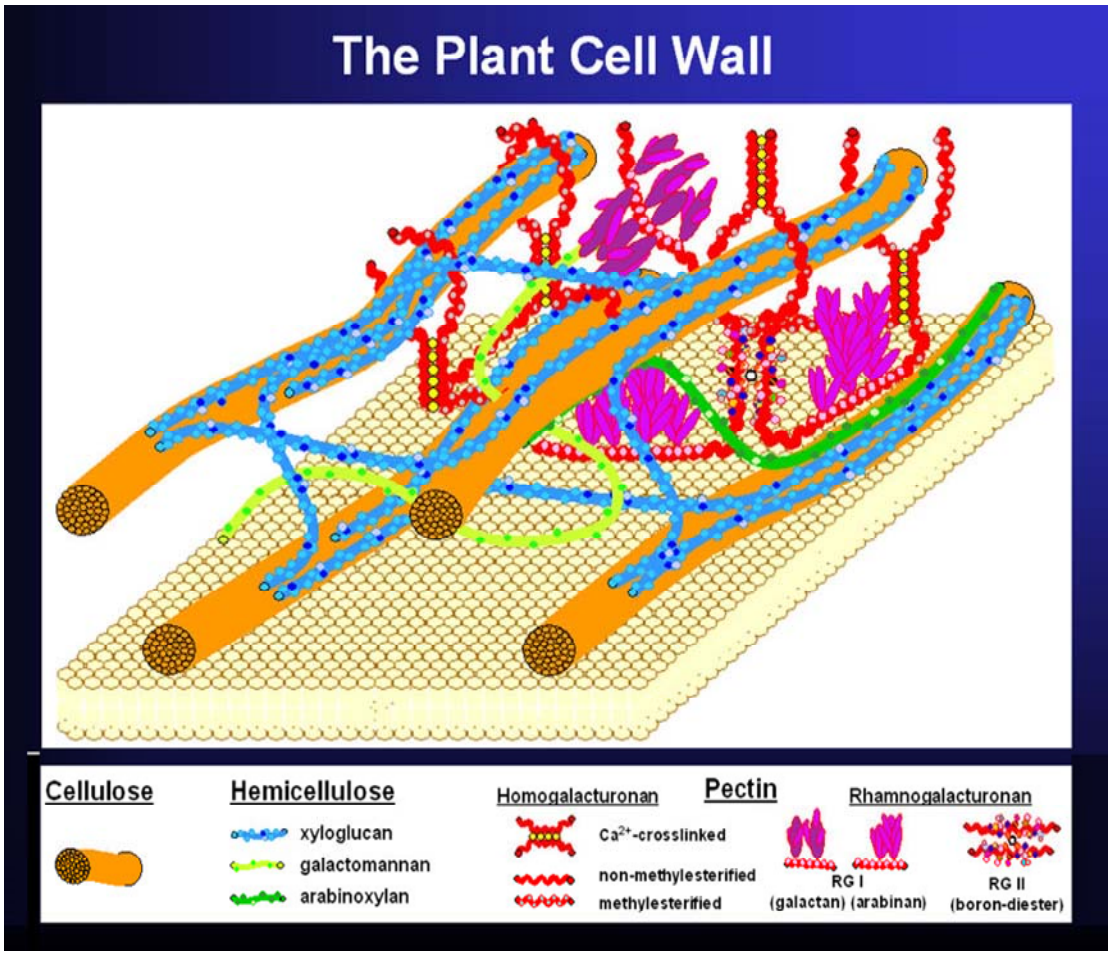

Figure 2.5 A simplified 'tethered-network' model of plant cell wall of an Arabidopsis leaf (Markus Pauly Lab website 2013) http://www.pmb.berkeley.edu

However a more realistic model of an Arabidopsis leaf cell wall is provided in Figure 2.6; from an article by Somerville *et al*. (2004) (Fig. 2.6). The figure shows, still representing the basic 'tethered-network model' that the cell wall is a much more intermeshed structure that contains a number of distinct polymer types which act as building blocks (e.g. cellulose, xyloglucan, mannans, proteins and pectins). It is also important to note the degree of inter-polymeric and intra-polymeric (covalent and non-covalent) cross-linking (i.e. calcium egg-boxes, borate, side chain links, hydrogen bonding, oxidative bridges etc.) that occurs, giving rise to higher level architecture. The model also presents xylans and lignins being;

present mostly in vascular tissue with secondary cell walls. In the case of grapes, being a fleshy berry fruit, these compounds would be only be present in very low abundance in these tissues.

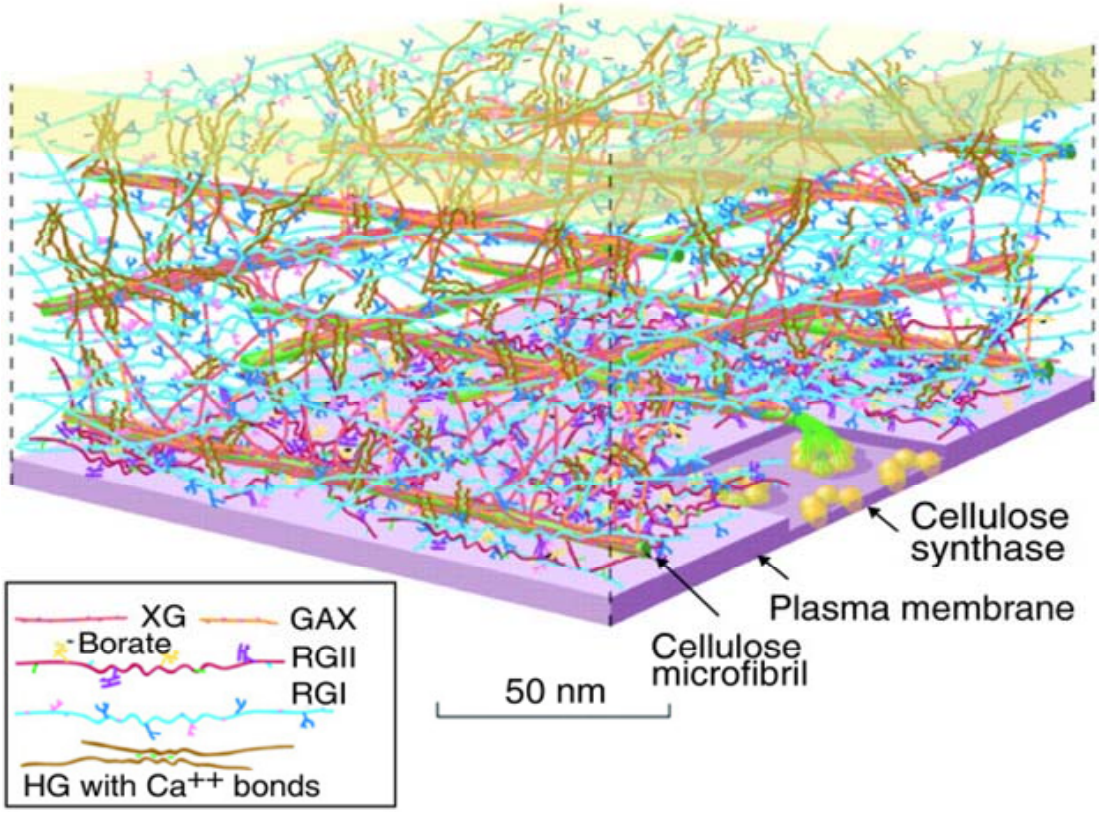

Figure 2.6 A more detailed cell wall model of an Arabidopsis leaf showing the main building blocks (i.e. polymers) and the intra- and inter-polymer cross-linking that is found. (by Somerville *et al*, 2004).

It is interesting to note that the tethered-network model has been challenged over the last decade based on the accumulation of evidence regarding the functioning of xyloglucan due to the *xxt xxt* double mutant of Arabidopsis (Cavalier *et al.* 2008) which lacks any detectable cell wall xyloglucan but still grows normally. Hence the 'tethering' role of xyloglucan has been questioned, but also the functioning of the xyloglucan endotransglucosylase/hydrolase (XTH) gene family (Fry *et al*, 1992, Hara *et al,* 2013) responsible for the production of enzymes associating with 'cutting and pasting' by XET enzyme action (Franková and Fry, 2013). The tethered-network cell wall model has been challenged in the literature by Cosgrove (2014), given the evidence against it especially the Cavalier *et al*. (2008) study. Park *et al*. (2015) have proposed the 'biomechanical hotspots model', (personal communication

with John Moore); however convincing evidence for a replacement model is still questionable at this early stage. Recently Kazuhiko Nishitani presented the conflicting models at the 8th Plant Biomechanics International Conference (held 30th November – 4th December in Nagoya, Japan, 2015) and showed how XTH and XET action data may be compatible with both models (see Fig. 2.7 from the abstracts book of the meeting, figure caption simplified from original).

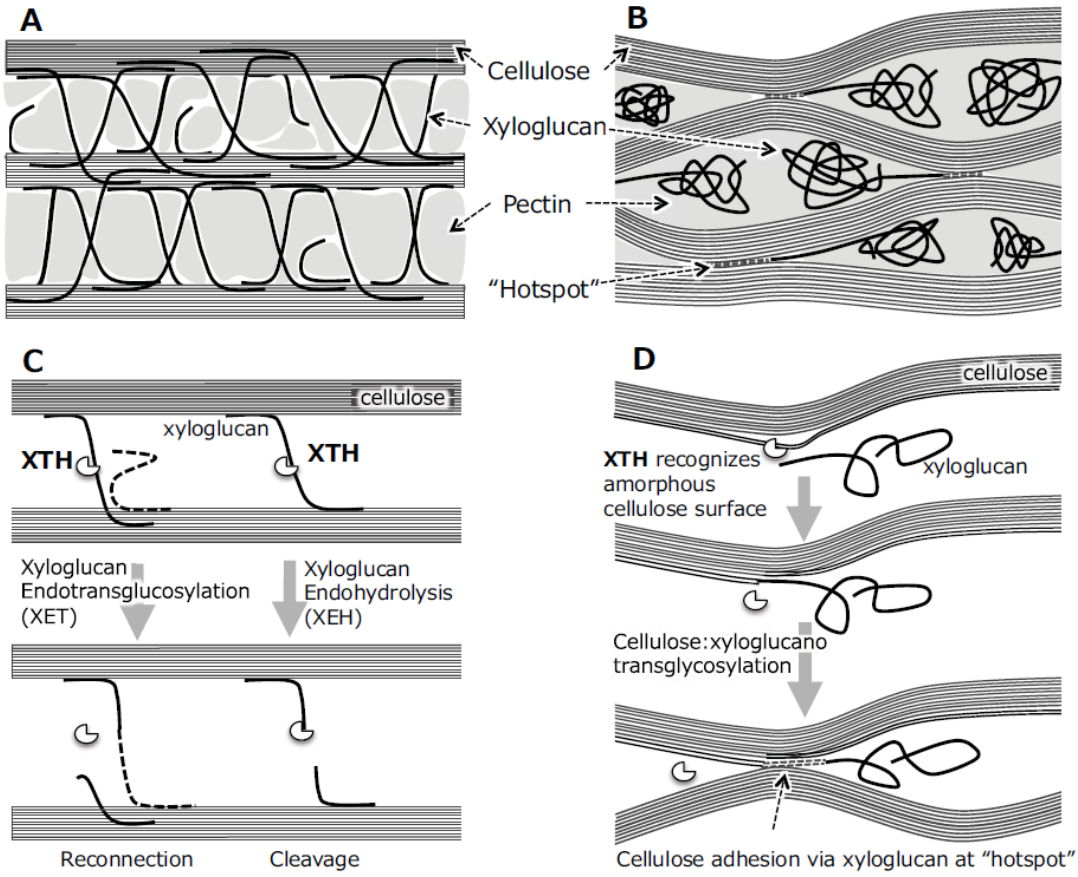

Figure 2.7 This model presented by Kazuhiko Nishitani (8th Plant Biomechanics International Conference, Nagoya, Japan) seeks to reconcile the (A) tethered-network model, (B) biomechanical hotspots model; with XTH functioning in (C) where XETs and XEHs 'cut and paste' the xyloglucan tethers; or (D) where XTH action functions to form 'tight adhesion' at cellulose and xyloglucan interaction sites (personal communication, John Moore).

Nevertheless the debates on the validity of either the 'tethered-network model' or 'biomechanical hotspots model' (Cosgrove 2014; Park and Cosgrove, 2015) do not influence the known compositional data on plant cell walls (Albersheim, 2010), and given the wide

acceptance of the tethered network model we use this as a basis for this thesis (clarified in later sections and chapters); here the main polymers are described as follows:

2.4.1 Cellulose

Cellulose is considered to be the backbone of primary cell walls (Pauly *et al*, 1999) and is synthesized by cellulose-synthases in the plasma membrane (Figs. 2.5-2.7). Cellulose is arranged in microfibrils and these are believed to provide the mechanical strength to the cell wall (Albersheim, 2010), it was also found to contain the regions of loosely arranged fibers and crystalline region which can resist enzymatic degradation as well as exist in amorphous state (Delmer, 1987). The microfibrils structure of cellulose is formed by many parallel homopolymer chains which consist of β-1,4-linked glucose monomers (Mutwil *et al*, 2008), and the degree of polymerization can be up to 6000 in higher plant cell walls (Delmer, 1987).

2.4.2 Hemicelluloses

Hemicelluloses are a 'catch-all' phrase for a heterogenic group of polymers (from primary and secondary cell walls) which have specific compositions that differs among various plant species, tissues, organs and cell types (Hayashi, 1989). In dicotyledonous plants, the main hemicellulose polymers are found to be xyloglucan (Hoffman *et al*, 2005), which consists of a backbone of β-1,4-linked glucan where 3 out of 4 glucose units are substituted with xylose at position 6 (e.g. the XXXG motif) (Rose *et al*, 2002). Other motifs are galactosylated and fucosylated such as XXFG and XLFG (Rose *et al*, 2002). Other polymers considered in this heteogenous group include those rich in xylose (e.g. xylans, arabinoxylans, glucuronoarabinxylans), mannose (e.g. mannans and galactomannans and those with alternating backbones; such as for mannose and glucose (e.g. galactoglucomannans) (Albersheim, 2010).

2.4.3 Pectin

Pectin is certainly recognized as the most complex of the plant cell wall polysaccharides (see Figure 2.8) (Albersheim, 2010). It consists of a large number of different monosaccharides and these sugars are linked to each other in various ways (Mohnen, 2008). The two main groups of plant cell wall pectins are homogalacturonans (HGs) and the rhamnogalacturonan polymers and side chains such as rhamnogalacturonan I (RGI) and II

(RGII) (Caffall and Mohnen, 2009, Atmodjo *et al*, 2013).

2.4.3.1 Homogalacturonan

Homogalacturonan (HG) consists of α-1,4-linked D-galacturonic acid (GalA) which forms a homopolymeric chain (Worth, 1967). In plants, some of GalA units can be methyl esterified to neutralise the charge of a region (i.e. block) of GalAs; in addition, the HG main chain can also be partially acetylated (Pilnik and Voragen, 1970). These ester groups change the solubility of HG, removal of methyl group can make the HG become more insoluble in acidic solution (Javis, 1982, 2011).

2.4.3.2 Rhamnogalacturonan I

Rhamnogalacturonan I (RGI) has a backbone of alternating repeating disaccharide units of rhamnose (Rha) and galacturonic acid (GalA) (up to 100 repeats)(Caffall and Mohnen, 2009). The rhamnoses can be substituted with side chains, including arabinans, galactans, arabinogalactans (type I and II) (McNeill *et al*, 1980, Mohnen, 2008) in various degrees. Arabinans consists of α-(1→5)-l-Ara chains with 2- and 3-linked arabinose or arabinan branching (Albersheim, 2010). Arabinogalactan type I contains β-(1→4)-d-Gal chains with α-(1→3)-l-Ara or arabinan branching at some of the Gal and arabinogalactan type II contain a β-(1→3)-d-Gal chain with β-(1→4)-d-Gal or arabinogalactan branching (Willats *et al*., 2001, Mohnen, 2008) however, it is still under discussion that if the GalA in RGI has been esterified with methyl ester (Schols and Voragen, 1994, Rihouey *et al*, 1995). RGI normally accounts 20-35% of pectin in plant (Albersheim, 2010).

2.4.3.3 Rhamnogalacturonan II

RG-II is usually a side chain attached to HG and consists of 12 different rare sugars, such as 2-O-methyl fucose,2-O-methyl xylose, aceric acid, 3-deoxy-D-lyxo-2-heptulosaric acid (KDO) and 3-deoxy-D-manno-2-octulosonic acid (DHA) (Albersheim, 2010). These sugar residues are interlinked by 20 different linkages (O'Neill *et al*, 1996). RG-II has a well preserved structure which is resistant to most of the pectinolytic enzymes. The content of RG-I in grape berry pectin was shown to be 3-fold higher than RG-II, but compositional analysis of red wine showed that there is more RG-II than RG-I in the wine soluble pectic polysaccharides (Vidal *et al*, 2001). This may be caused by the degradation of RG-I in wine fermentation processes. (Buffetto *et al*, 2014). However, RGII was not the target to study in this study, as we do not

have characterised monoclonal antibody for detecting this polymer in the analysis.

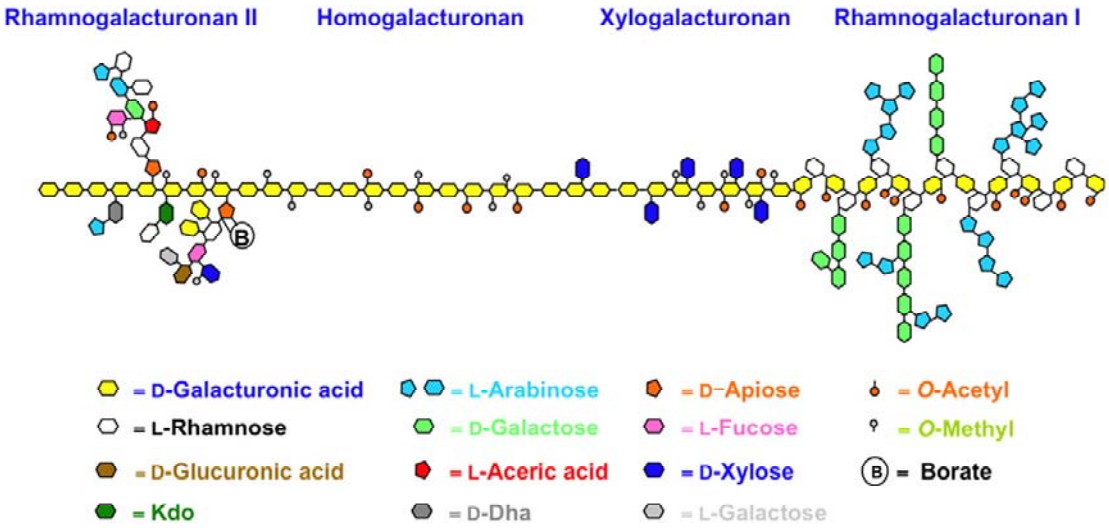

Figure 2.8 The schematic image of pectin main chain which generally supports the "smooth and hairy region model" (Harholt *et al*, 2010)

2.4.4 Cell wall glycoproteins

Beside the polysaccharides, plant cell walls also contain glycoproteins, and they are involved in many biological functions, such as growth, defense, environmental sensing and signaling (Hijazi *et al*, 2014). Two most commonly studied structural cell wall proteins are arabinogalactan proteins (AGPs) and extensins (Showalter, 1993).

2.4.4.1 AGPs

AGPs are rich in serine and hydroxyproline, and are decorated with arabinogalactan type II side chains (Tan *et al*, 2012). AGPs were found involved in a variety of physiological processes including reproductive development, embryogenesis, signaling, cell expansion and defense (Seifert and Roberts, 2007, Tan *et al*, 2012, Hijazi *et al*, 2014). In cell wall, AGPS were suggested to be covalently linked to pectin (Immerzeel, 2005).

2.4.4.2 Extensins

Extensins are structural proteins and are found to be highly repetitive HRGPs showing similar features as collagen that contain Tyr cross-linking motifs (Hijazi *et al*, 2014). In plants many unrelated proteins contain extensins repeat domains which makes the studying of the functions of extensins quite difficult (Lamport *et al*, 2011, Borassi *et al*, 2015).

2.5 Pectin polymer cross-linking

Pectin, as the most complex of natural glycopolymers, has the potential for many types of cross-linking interactions (Caffall and Mohnen, 2009). Firstly, the free carboxyl groups of HG were suggested to form an “egg-box” via calcium bridges in order to increase the rigidity of pectin (Grant *et al*, 1973, Schols and Voragen, 2002). These egg-boxes were found to be abundant in the middle lamella, especially at the intercellular junctions (Bush *et al*, 2001); secondly, the larger side chains RG-II can form dimers through a borate di-ester cross-links (Chormova and Fry, 2015), and such dimers have been implicated in a number of biological functions, such as structure and development (Fleischer *et al*, 1999, Bar-Peled *et al*, 2012), and two HG chains may link to each other through this RGII dimer as RGII has been shown covalently linked to the HG backbone as a side chain (Caffall and Mohnen, 2009). Pectin has not been found to be directly attached to the cellulose *in vivo*, however, the side chains (e.g. arabinans) on RGI was found to be associated with cellulose microfibrils, presumably via a hydrogen bonding using *in vitro* methods (Zykwinska *et al*, 2005, Marcus *et al*, 2008).However xyloglucan has been shown to be attached to pectin by presumably covalent means (Popper and Fry 2005; 2008).

Pectins are difficult polymers to study due to their heterogeneous natural complexity (Caffall and Mohnen, 2009). Different models have been developed to describe the associations among these pectic polymers. The “smooth and hairy region model” was supported by many studies, it suggested HG is interspersed with RGI to form the pectin backbone structure, and those neutral sugar side chains (i.e. arabinans and galactans) are attached to RGI (see Fig. 2.8, De Vries *et al*, 1982, Schols and Voragen, 1996, Coenen *et al*, 2007). In this model, the HG was called the “smooth region” and RGI was called the “hairy region”. However, a problem was pointed out regarding this model due to the low concentration of rhamnose that cannot explain the degrees of polymerization found (Vincken *et al*, 2003; Zhan *et al*, 1998, Prade *et al*, 1999). To solve this issue, another model was suggested where only RGI is the main backbone of pectin, with the arabinans, galactans and HGs attached as side chains (Vincken *et al*, 2003). More recently, a model combining these two ideas was developed, in which the HG is presented in the backbone with RGI, but also as side chains to RGI (Ralet and Thibault, 2009, Schols *et al*, 2009).

2.6 Methods for studying the plant cell wall

The analysis of plant cell walls is challenging due to its complex structure and dynamic nature (Popper, 2011). As the plant cell contains various biological molecules (i.e. nucleic acids, lipids, polyphenols, proteins etc.), the first step before analysis can begin is to prepare an extract enriched for cell wall polymers. To achieve this, a series of organic solvents are optimized to perform the sequential washing the hand ground (i.e. traditionally) or milled (i.e. efficient and more reproducible) frozen (liquid nitrogen) plant samples (see Moore *et al*, 2014), after freeze-drying this results in alcohol insoluble residue (AIR) which can be used for further chemical and biochemical analysis (Fry, 1988). A work-flow has developed (which can be optimized for high-throughput analysis) for analyzing the same AIR samples (see Figure 2.9) from a set of experiments which are divided into multiple directions (four in this case) for specific analytical techniques; these being (1) gas chromatography methods for sugar composition after acid hydrolysis (York *et al*, 1985); (2) ATR-FT-MIR spectroscopy for functional group chemistry (Kacurakova *et al*, 2000); (3) CoMPP (Carbohydrate Microarray Polymer Profiling) for polymer epitope abundance (Moller *et al*, 2007) and (4) mass spectrometry analysis after degradative techniques (usually enzymatic) (Lerouxel *et al*, 2002). Thereafter the datasets generated need to be analysed using various univariate and multivariate statistical approaches (Eriksson *et al*, 2013).

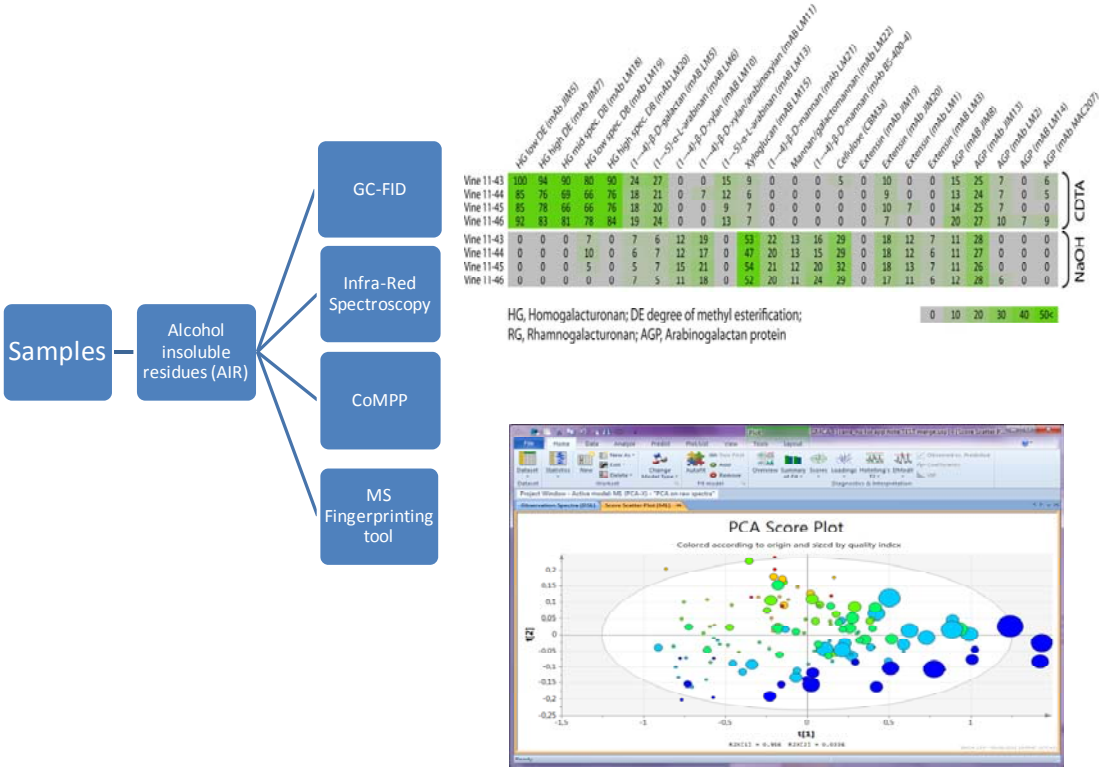

Figure 2.9 The high-throughput approach combining a series of cell wall analytical techniques (e.g. gas chromatography for sugar composition; FT-IR analysis for functional group chemistry; CoMPP for specific polymer epitope abundance (e.g. Heatmap); and OLIMP MS fingerprinting) for multivariate data analysis (e.g. PCA score plot) of many samples in scientific and industrial scale experiments (e.g. wine fermentations).

Historically, the primary tool for plant cell wall analysis is gas chromatography of sugar monomers released from acid hydrosylates of AIR which was developed by in by Albersheim *et al*. (1967). However, this bulk cell wall sugar chemistry information cannot fully answer questions at the polymer level; even though there have been studies that have worked on the prediction of polymer information from monosaccharide compositions (e.g. Gorshkova *et al*, 1996, Nunan *et al*, 1997, Arnous & Meyer, 2009), this is always inferred and not directly assessed. Cell wall oligosaccharides released by partial acid hydrolysis or enzymatic degradation can be analysed using a number of techniques, such as; (1) paper chromatography and paper electrophoresis (Fry, 1988; 2010), (2) Oligosaccharide mass profiling (OLIMP) combined with enzyme treatment where released material is analysed using matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) ((Lerouxel *et al*., 2002); as well as (3) polysaccharide gel electrophoresis

(PACE) which separate the derivatized oligosaccharides on polyacrylamide gels (Goubet *et al*, 2002). However, each of these analytical chemistry-based techniques have their own set of advantages and disadvantages; the use of a combination of techniques can provide the data from different angles to generate a more complete description of the cell wall compositional and structural information (Fig. 2.9).

On the polymer level, the use of monoclonal antibodies (mAbs) and carbohydrate binding module (CBMs) combined with high throughput microarray provides a fast and cost-effective way for cell wall profiling (Moller *et al*, 2007). This approach, termed comprehensive microarray polymer profiling (CoMPP), is shown in Fig. 2.10. Briefly, the process includes the extraction of AIR (using a sequential extraction into a CDTA and NaOH fraction), these fractions are then printed on nitrocellulose membranes and probed with mAbs and CBMs followed spot quantification using image analysis software. CoMPP is versatile in that new sets of mAbs and CBMs can be included or excluded providing a customizable profiling tool (Sørensen and Willats, 2011). The mAbs and CBMs commonly used in our analyses are provided in Table 2.1.

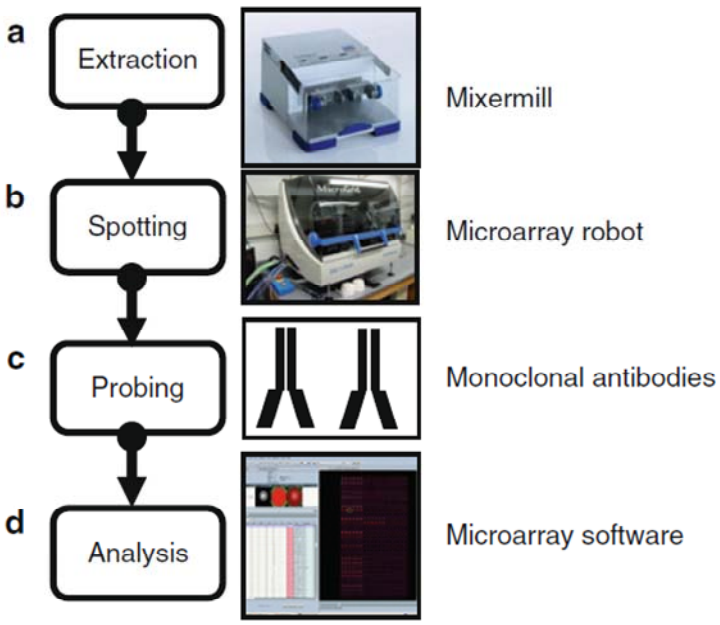

Figure 2.10 The standard process of comprehensive microarray polymer profiling (CoMPP) as illustrated and described in (Sørensen and Willats, 2011.)

Table 2.1. mAbs and CBMs used in CoMPP (Paul Knox Cell Wall Lab http://www.personal.leeds.ac.uk/~bmbjpk/ and http://www.plantprobes.net/index.php)

Category	mAbs/CBMs	Epitope recognition	Reference
HG	JIM5	HG with a low DE (mAb JIM5)	Knox et al, 1990; Clausen et al, 2003
	JIM7	HG with a high DE (mAb JIM7)	
	LM18	HG Partially methylesterified (mAb LM18)	Verhertbrugger et al, 2009a
	LM19	HG Partially methylesterified (mAb LM19)	
	LM20	HG Partially methylesterified (mAb LM20)	
	2F4	HG Ca2+ crosslinked (mAb 2F4)	Liners et al, 1989
	LM8	Xylogalacturonan (mAb LM8)	Willats et al, 2004
RGI	INRA-RU1	Backbone of rhamnogalacturonan I (mAb INRA-RU1)	Ralet et al, 2010
	INRA-RU2	Backbone of rhamnogalacturonan I (mAb INRA-RU2)	
RGI side chains	LM5	(1→4)-β-D-galactan (mAb LM5)	Jones et al, 1997
	LM6	(1→5)-α-L-arabinan (mAb LM6)	Willats et al, 1998
	LM13	Linearised (1→5)-α-L-arabinan (mAb LM13)	Verhertbrugger et al, 2009b
Mannan	LM21	(1→4)-β-D-(galacto)(gluco)mannan (mAb LM21)	Marcus et al, 2009
	LM22	(1→4)-β-D-(gluco)mannan (mAb LM22)	
Glucan Xyloglucan	BS-400-2	(1→3)-β-D-glucan (mAb BS-400-2)	Meikle et al, 1991
	LM15	Xyloglucan (XXXG motif) (mAb LM15)	Marcus et al, 2008
	LM25	Xyloglucan / unsibstituted β-D-glucan (mAb LM25)	Pedersen et al, 2012
Xylan/cellulose	LM10	(1→4)-β-D-xylan (mAb LM10)	McCartney et al, 2005
	LM11	(1→4)-β-D-xylan/arabinoxylan (mAb LM11)	
	CBM3a	Celulose (crystalline) (CBM3a)	Tormo et al, 1996
Extensins	LM1	Extensin (mAb LM1)	Smallwood et al, 1995
	JIM11	Extensin (mAb JIM11)	Smallwood et al, 1994
	JIM20	Extensin (mAb JIM20)	
AGP	JIM8	AGP (mAb JIM8)	McCabe et al, 1997
	JIM13	AGP (mAb JIM13)	Knox et al, 1991; Yates et al, 1996
	LM14	AGP (mAb LM14)	Moller et al, 2008
	LM2	AGP, β-linked GlcA (mAb LM2)	Smallwood et al, 1996

CoMPP has provided an effective way to profile and compare the differences between the cell wall samples, this is very useful in many applications. When combined with our workflow (Fig. 2.9) it has proved useful in validation studies on tobacco (Nguema-Ona *et al*. 2012) and grapevine leaves (Moore *et al*. 2014a); and grape berries (Moore *et al*. 2014b). CoMPP has been useful in applications such as studying comparative of differences in plant tissues and organs (Zhang *et al*, 2013), the study of plant cell wall evolution study (Fangel *et al.*, 2012), as well as the changes effected on cell walls during defense priming against potential pathogens (e.g. fungi) (Nguema-Ona *et al*, 2013).

2.7 Importance of plant cell wall studies in grape and wine research

Plant cell walls play various important roles in fields of grapevine physiology (e.g. ripening) and maceration during winemaking (see Moore *et al*, 2014b and Zietsman *et al*, 2015). As stated earlier the major challenge in winemaking is achieving an effective and efficiently

regulated extraction of beneficial oenological compounds from the berry. However, as most of these compounds are found to be localized in berry skins (shown in Fig. 2.11), long maceration times; alternative methods of wine enzymes are needed. Thus, many studies have been conducted to investigate the correlation between the cell wall compositional change and the release of these molecules (mainly by enzymes) (Ortega-Regules *et al*, 2008, Arnos and Meyer, 2010, Bindon *et al*, 2012, 2014, Romero-Cascales *et al*, 2012, Hernández-Hierro *et al*, 2014), but have resulted in relatively little in the way of new findings, e.g. (1) the extractability of these beneficial compounds are different among the cultivars as they have different thickness of the skin; (2) the cellulose and pectin content are suggested to be correlated to the anthocyanin extraction. Some research work has looked at the influence of berry ripeness (i.e. Cabernet Sauvignon and Tempranillo) on the general extractability, and have suggested there is a positive link between these two factors (i.e. more ripe berries extract more easily) (Gil *et al*, 2012). This is not a surprise, as a number of studies on the berry ripening have evaluated the changes of pectin and cellulose content during the ripening process (Nunan *et al*, 1997, Moore *et al*, 2014b). Furthermore, the studies performed on over-ripe Shiraz berries by Bindon *et al.* (2014) and later on Pinotage a two different ripeness levels (Zietsman *et al*, 2015a) have suggested that a higher cell wall porosity may be caused by the degradation of pectin polymers during the winemaking. In summary, previous studies have shown that the ripeness of grape berry often positively correlates to beneficial compound extraction, but can also be influenced by cultivar variation, the vintage effect, vine physiology and environmental factors. However a major weakness of most of these studies is that the information generated is mainly on the monosaccharide level, which can only provide indirect data on cell wall polymer composition and architecture..

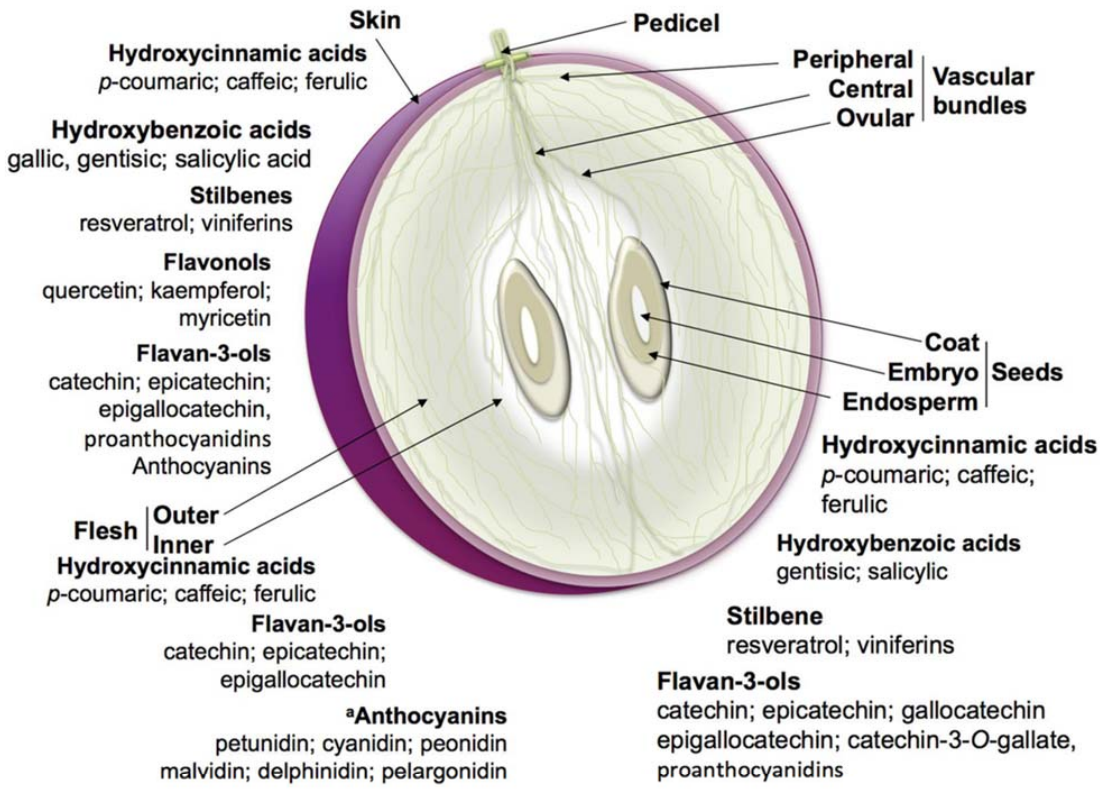

Figure 2.11 The main beneficial compounds localized in the berry tissues (Teixeira *et al*, 2013).

Although our knowledge of cell walls in grapes and wines still limited; polysaccharides in wine (via polysaccharide chemistry research over many years) have attracted significant attention regarding their contribution and impacts on the winemaking process (Dufour and Bayououe, 1999, Vidal *et al*, 2004, Chalier *et al*, 2005). Rhamnogalacturonan II (RG II) is reported to be the major grape-derived pectic polysaccharide (AGPs are also major components present in primarily red wine (Vidal *et al*, 2003); this has often been attributed to the lack of maceration enzymes able to degrade such a complex polymer (Pellerin *et al*, 1996). In its dimer form it strongly enhances colloidal particle size suggesting co-aggregation between the polysaccharides and tannin (Riou *et al*, 2002). During wine fermentation, the degrading enzymes break cross-links within the cell wall and reduce the ability of these pectic polysaccharides which bind to tannins (Doco *et al*, 2007). However, the excessive extraction of tannins into the wine could also produce undesirable astringent characters which need fining to improve wine quality (Fia *et al*, 2014). RG II has been proven to be a good fining agent to prevent the aggregation of tannins and is also able to contribute to wine colour stability and aging (Riou *et al*, 2002). Hence clearly grape-derived polysaccharides in

wine, on balance, probably exert more positive effects, particularly in the case of red wine.

Wine polysaccharides can be divided into two main groups: those originating from the grape cell wall, such as homogalacturonan oligomers (HG), Rhamnogalacturonan I and II (RG I and RG II), arabinans (A), arabinogalactans (AG), arabinogalactan proteins (AGP) (Vidal *et al*, 2003); and those derived from the microorganisms involved in the wine fermentation and processing, such as the mannoproteins (MP) released from yeast during the alcoholic fermentation and ageing on lees (Vernhet *et al*, 1999). During the entire wine fermentation process, these polysaccharides interact with each other or react with other molecules and contribute to the aroma and mouthfeel of the final wine (Moore and Divol, 2011). Not all the polysaccharides show the same behavior in wine as this depends on a number of factors, such as the quantity of polysaccharides compounds, as well as their structure, composition and distribution through the process (Vernhet *et al*, 1999, Vidal *et al*, 2004). A number of studies have been conducted on the contribution of each class of polysaccharide on defined wine characteristics (Chalier *et al*, 2005; Vidal *et al*, 2004; Dufour and Bayououe, 1999). They have showed that AGPs have great influence on the filtration during the wine making processes (Dufour and Bayououe, 1999), MPs could reduce the protein haze of white wines (Chalier *et al*, 2005) and RGI and RGII polysaccharides inhibit hydrogen tartrate crystallization (Vidal *et al*, 2004). The limitation in many of the current wine polysaccharide studies is that they are inferred from monosaccharide composition information (Apolinar-Valiente *et al*, 2014, Gil *et al*, 2015) so newer techniques are needed (as discussed in section 1.6 of this chapter), especially if more significant insights are to be gained from the role of enzymes in winemaking.

2.8 Pectinases

Considering the importance of the grape berry cell wall in winemaking (Moore and Divol, 2011), understanding more fully its degradation, the contribution of its derived polymers to the final wine's quality, and the efficacy plus commercial enzyme preparations are needed. As many previous studies have stated that berry cell wall is rich in pectin (Saulnier and Thibault, 1987, Lecas and Brillouet, 1994, Ortega-Regules *et al*, 2008), most enzyme

preparations on the market claim that pectinases are the main enzymes in these "cocktails" (http://www.laffort.com/en/products/enzymes). Many enzymes are involved in the pectin degradation of grape wine matrices, (Fig. 2.12). these enzymes can be classified into three main categories, namely; (1) glycoside hydrolases (E.C. 3.2.1, e.g. endo-polygalacturonase); (2) polysaccharide lyases (E.C. 4.2.2, e.g. Pectin lyase) and (3) carbohydrate esterases (E.C. 3.1.1, e.g. pectin methyl esterase) (Ranveer *et al*, 2005).

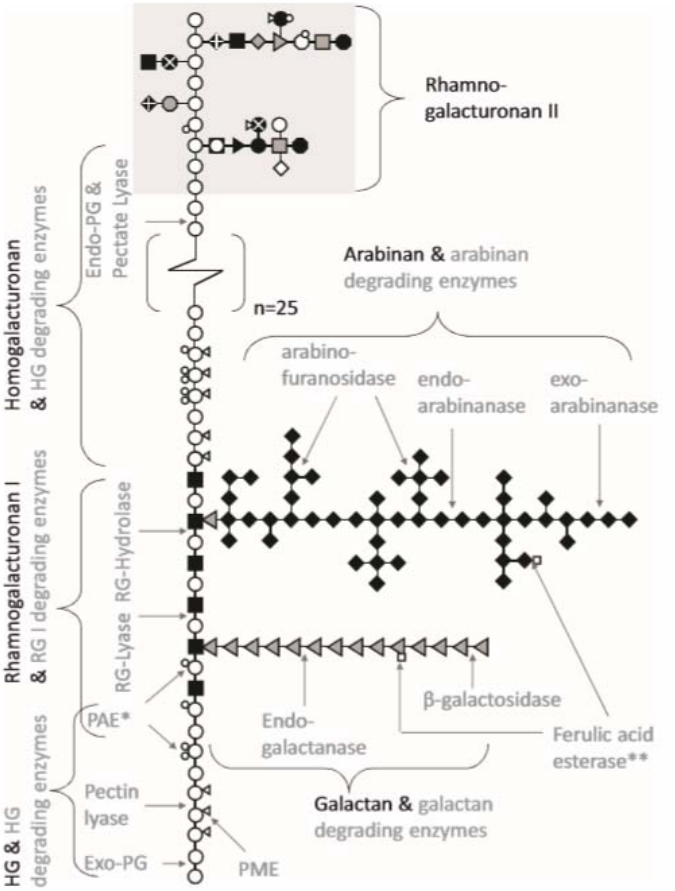

Fig. 2.12 The pectin structure (the smooth and hairy region model) and the mode of action of the pectinolytic enzymes. White circle: galacturonic acid, small white triangle: methyl-ester group, small white circle: acetyl groups, black square: Rhamnose, gray triangle: galactose, black diamond: arabinose, small white square: ferulic acid group. RGII is in the gray box. (from Kuhnel, 2011)

Five of these enzymes are able to work on degrading the HG main chain; these include the endo-polygalacturonases (EPG) and exo-polygalacturonases which cleave the bond between two un-esterified GalAs, but work on different locations on HG backbone (Bonnin *et al*, 2014). In contrast, pectin lyases (PL) have higher affinity to cleave the bonds between two esterified GalA residues (Schols *et al*, 2009). Pectin methyl esterases and pectin acetyl esterases will

work on removal of the ester groups from the HG main chain (Schols *et al*, 2009; Levesque-Tremblay *et al*, 2015). As another part of pectin main chain, RGI can be degraded by two enzymes: RG-lyases or RG-hydrolases (Mutter *et al*, 1998). For the side chains of RGI, arabinan can be degraded by a number of enzymes, include arabinofuranosidases and exoarabinanases which release the arabinose from the non-reducing ends of arabinan polymers (Chavez Montes *et al*, 2008), while endoarabinanases hydrolyse the arabinan chain backbone (Dunkel and Amado, 1995). There are two enzymes (endo-galactanases and β-galacosidases) that work on cleaving the galactan at different positions (Schols *et al*, 2009).

However, these pectin-degrading enzymes are variable in action and may be present in different compositions in the products, as they are 'considered' tailored for different applications (see Table 2.2 for a useful summary of well-known and widely used enzymes). However without a clearer understanding of grape berry cell wall architecture; and also how commercial enzymes and pure recombinant enzymes (acting singly or in combination) act under winemaking conditions; much of or knowledge is overly reliant on a 'black box' approach, without testable hypotheses.

Table 2.2. The properties and applications of some commercial available enzyme preparations for winemaking on the market (http://www.laffort.com/en/products/enzymes and http://www.oenobrands.com/en/our-brands/rapidase-wine-enzymes).

Commercial enzyme preparation	Properties	Application
Lafase HE Grand Cru	Pectolytic enzyme preparation, purified in CE for the production red wines that are rich in colouring matter and structured tannins, destined for ageing.	Maceration
Lafase Fruit	Purified pectolytic enzyme preparation for the production of fruity, colourful and round red wines.	Maceration
Lafase Clarification	Pectolytic enzyme preparation for must and wine clarification.	Clarification
Lafazym Press	Pectolytic enzyme preparation purified in CE for optimising pressing and extraction of aroma precursors during the production of white and rosé wines.	Pressing
Extralyse	Preparation of ß-(1-3) beta-glucanases and pectolytic enzymes, purified in CE for the improvement of wine filterability and ageing on lees.	Ageing
Lafazym Aroma	Betaglucosidase and pectinase preparation for revealing terpenic varietal aromas from their precursors during the production of aromatic white wines.	Ageing
Lafase XL Extraction	Liquid enzymatic preparation for red wine maceration and clarification	Extraction and clarification
Rapidase Extra color	Fast, early color extraction in red grape maceration	Maceration
Rapidase Batonnage	Fast and early release in ageing on lees of molecules contributing to mouthfeel	Ageing

As discussed previously, the analysis of enzyme action is often based on monosaccharide analysis (Apolinar-Valiente *et al*, 2014), or indirect parameters (such as the release of beneficial compounds e,g, pigments and tannins) (Ortega-Heras *et al*, 2012, Romero-Cascales *et al*, 2012) when using the commercial enzyme preparations (see Table 2.2). Recently newer approaches have been used which involved a more direct study on polymer level (based on CoMPP and polymer epitope abundance) which has provided much more insight into the complex cell wall degradation in wine conditions (Zietsman *et al.* 2015; first paper in wine with the recent developed cell wall approach). In this study Zietsman *et al*. applied three commercial enzyme preparations on berries harvested at two viticultural ripeness levels, and compared the information acquired from CoMPP analysis between the enzyme treated skin samples to untreated controls using a multivariate data analysis approach They found novel information regarding the manner in which the ripeness levels can influence the cell wall degradation process at the cell wall polymer level

2.9. Concluding remarks

In summary, the current wine industry is still facing a number of challenges where enzymes are important aids, including (i) the balance of the juice yield with consistent quality, (ii) more effective maceration techniques and (iii) enhanced clarification while (iv) increasing the diversity of wine styles available. However without a clearer understanding of grape berry cell wall composition and architecture; linked with the action of wine enzymes; much of the potential of enzymes in winemaking is left untapped and under-utilised.

In order for enzymes to be used efficiently we need a more comprehensive study of the complex nature of the grape berry cell wall as we are still reliant on partial, dated and indirect information inferred from other plant species. It is therefore crucial that a more comprehensive investigation on the grape berry cell wall changes during winemaking in the context of enzymatic degradation is performed. In this study, Cabernet Sauvignon, as one of the most widely planted cultivars, was chosen as model commercial and scientific system for investigation.

Hence, the application of high-throughput validated cell wall profiling tools (e.g. glycan microarray, i.e. CoMPP) in conjunction with enzymes; and multivariate data analysis, should provide significant new insights into wine grape cell wall research and simultaneously move the field of study forward into new directions for further research. This should also offer the opportunity to achieve a controllable and customized winemaking through the rational applications of enzymes in specific winemaking scenarios.

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Chapter 3

Research results

Dissecting the polysaccharide-rich grape cell wall matrix during the red winemaking process, using high-throughput and fractionation methods

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Dissecting the polysaccharide-rich grape cell wall matrix during the red winemaking process, using high-throughput and fractionation methods

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Abstract

Limited information is available on grape wall-derived polymeric structure/composition and how this changes during fermentation. Commercial winemaking operations use enzymes that target the polysaccharide-rich polymers of the cell walls of grape tissues to clarify musts and extract pigments during the fermentations. In this study we have assessed changes in polysaccharide composition/turnover throughout the winemaking process by applying recently developed cell wall profiling approaches to both wine and pomace polysaccharides. The methods included gas chromatography for monosaccharide composition (GC-MS), infra-red (IR) spectroscopy and comprehensive microarray polymer profiling (CoMPP) using cell wall probes. CoMPP performed on the concentrated soluble wine polysaccharides showed a fraction rich in rhamnogalacturonan I (RGI), homogalacturonan (HG) and Arabinogalactan proteins (AGPs). We also used chemical and enzymatic fractionation techniques in addition to CoMPP to understand the berry deconstruction process more in-depth. CoMPP and gravimetric analysis of the fractionated samples showed that the fermentation-derived pomace could be divided into a pectin-rich fraction (pulp tightly-bound to skins) containing HG, RGI and AGPs; and secondly, a xyloglucan-rich fraction (mainly skins). Interestingly this fraction was found to include pectins consisting of tightly-associated and highly methyl-esterified HG and RGI networks. A unique aspect is datasets suggesting that enzyme-resistant pectin polymers 'coat' the inner xyloglucan-rich skin cells. This data has important implications for developing effective strategies for efficient release of favourable compounds (pigments, tannins, aromatics, etc.) from the berry tissues during winemaking. This study provides a framework to understand the complex interactions between the grape matrix and carbohydrate-active enzymes to produce wine of desired quality and consistency.

Keywords

Cell wall

Fractionation

Grape pomace

Profiling

Pectin

Xyloglucan

Winemaking

Abbreviations

AIR: alcohol insoluble residue

AGP: arabinogalactan protein

CoMPP: comprehensive microarray polymer profiling

CBM: carbohydrate binding module

CDTA: cyclohexanediamine-tetra-acetic acid

EPG: endo-polygalacturonase

FT-IR: fourier transform – infrared spectroscopy

GC: gas chromatography

HG: homogalacturonan

mAb: monoclonal antibody

PME: pectin methyl esterase

RG: rhamnogalacturonan

Introduction

Grape berry composition has a direct influence on final wine quality (Hernandez-Hierro *et al.,* 2014). The physical breaking of the grape berries during the crush and the subsequent degradation of berry tissues through chemical and enzymatic reactions form the must (grape juice), the cap (mostly skins and pulp, which we define as de-seeded pomace) and sediment (mostly pulp and seeds) (Gonzalez-Neves *et al.,* 2010), which provides the fermentation matrix for wine production. The release of grape-derived compounds (particularly from the skins) contributes to the specific wine-style and the perceived quality of the wine (Busse-Valverde *et al.,* 2011). Wine industries apply commercial enzyme blends (mainly crude enzyme mixtures from fungi such as *Aspergillus* spp.) during alcoholic fermentation to aid the winemaking process and enhance quality parameters. For example, in red wine fermentations, enzyme mixes are included to support maceration (e.g. leaching of phenolic compounds from berry skin into must) (Canal-Llauberes, 1993). Enzymes are added to both red and white wine fermentations for the purpose of clarifying the wine, decreasing viscous polymers and improving free-run juice/wine volumes (Canal-Llauberes, 1993).

In essence, these enzymes are added to "work on" the berry tissues to disrupt and/or weaken the cell walls that contain the compounds of interest in the various cells of the berry skin and pulp tissues. These enzyme blends therefore contain a number of carbohydrate active enzymes (these include amongst others endo-polygalacturonase (EPG), pectin-lyase (PL), arabinanase, galactanase, rhamnogalacturonase (RGase), endo-glucanase (EG), xyloglucanase (XEG) and cellulases) which actively assist the breakdown of the grape cell walls of berry tissues, favouring the release of important compounds such as sugars, organic acids, anthocyanins, tannins and monoterpenes (Romero-Cascales *et al*, 2012). The structure(s) of the grape tissue cell walls are complex and still poorly understood at the polymer architectural level. Currently, we know that the grape berry skin cell walls consist of cellulose, hemicellulose and are particularly rich in pectin (Lecas and Brillouet, 1994; Moore *et al.*, 2014a; Saulnier *et al.*, 1987; Vidal *et al.,* 2001 and Zietsman *et al.*, 2015). This pectin component contains a number of polymers (homogalacturonan (HG), rhamnogalacturonan I (RGI), side chains such as arabinans and galactans, rhamnogalacturonan II RGII and arabinogalactan-proteins (AGPs)) (Arnous and Meyer, 2009; Moore *et al.*, 2014a; Ralet *et al.*,

2014) and was proposed to be associated with other cell wall polymers (cellulose and hemicellulose) (Doco *et al.*, 2003). The HG and RG1 backbones of plant pectins are often acetylated and methylesterified to various degrees while the RG1 side chains include various lengths of linear and branched galactans, arabinans and arabinogalactans (Fry, 2011). These different cell wall components undergo compositional changes during berry development (ripening) with pectin and hemicelluloses both showing degrees of depolymerisation (Moore *et al.,* 2014a; Nunan *et al.,* 1998). The ripeness levels of harvested grapes correlated with a greater degree of depolymerisation with overripe (27 Brix^{o}) versus ripe (23 Brix^{o}) in a study performed on Pinotage (Zietsman *et al.,* 2015). It was found that wine maceration enzymes were useful in ripe grapes where skin cell walls were much more intact than in overripe grapes where significant tissue polymer degradation had already taken place (Zietsman *et al.,* 2015).

Recent work on grape-and wine-derived cell wall polysaccharides almost exclusively rely on monosaccharide compositional analysis (i.e. total polysaccharide hydrolysis followed by chromatography of the main monomeric sugars released) (Arnous and Meyer, 2009; Bindon *et al.*, 2012; Mendes *et al.*, 2013). The advantage of using the CoMPP (Comprehensive Microarray Polymer Profiling) methodology (Moller *et al.*, 2007) is that polysaccharides are directly identified by virtue of their epitope abundance, albeit semi-quantitatively in a profiling approach (Persson *et al.,* 2011). Using monosaccharide composition analysis (York *et al.*, 1985) and CoMPP (Moller *et al.*, 2007) in conjunction with ATR-FT-MIR spectroscopy (Moore *et al.,* 2014b) allows for a more complete picture to be obtained of cell wall composition and turnover. These higher-throughput methods have been successfully applied in a comparative grape ripening study (Moore *et al.,* 2014a) and to monitor the effects of enzyme addition on grape skin cells walls during wine fermentation (Zietsman *et al.,* 2015). For example, in the work by Zietsman *et al.* (2015) it was observed that partial unraveling of skin cell walls occurred during fermentation demonstrating the value of the approach in detecting more subtle cell wall structural alterations than that achievable using a complete acid hydrolysis approach (Arnous and Meyer, 2009).

The aim of this study was to characterise the different matrices obtained from Cabernet Sauvignon grapes (Moore *et al.,* 2014a) undergoing a standard red winemaking procedure by

applying these higher-throughput methods (Moore *et al.,* 2014b). These matrices include the de-seeded pomace (processed to alcohol insoluble residue, AIR) at three different stages of fermentation (Brix^{o} 24, 12 and 0) and the final wine (concentrated to obtain the main polysaccharide constituents) obtained. Further to this a unique aspect of the study is the combination of profiling methods (Moore *et al.,* 2014b) with chemical and enzymatic fractionation protocols applied to the de-seeded pomace AIR in order to obtain an in-depth characterisation of the grape berry polysaccharide matrix as it underwent enzyme-assisted maceration and alcoholic fermentation.

2. Experimental

2.1. Grape sampling and processing during winemaking

The grape samples (*Vitis vinifera* cv. Cabernet Sauvignon) used in this study came from the Welgevallen experimental vineyard of the Department of Viticulture and Oenology, Stellenbosch University, South Africa. The vineyard is situated (33^{o}56'42"S, 18^{o}51'44"E), close to the Eerste River and composed of alluvial soils with light to medium texture and is arranged in a north-south row orientation. Vines are trained on a seven-wire vertical trellis system and are drip-irrigated. The berries have been harvested when the average Brix^{o} was around 24 (sugar content 275 g/l), ca. 150 kg of berries were harvested from 80 vines in total. Red wine was fermented followed the procedure in Zietsman *et al.* (2015). Briefly, the berries (3 biological repeats, 50 kg each in 50 litres buckets) were de-stalked and pressed and the must inoculated with *Sacchromyces cereviseae* VIN13 (Anchor Yeast, Cape Town, South Africa) at 0.2 g/l (following the manufacturer's suggestions). The DSM Rapidase pectinolytic clarification enzyme blend (mainly containing endo-polygalacturonases according to the product data sheets, http:\\www.dsm-foodspecialities.com) was added, following the recommended dosage for easier filtration and clarification at the end of fermentation. The fermentation ran for 8 days at 25 ^{o}C until the dry (sugar level determined by using the hydrometer, CDS Vintec, Germany). The residual sugar was lower than 5 g/l. The de-seeded pomace (grape residues) were sampled at 3 fermentation stages, i.e. at the start (Brix^{o}24, alcohol ca. 0 % v/v), middle (Brix^{o} 12, alcohol ca. 7 % v/v) and end (Brix^{o} 0, alcohol ca. 14 %

v/v) of fermentation. Pomace samples were collected by separating the cap from the seeds and must/wine. Then flash-frozen in liquid nitrogen and stored at -80^{o}C for later analysis, whereas the wine was racked and stored at -4 ^{o}C until the wine polysaccharides were concentrated and analysed.

2.2. Wine concentration and polysaccharide enrichment

The wine samples (alcohol ca. 14 % w/w) were filtered using an Amicon ultrafiltration system (Millipore, South Africa) with a 5 kDa cut-off filtration membrane (Sigma-aldrich, South Africa) to reduce ca. 80% of the original volume. Ice-cold absolute ethanol was then slowly added to the concentrated wine to precipitate the soluble polysaccharide polymers overnight; the precipitated residue was subsequently washed in organic solvents according to Moore *et al.* (2014b).

2.3. Cell wall material isolation and fractionation from pomace

Pomace (de-seeded) from each of the three stages was milled in liquid nitrogen using a Retsch Mixer Mill (30 round/minute, 30 second, Retsch, Haan, Germany). The liquid nitrogen cooled milled material was plunged into boiling 80% v/v aqueous ethanol for 15 minutes and then cooled, followed by centrifugation at 4000 rpm for 5 minutes. The pellets were sequentially washed in ethanol, methanol, chloroform and acetone according to the protocol used in Moore *et al*., (2014b) and Nguema-Ona *et al*., (2012), the liquid to solid ration is generally three to one (v/v) for each wash. This is noticeably different to Zietsman *et al*, 2015 who also used buffered phenol in the processing procedure for the grape skins. Processed samples were then re-suspended in a small volume of MilliQ water (just enough for re-suspend the samples) and freeze-dried to yield a powder-like material termed the alcohol insoluble residue (AIR) which were used in subsequent analytical steps.

2.4. Fractionation of pomace using chemical and enzymatic procedures

The pomace samples taken throughout the fermentation were fractionated chemically, whereas the pomace sample taken at the start of fermentation was both chemically and enzymatically fractionated; all the fractions generated were used for analysis according to

Selvendran *et al*., (1985). The fractionation scheme is provided in the legends of the relevant figures for ease of interpretation. The Enzymes used include Endo-polygalacturonanase (880 U/mg), *endo*-1,4-β-D-glucanase (70 U/mg) and xyloglucanase (130 U/mg), all enzymes from Megazyme.

2.5. Gas chromatography for monosaccharide composition analysis

The composition of nine main monosaccharides (Arabinose, Ara; Rhamnose, Rha; Fucose, Fuc; Xylose, Xyl; Mannose, Man; Galacturonic acid, GalA; Galactose, Gal; Glucose, Glc and Glucuronic acid, GlcA) of both pomace cell wall AIR, as well as the fractionated AIR samples were analysed as their trimethyl-silyl-glycosides derivatives after hydrolysis, using a gas chromatography method modified from York, Darvill *et al*. (1985). Briefly, the samples were hydrolysed with TFA (2 M) for 2 h at 110 ^{o}C; the liberated monosaccharides were converted to their methoxy form using 1 M methanolic HCl at 80^{o}C for 16 h. Silylation was performed to produce the derivatives which then be dissolved in cyclohexane. Gas chromatograph (Hewlett Packard 5890 series II) coupled to mass spectrometry was used to separate and analyse the various derivatives obtained. Each sample was analysed in triplicate. The peak of each sugar was integrated and quantified according to the retention time and the equation established by analysing sugar standards (5 concentration levels) from GC.

2.6. Infra-Red (IR) spectroscopy

AIR sourced from grape berry pomace were analysed using a NEXUS 670 Fourier Transform Infrared Spectroscopy instrument (Thermo Scientific, MA, USA) containing a Golden Gate Diamond ATR (attenuated total reflectance) accessory with a type II diamond crystal, the spectra between 4000 and 650 cm^{-1} were recorded with a Geon-KBr beam splitter and DTGS/Csl detector. Each measurement consisted of 128 co-added scans and the spectral data were processed using Unscrambler^{TM} (Camo$^{®}$ Inc., USA) and SIMCA (Umetrics).

2.8. Comprehensive Microarray Polymer Profiling (CoMPP) analysis

CoMPP employs a set of monoclonal antibodies and carbohydrate binding modules to detect the glycan epitopes of different cell wall polymers (Figure 1C), and showed their

relative abundance in the heatmap. It provides the information on polysaccharide occurrence directly rather than using monosaccharide composition to indirectly infer polymer presence. In this study, grape pomace AIR and their gravimetric fractions were used to perform the CoMPP analysis as described in Moller *et al*. (2007). All analysis were done in triplicate and the values shown in the heatmap represent the mean values, the highest signal was always set to 100 and all other data adjusted accordingly.

2.9. Univariate statistics

Univariate statistical analysis was done (ANOVA, with P = 0.05) under the guidance of Prof. Martin Kidd from the Centre for Statistical Consultation at Stellenbosch University using Statistica 10 (StatSoft Southern Africa - Analytics, Sandton, South Africa).

3. Results and discussion

3.1 Profiling of total AIR of berry pomace and wine

Gas chromatography (GC) techniques (GC-FID and GC-MS) have been predominantly employed to determine the composition of cell wall derived monosaccharides in grapevine and wine, as well as follow their turnover during grape berry development (Brummell, 2006; Vicens *et al.*, 2009; Moore *et al.*, 2014a), following wine fermentation (Dols-Lafargue *et al.*, 2007) and during the aging of red wine (Doco *et al.*, 1999). In this study, GC-MS has been performed on total AIR sourced from grape de-seeded pomace sampled throughout the fermentation process, as well as the concentrated wine soluble polysaccharides in order to acquire information on bulk glycopolymer changes during winemaking. The unique aspects of this study include evaluation of the unravelling (Zietsman *et al.*, 2015) of the berry tissues (fermented matrices at different stages) analysed using a useful combination of profiling techniques that have now been established for grape berry cell wall analysis (Moore *et al.*, 2014b). These techniques were applied in this study to analyse (1) the AIR sourced from grape pomace after crushing and during fermentation and maceration; (2) the polymeric composition in the final wine; and (3) the fractionation of the grape pomace AIR (chemically and enzymatically). The application of CoMPP methodology in combination with chemical

fractionation has not been used to study berry cell walls before and provided informative insights as outlined below.

The monosaccharide composition (w/w in AIR) analysis of grape pomace AIR stages post-crush revealed an abundance of GalA (ca. 280-240 ug/mg) for all three fermentation stages analysed (Figure 1A), with the presence of Ara (ca. 58 ug/mg), Gal (ca. 53 ug/mg) and Rha (ca. 13 ug/mg) at the start of the fermentation, (i.e. fresh grapes crushed but not fermented). This confirms that the grape pomace matrix is pectin-rich, consisting of homogalacturonan (HG), rhamnogalacturonan I (RGI), RGI side chains and probably low levels of RGII (although RGII was not explicitly tested for). The presence of other sugars (Xyl ca. 30 ug/mg, Man ca. 33 ug/mg, Glc ca. 21 ug/mg, and Fuc ca. 20 ug/mg) at low levels suggested that xyloglucan, mannan, xylan and glucans may also be present. Fermentation (in the presence of macerating enzymes) caused a marked decrease of GalA, Gal and Ara and a slight increase in Xyl and Glc, indicating that de-pectination had occurred.

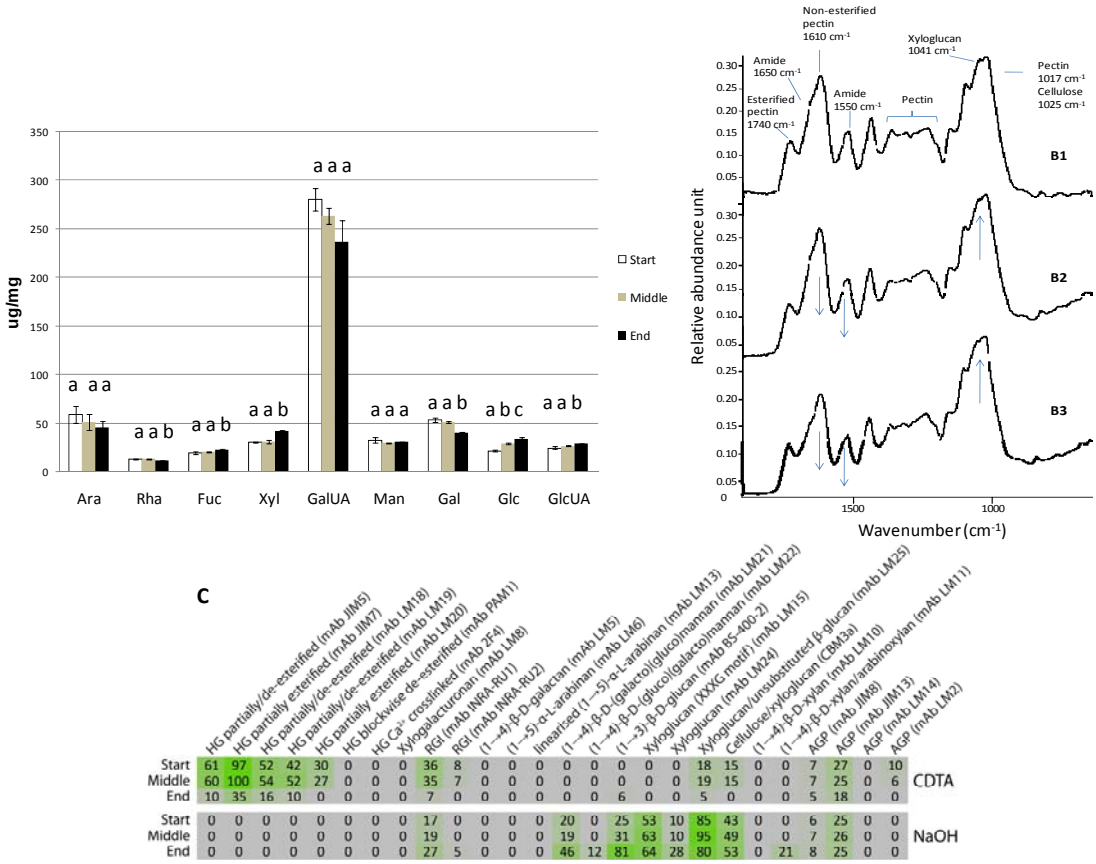

Figure 1. Analysis of alcohol insoluble residues (AIR) sourced from pomace from three fermentation stages (start, middle and end). **A:** Monosaccharide composition analysis (w/w in AIR, expressed in

ug/mg). Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats; different letters (a, b and c) indicate a significant difference for a specific sugar, between the different stages (95% confidence level, ANOVA). B: Fourier transform infrared (FT-IR) spectra generated from the AIR samples at different fermentation stages, start (B1), middle (B2) and end (B3). C: CoMPP (Comprehensive Microarray Polymer Profiling) analysis; the heatmap shows the relative abundance of plant cell wall glycan associated epitopes present in grape pomace material and colour intensity is correlated to mean spot signals. Sequential extractions were carried out with CDTA and NaOH; and the extracted material spotted onto nitrocellulose which was probed with sets of antibodies and carbohydrate binding modules. The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data set was set to 100 and other data adjusted accordingly. A cut off value of 5 was imposed.

Supporting these data; ATR-FT-MIR spectroscopy which provides a fast and convenient method to detect polysaccharide functional group chemistry, provided a similar overall picture of fermentation with enzyme induced perturbations (Figure 1B). Inspection of the wavescan spectral profiles revealed many overlapping resonance bands associated with cell wall polymers (Kacuráková *et al.*, 2000) at each fermentation stage (Figure 1B). All spectra consisted of a number of maxima; these being ascribed to esterified pectin (1740 cm^{-1}), amide (1650 cm^{-1}), non-esterified pectin (1610 cm^{-1}), and amide (1550 cm^{-1}), and a maxima which contains a number of polymers (xyloglucan, 1041 cm^{-1}, cellulose, 1017 cm^{-1} and pectin at 1025 cm^{-1}). It was also noted that all spectra were very similar to each other which confirmed the similar monosaccharide composition profile of the total AIR (Figure 1A). It also reflects that bulk chemistry did not alter dramatically in the de-pectinated pomace AIR. Figure 1B shows the representative spectra from the three stages (start B1, middle B2 and end B3). Visual inspection indicates a decrease in the spectra from middle to end of fermentation in the region 1600-1630 cm^{-1}. This region is assigned to non-esterified pectin, therefore indicating that de-pectination occurred in later stages of the fermentation, as was observed with the GC-MS analysis (Figure 1A). There was no significant change in the 1740 cm^{-1} maxima between stages, suggesting that de-esterification did not take place to any great extent during the fermentation. Additional differences noted in the spectra include a decrease during fermentation in the region 1650 $cm^{-1}$and 1550 cm^{-1} which correspond to the amide groups (bands I and II) present in proteins. A similar increase was found in the maxima regions 1041-1025 cm^{-1} which correlates with the cellulose-xyloglucan network. This data

might suggest that pectin-protein polymers were being peeled off during the fermentation process, leading to the exposure of the xyloglucan-cellulose polymers for detection by ATR-FT-MIR spectroscopy.

The information obtained using gas chromatography and infrared spectroscopy provides data on bulk chemical composition and based on these data we could infer that some pectin and protein polymers had been extracted from the pomace during the wine fermentation, similar to the results found by Zietsman *et al.*, (2015). However these methods do not permit accurate identification of the polysaccharides undergoing these changes in the pomace and wine matrix, respectively. The use of CoMPP methodology, which has now gained more widespread use and exposure in the plant cell wall community (Moller *et al.*, 2007; Nguema-Ona *et al.*, 2012; Moore *et al.*, 2014a; Moore *et al.*, 2014b; Zietsman *et al.,* 2015), provides direct analysis of the polysaccharides by virtue of their glycan epitopes (e.g. the mAb LM15 recognises XXXG motif in xyloglucan, Albersheim *et al.*, 2011) rather than the monosaccharides produced after acid hydrolysis (e.g. GC-MS). The method is based on the use of monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) to probe AIR samples in a microarray format, the data is reported based on the relative epitope abundance of different polymers in the samples. The methodology also consists of fractionating the AIR samples into a CDTA-soluble fraction first and thereafter a NaOH-soluble fraction which are printed and then probed with mAbs and CBMs (Figure1C) in order to produce a visual heatmap. The heatmap in Figure 1C shows datasets for pomace obtained throughout the fermentation process, fractionated with CDTA and NaOH. For the CDTA fractions sourced from total AIR (pomace) the main epitopes present include those of HG (recognised by mAbs JIM5, JIM7, LM18, LM19 and LM20), xyloglucan (mAb LM25), cellulose (CBM3a) and AGPs (mAbs JIM8, JIM13, LM2). Two new mAbs INRA-RU1 and INRA-RU2 (developed in INRA, Nantes, Ralet *et al.*, 2010) which both recognise the backbone of RG I was also used in this analysis. The mAb INRA-RU1 showed a strong signal in the CDTA fraction. The relative abundance of all of the HG epitopes decreased markedly from the middle-to-the-end of fermentation (Figure 1C) suggesting that the degradation of pectic polysaccharides has taken place. Further to this, the mAb INRA-RU1, mAb LM25, CBM3a and

mAb JIM13 also showed a similar decrease in epitope abundance (Figure 1C). The NaOH fraction was composed of a significant amount of hemicellulose, based on the binding of mAbs LM21 (mannans), BS-400-2 (glucans); as well as mAbs LM15, LM24 and LM25 which all recognise xyloglucan. Cellulose was also shown to be present by the binding of CBM3a. Interestingly, the mAb INRA-RU1 which binds to RG1 and the mAbs JIM8 and JIM13 for AGP epitopes were also present in this fraction. The mAbs LM21 (mannans), BS-400-2 (glucans) and LM24 (xyloglucan) all showed a marked increase from the middle-to-end of fermentation. Overall it was interesting to note that RG1 (mAb INRA-RU1) and AGP (mAb JIM13) epitopes were present in both the CDTA and NaOH fractions, possibly suggesting that two populations of these polymers exist in the grape berry cell wall. The high abundance (increase from middle to end of fermentation) of mAb BS-400-2 (glucans) likely suggests the formation of callose during the wounding and maceration of grapes during winemaking; this was also found in Zietsman *et al.*, (2015). It is possible that the glucans could also be yeast derived, due to yeast settling after fermentation. Another interesting observation was although GalA did not decrease dramatically, but there was a significantly larger drop in abundance for most HG epitopes at the end of fermentation. When Zietsman *et al.*, (2015) employed different commercial enzyme preparations under winemaking conditions (albeit in Pinotage grapes) and analysed those treated skin AIR samples after fermentation using CoMPP, it was found that the abundance of HG of enzyme treated pomace was higher than those of non-enzyme-treated pomace at the end of fermentation. Zietsman *et al.*, (2015) proposed a hypothesis whereby unravelling of the cell wall by enzyme treatment increased the exposure of the “hidden” HG epitopes. In this case enzyme treatment and analysis revealed a removal of HG epitopes at the end of fermentation consistent with a process of de-pectination.

During the crush and subsequent fermentation a significant amount of pulp and soluble polysaccharides and proteins are known to be released into the aqueous milieu which is separated from the pomace at the pressing stage of red winemaking. In order to analyse the wine-soluble polysaccharides present in the final wine at the end of fermentation a concentration and precipitation approach was used. Wine was concentrated by ultra-filtration and precipitated with ice-cold ethanol (Dols-Lafargue *et al.*, 2007); the

extraction yield of soluble polysaccharides was ca. 400 mg/litre, which corresponded with similar results in other studies on different cultivars (Merlot and Tempranillo) fermented in the absence of commercial maceration enzymes (Dols-Lafargue *et al.*, 2007; Guadalupe and Ayestaran, 2007). GC-MS analysis on the wine polysaccharides revealed a substantial amount of GalA (ca. 200 ug/mg), as well as Ara (ca. 65 ug/mg) and Gal (ca. 72 ug/mg) (Figure 2A), showing that the wine soluble polysaccharides extracted from berry tissue cell walls were rich in pectin components (HG, RGI and probably RGII, Vidal *et al.*, 2003). Interestingly, the most abundant monosaccharide was mannose (ca. 205 ug/mg), which could originate from mannans in the grape and yeast, as well as mannoproteins present in yeast cell walls (Zietsman *et al.*, 2015). Similar results concerning mannose were found in several previous studies on other cultivars (Merlot in Dols-Lafargue *et al.*, 2007; Tempranillo in Guadalupe and Ayestaran, 2007; Carignan noir in Doco *et al.*, 1999). In our data, the concentration of Fuc, Xyl, Glc and GlcA (< 30 ug/mg) was very low, albeit present in the wine soluble polysaccharides, in addition to the abundant pectins. The corresponding CoMPP analysis (Figure 2B) on the wine soluble polysaccharides showed that the CDTA fraction displayed epitopes associated with HG (mAbs JIM5, JIM7, LM18 and LM19), RG1 (mAb INRA-RU1 and RU2), xyloglucan (mAb LM25) and also AGPs (mAb JIM8, JIM13 and LM2). This the first report as far as we are aware of showing direct evidence of the presence of HG in wine soluble polysaccharides by using CoMPP; the pre-concentration step might have been a positive contributing factor, as was the sensitivity afforded by CoMPP probes. It confirmed several previous studies which showed that pre-concentration avoids under-estimation of HG in red wine (Doco *et al*., 1999; Dols-Lafargue *et al.*, 2007). In contrast the NaOH fraction showed only epitopes associated with mannans (mAb LM21), xyloglucan (mAbs LM15 and LM25), cellulose (CBM3a) and AGPs (mAbs JIM8 and JIM13). Thus the analysis of total wine polysaccharides generally supports the monosaccharide composition data (Figure 2A) which indicated an abundance of HG (ca. 200 ug/mg GalA), RG1 and AGPs (presence of Rha, Gal and Ara). The high Man content (ca. 205 ug/mg) was not reflected in the CoMPP dataset, supporting the hypothesis that the mannose is probably of yeast origin (i.e. mannoproteins, Moore and Divol, 2011).

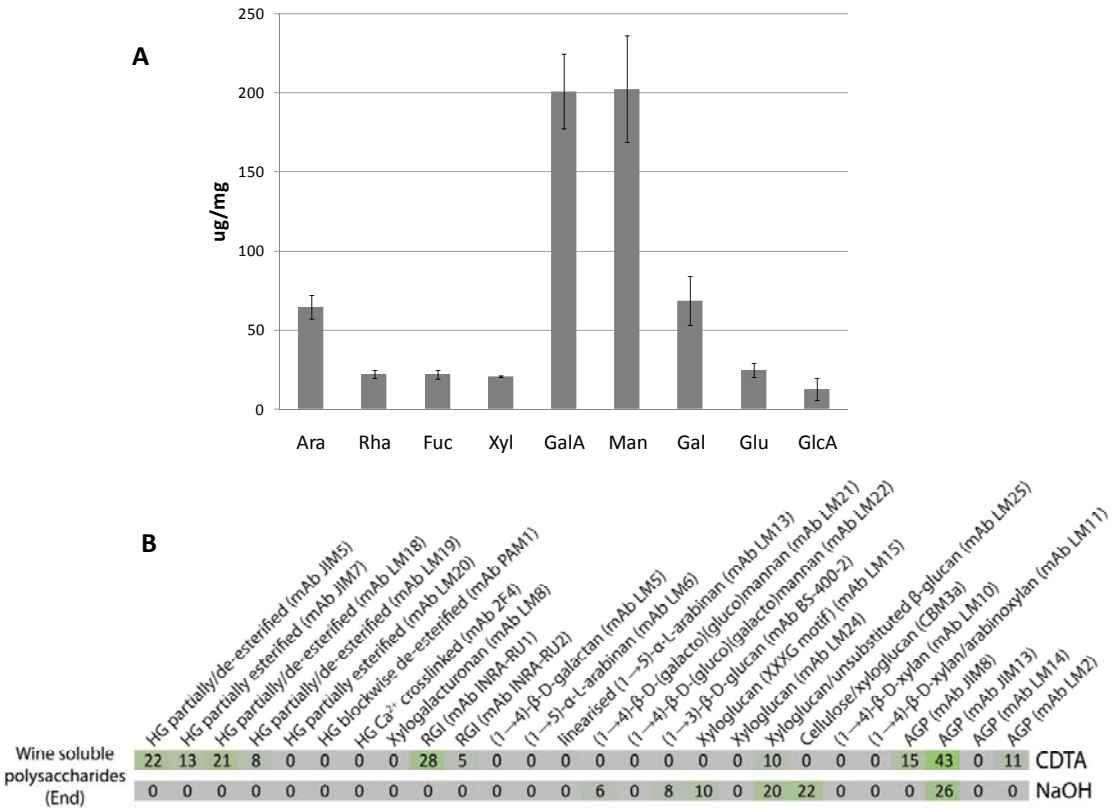

Figure 2. Analysis of alcohol insoluble residues (AIR) from soluble polysaccharides in concentrated wine (20% original volume) at the end of fermentation (ca. 14% alcohol, ca. 5 g/l residual sugar). **A:** Monosaccharide composition analysis (w/w in AIR, ug/mg). Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats. B: CoMPP (comprehensive Microarray Polymer Profiling) analysis; the heatmap is a sub-map of figure 1C, thus, the epitope abundance was adjusted according to the highest signal in the heatmap in figure 1C.

3.2 Chemical fractionation of pomace AIR

CoMPP provides in-depth datasets on polysaccharide abundance, however, the multi-layered complex architecture of the cell wall may result in partial masking of epitopes in total AIR (e.g. pectin masking hemicellulose and cellulose polymers, Marcus *et al.*, 2008) even though CDTA and NaOH fractionations are applied. We felt further analysis was warranted on the pomace AIR samples. The total grape pomace AIR (one gram from 3 fermentation stages) were fractionated chemically (using a similar procedure to Selvendran *et al.*, 1985) (fractionation scheme shown in Figure 3) to provide additional information on the content and composition of the various grape pomace cell wall layers. Gravimetric data (expressed as percentage dry weight) of chemical fractions is shown in Figure 3, however, the

total mass of all fractions from each fermentation stage are all over 1 gram (1.2 g at start, 1.18 g at middle and 1.12 g at the end) which means the extra chemicals kept in the fraction(s). It is important to note that sodium carbonate addition may have resulted in the formation of oligomeric carbohydrate salts of calcium and sodium which were large enough to be retained by the 5 kDa membrane and/or the salts precipitated during the dialysis due to the 4ºC temperature regime used. We are of the opinion that the rather high sodium carbonate fraction weight may be due to these reasons and may not accurately reflect the polysaccharide content. Berries, and hence pomace, should at ripe stages (e.g. Brix 24) have a significant amount of de-polymerised pectin (Vicens *et al.*, 2009). A large amount of de-polymerised pectin is probably removed during the crushing and fermentation stages ending up in the wine (see Figure 2A and 2B). The hot sodium acetate buffer extracted ca. 18% (0.22 g), the chelating reagent (CDTA) extracted only ca. 2.7% (0.03 g), the Na_2CO_3 fraction comprised ca. 34% (0.41 g) of the AIR. It was noted that the composition of the hot buffer (ca. 18-11%, 0.22-0.12 g) fraction decreased, whereas the KOH fraction (ca. 13-19 %, 0.16-0.21 g) increased markedly, in pomace AIR through the fermentation. The residue consisted mainly of cellulose (data not shown).

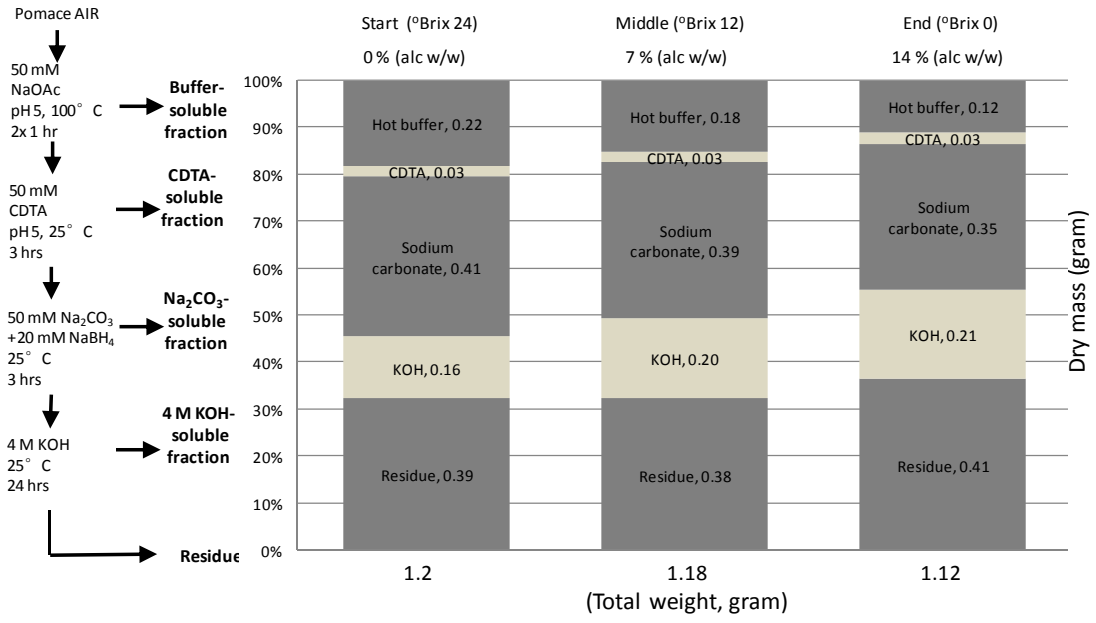

Figure 3. Chemical fractionation flow diagram and the gravimetric analysis of the chemically-extracted fractions from grape pomace AIR sourced from different fermentation stages (start, middle and end).

In order to ascertain the main wall polymers in each fraction, GC-FID was performed on the fractions to quantify the monosaccharide composition (Figure 4). The hot buffer fraction (consisting of 18 to 11% (w/w) of the total AIR) was found to be pectin-rich with a slight decrease in GalA extracted from middle (ca. 300 ug/mg) to the end (ca. 250 ug/mg) of fermentation. Ara and Gal also comprised relatively higher proportions (ca. 80 ug/mg and 60 ug/mg) compared to other less abundant sugars (Rha ca. 40 ug/mg, Fuc ca. 20 ug/mg, Xyl ca. 50 ug/mg, Man ca. 50 ug/mg). The CDTA fraction only accounted for ca. 2.7 to 2.5% of the total dry weight (Figure 3) and was comprised of elevated GalA levels (140 ug/mg), suggesting some pectin was extracted. Again de-esterified and de-polymerised pectin was most probably removed during the crushing and fermentation (with enzyme addition) already. The sodium carbonate fraction (accounted for ca. 34 to 31% (w/w) of AIR) consisted of almost equi-molar % levels of all nine monosaccharides with Xyl, Man, GalA, Gal, Glc and GlcA; compared to Ara, Rha and Fuc which were slightly lower. The KOH fraction represented ca. 13 to 19% (w/w) of the total AIR. These fractions showed high levels of Xyl (ca. 170 ug/mg) and Glc (ca. 220 ug/mg) suggesting that the main polysaccharide in the KOH fraction was probably xyloglucan with additional hemicellulose-related polymers. The presence of Ara (ca. 90 ug/mg) and Gal (ca. 70 ug/mg), suggests either branched pectin (RGI side chains) and/or AGPs present. The increase of Man (ca. 50 to 80 ug/mg) and Glc (ca. 220 to 310 ug/mg) suggested that glucomannans are also present, similar to the findings of Mendes *et al.*, (2013) when they worked on the Portuguese cultivar Touriga Nacional pomace.

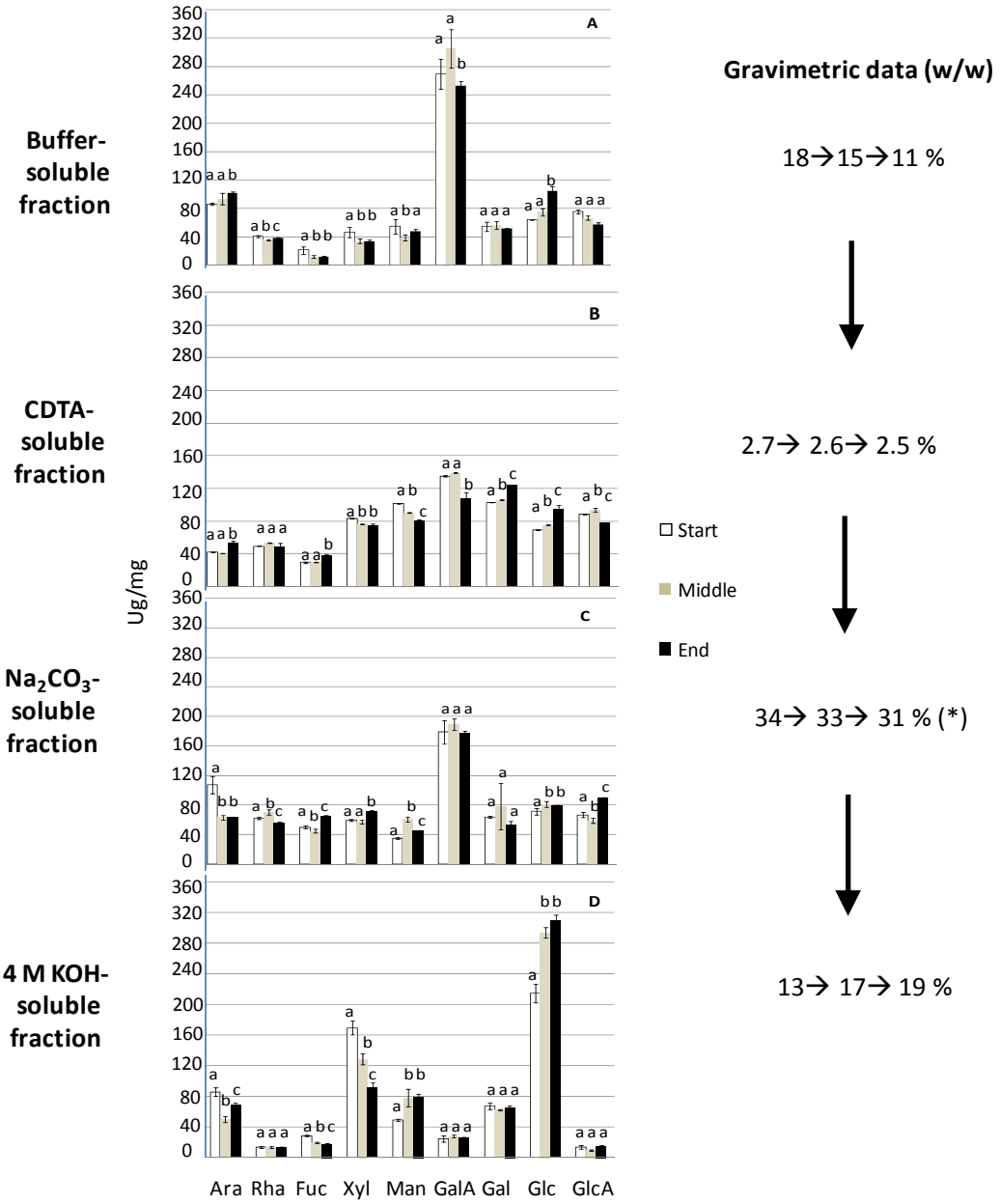

Figure 4. Monosaccharide composition (w/w in AIR, ug/mg) of chemically extracted fermentation-derived pomace fractions (hot buffer extract, CDTA extract, Na_2CO_3 extract, KOH extract). Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose, GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats; different letters indicate a significant difference for a specific sugar, between the different stages (95% confidence level, ANOVA). (*) indicates the gravimetric data of sodium carbonate fraction, which is considered an over-estimated value due to the presence of oligomeric salt.

In addition to the monosaccharide composition analysis by GC-FID; CoMPP was used to analyse all of the fractions so as to provide information on polysaccharide occurrence (Figure 5). Each fraction from the chemical fractionation process was extracted sequentially with

CDTA and NaOH, spotted onto membranes and then probed with a series of antibodies to create a heatmap. The heatmap in Figure 5 shows each fraction (i.e. Hot Buffer; CDTA, Sodium Carbonate and KOH); taken from the start, middle and end of fermentation; and further sub-divided into a CDTA and NaOH fraction following the CoMPP methodology (Moller *et al.*, 2007). Further to these four sections of the CoMPP probe sets were categorised; column A for HG epitopes, column B for RGI and side chains mainly, column C for xyloglucan, cellulose, mannans and xylans, and column D for AGP epitopes.

The Hot Buffer fraction, accounting for 18 to 11% (w/w) of the total AIR , showed (Figure 5) that the CDTA extracted HG which was detected by JIM 5, JIM7, LM18 and LM19; RGI (INRA-RU1 and INRA-RU2); arabinans (LM6 and LM13); galactans (LM5); glucans and xyloglucan (BS-400-2 and LM25) and AGPs (JIM8, JIM13, LM14 and LM2). Most signals showed a decreasing trend as fermentation proceeded (confirming the GC-MS datasets (GalA levels in Figure 4); except the glucan probe (BS-400-2) which increased and the xyloglucan mAb LM25 which remained constant. For the NaOH extract of the Hot Buffer fraction probes for mannans (LM21), glucans (BS-400-2), xyloglucan (LM15 and LM25) and an AGP epitope recognised by mAb JIM13 was highlighted. Most probes showed an increasing trend as fermentation proceeded. The CDTA fraction, only accounting for ca. 2.7 to 2.5% of the total dry weight revealed a number of epitopes, including HG (JIM5, JIM7, LM18, LM19 and LM20), RGI (INRA-RU1 and INRA-RU2, Ralet *et al.*, 2010), AGP (JIM8 and JIM13) which also seemed to corroborate the GC datasets (Figure 4 showing elevated levels of GalA). The NaOH extract of the CDTA fraction revealed various hemicellulose epitopes (LM21 for mannans, LM15 and LM25 for xyloglucan, and BS-400-2 for glucans). The sodium carbonate fraction (Figure 5), which we believe is a significant over-estimation at ca. 30% (w/w) due to salt contribution to the gravimetric data, only really showed epitopes for RG1 (mAb INRA-RU1) and AGPs (mAb JIM8 and JIM13) in the CDTA extract. The NaOH extract only showed some xyloglucan (mAb LM25) and cellulose (CBM3a) present. Finally the KOH fraction, contributing ca. 13 to 19% (w/w) of the total AIR was found to be particularly rich in a range of epitopes (Figure 5). The CDTA extracts revealed the presence of pectin epitopes specifically HG (mAbs LM18 and LM19 mainly) and RGI (mAbs INRA-RU1, INRA-RU2, mAbs LM5, LM6 and LM13). The

presence of LM18 and LM19 signals suggested de-esterification by the KOH and this is supported by the observation of two epitopes (HG blockwise de-esterified, PAM1 and Ca^{2+} cross-linked, 2F4) which did not show up in the earlier fractions. It is known that grapes have a high degree of methyl esterification of pectins in ripe grape berries (ca. 50%, Nunan *et al.*, 1998) which must have undergone de-esterification (saponification) through the KOH addition, and then the formation of Ca^{2+} bridges between the de-esterified HG chains. A rich abundance of mannan (LM21 and LM22), glucan (BS-400-2), xyloglucan (mAbs LM15, LM24 and LM25), xylan (LM10 and LM11) and cellulose (CBM3a) were found in the CDTA and NaOH extracts. Finally all four AGP epitopes (mAbs JIM 8, JIM13, LM2 and LM14) were recognised in the KOH fraction. In general, most probe signals showed a decreasing trend in intensity as fermentation proceeded. In essence this CoMPP dataset strongly supports the gravimetric (Figure 3) and the GC-MS data (Figure 4) which shows that the Hot Buffer and KOH fractions are the major fractions obtained from pomace; with the exception of the cellulose-rich residue and the salt-rich sodium carbonate fraction. Hence, pomace can be divided into a pectin-rich fraction (residual pulp) and a hemicellulose-rich (xyloglucan) component. A new insight from the CoMPP however is the presence of numerous pectin epitopes present (although in low total sugar contribution; see Figure 4D) which appear to associate strongly with the cellulose-xyloglucan network.

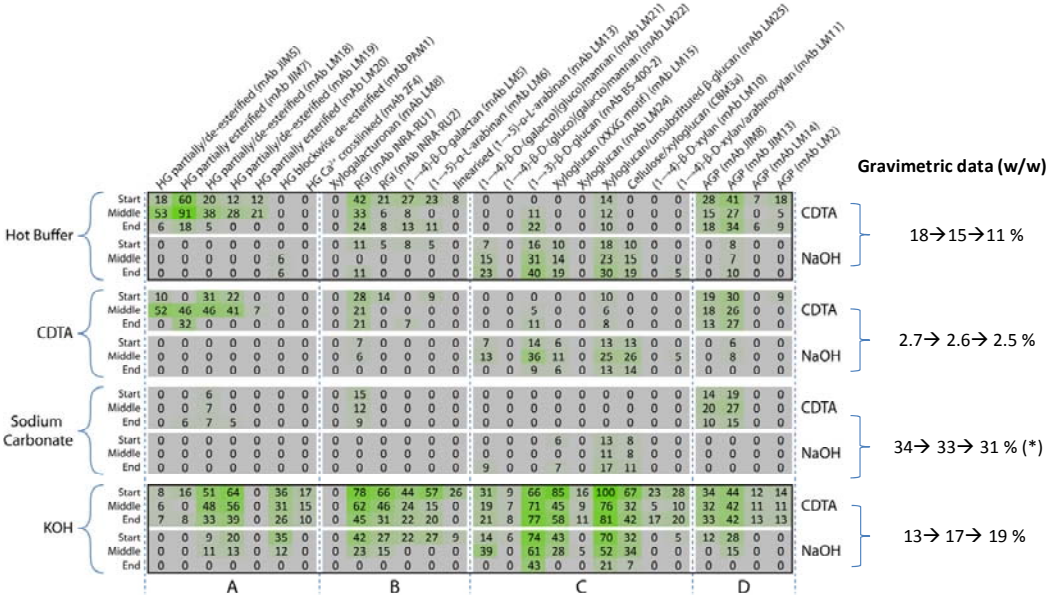

Figure 5. CoMPP (Comprehensive microarray polymer profiling) analysis of chemical-extracted fractions from fermentation-derived pomace AIR at three fermentation stages. The heatmap shows

the relative abundance of plant cell wall glycan associated epitopes present in grape pomace material and the colour intensity is correlated to mean spot signals. Sequential extractions were carried out with CDTA and NaOH; and the extracted material spotted onto nitrocellulose which was probed with sets of antibodies and carbohydrate binding modules. The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data set was set to 100 and all other data adjusted accordingly. A cut off value of 5 was imposed. (*) indicates the gravimetric data of sodium carbonate fraction, which is considered an over-estimated value due to the presence of oligomeric salt. Further to this four sections of the CoMPP probe sets were categorised; column A for HG epitopes, column B for RGI and side chains mainly, column C for xyloglucan, cellulose, mannans and xylans, and column D for AGP epitopes.

3.3 Enzymatic fractionation of pomace AIR

To further dissect the pomace compositional structure, we have chosen to use an enzymatic fractionation technique employed previously and validated on grapevine leaves (Moore *et al.*, 2014). Enzymes offer a means to precisely 'cut-out' components of the cell wall matrix allowing for more accurate identification of the presence of specific polymeric networks (e.g. xyloglucans) independently of the CoMPP analysis (Persson *et al.*, 2011). Enzyme fractionation of the berry pomace AIR (see Figure 6 for a flow diagram for the process) involved two sequential extractions; firstly endo-polygalacturonase (EPG, Megazyme, Ireland) in sodium acetate buffer (pH 5) and then followed by another round of EPG digestion but in a CDTA buffer. The de-pectinated material obtained following the two rounds of EPG as treatment was then split and subjected either (1) to Endo-glucanase (EGII, from *Trichoderma longibrachatum*) treatment or a (2) xyloglucanase (XEG5, from *Paenibacillus sp.*) treatment. The soluble polysaccharide material obtained from each of the enzyme treatments was subjected to monosaccharide compositional analysis (Figure 6). Compositional analysis of the NaOAc-EPG fraction (Figure 6A) revealed ca. 210 ug/mg of GalA which confirmed the pectin-rich matrix found previously (Figures 1A, 4A and 5), Further treatment with the CDTA-EPG fraction (Figure 6B) only revealed 130 ug/mg of GalA, along with similar or lower levels for the other monosaccharides. This is in line with the ineffective CDTA fractionation of grape pomace, accounting for only ca. 2.7% (w/w) total AIR (Figure 3) and the presence of some GalA (Figure 4B). This supports our assertion that most of the de-esterified and de-polymerised pectin of ripe grapes is removed to and partially degraded in the final wine during the fermentation (Figure 2). De-pectinated pomace subjected to

endo-glucanase treatment (EGII), yielded a higher composition of Glc (260 ug/mg) and Man (90 ug/mg), suggesting the presence of glucomannans, in support of the study of Doco *et al.* (2002) and confirming the datasets acquired from CoMPP on the hemicellulose (NaOH) fraction (Figure 5) from the chemical fractionation. When treated with XEG5, de-pectinated extract revealed Xyl (ca. 120 ug/mg) and Glc (ca. 220 ug/mg) as major sugars, suggesting that xyloglucan is a predominant polymer of grape pomace cell wall hemicellulose, in line with the data presented in Figure 4A and Figure 5.

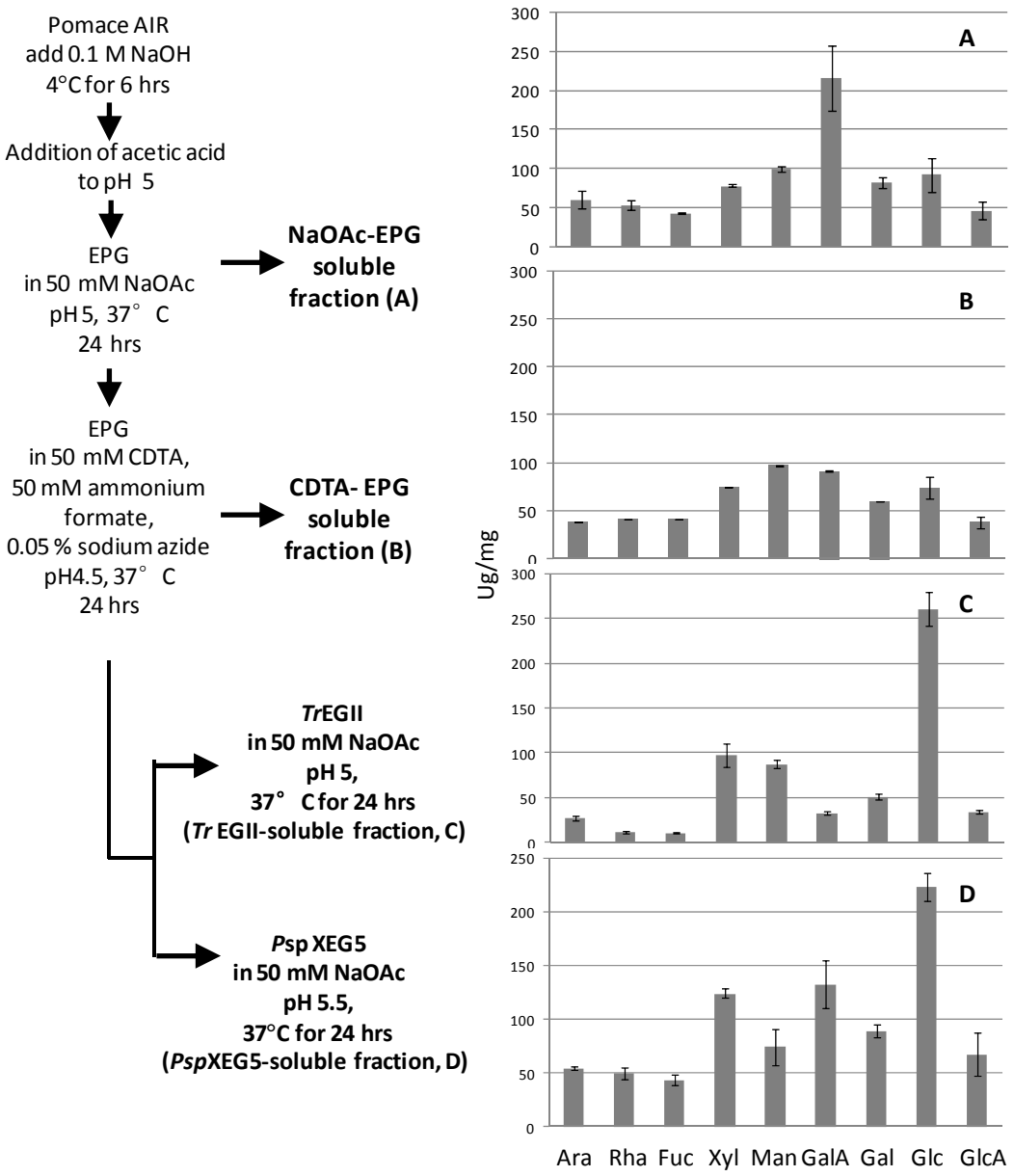

Figure 6. Enzymatic fractionation flow diagram and the monosaccharide composition analysis (w/w in AIR, ug/mg) of each fraction from pomace AIR at the start of fermentation. A: Fraction resulting after EPG digestion of previously saponified AIR (NaOAC-EPG fraction); B: Fraction resulting after EPG digestion in a presence of chelating regent (CDTA-EPG fraction); C: Fraction resulting after EGII digestion of de-pectinated residue produced after NaOAC-EPG and CDTA-EPG treatment; D: Fraction resulting after XEG digestion of de-pectinated residue produced after NaOAC-EPG and CDTA-EPG treatment.

4. Conclusion

In this study, the Cabernet Sauvignon grape berries have been taken through the standard red wine fermentation, and both the wine and the pomace AIR had been profiled by using a

high-throughput approach including several cutting-edge techniques, whereas the pomace AIR was comprehensively analysed using chemical and enzymatic fractionation. The datasets gained from those techniques have generally supported each other and provide more in-depth information on the complex nature of the grape berry cell wall and the changes it undergoes during enzyme-assisted maceration and winemaking.

Firstly, it is clear that crushed and macerated grapes lose a significant amount of pectin and AGPs to the must and wine. The pectin components of pulp are probably significantly de-esterified and de-polymerised; and extract easily with enzyme addition. The presence of AGPs has been well known in the literature (Vidal *et al.*, 2003), however the effect of concentration with the sensitivity of CoMPP has revealed for the first time that HGs and RG1 components are also present in low amounts in the final wine. The addition of enzymes at this early stage of fermentation provides for the large increase in volume of the juice; containing mainly sugars and acids from the pulp cell vacuoles of the berries once crushed. But red winemaking necessitates leaving the 'cap' (skins and pulp) and 'sediment' (seeds) in contact with the must for the full duration of fermentation which can last many days until alcohol levels peak. During this time winemakers hope to extract significant colour pigments, tannins and flavour compounds; deriving from grape skins (including the aroma contribution by yeast metabolism), to produce wine of desired style and quality for bottling or further ageing processes.

Here the fractionation data obtained is very interesting; we find two major fractions in fermentation-derived pomace. The first being a pectin-rich more easily extractable water soluble polysaccharide fraction containing mainly HGs, RG1 and side chains; AGPs and some xyloglucan; and glucans, presumably callose from the wounding process (i.e. crushing). This fraction is also methyl-esterified and/or acetyl-esterified; which probably becomes more soluble as the alcohol content increases. As fermentation proceeds this fraction reduces presumably as the enzymes and contact time leads to these polymers moving into the wine matrix. The second fraction is particularly interesting; this fraction contains an abundance of xyloglucan but also mannans, glucans and xylans. Xyloglucan appears to dominate this

fraction based on the monosaccharide compositional data but also the enzyme work performed. What is intriguing is the abundant pectin epitopes present here detected by CoMPP; de-esterified pectin HGs and methyl-esterified HGs (that saponify on the addition of KOH to form calcium egg-box epitopes recognised by 2F4 and PAM1) was present. Furthermore RG1 backbones (INRA-RU1, INRA-RU2) with arabinan (LM6) and galactan (LM5) side chains; as well as strong signals for all four AGP epitopes (mAbs JIM8, JIM13, LM14 and LM2) were detected. We propose that these pectin and AGP epitopes 'coat' or covalently cross-link with the xyloglucan-rich hemicellulose matrix (Tan *et al.*, 2013). This idea is not novel; Keegstra *et al.*, 1973 proposed a xyloglucan-RGI linkage model, and various other models have been suggested (McCann and Roberts, 1991; Carpita and Gibeaut, 1993). Over the last decade this type of linkage has been proposed in a number of different plant tissues (cotton in Fu and Mort, 1997; rose suspension cells in Thompson and Fry, 2000; wine polysaccharides in Vidal *et al.*, 2003; pea shoots in Abdel-Massih *et al.*, 2003). It has recently been shown in wheat grains that pectin domains are found in various spatial locations while it was generally accepted that arabinoxylans and glucans were the only major polymers present (Chateigner-Boutin *et al.*, 2014). Zietsman et al. (2015) has shown that grape skin cell walls show a very similar trend in de-pectination using sequential treatment with pectinases to achieve effective deconstruction in order to get at the underlying hemicellulose-rich cells.

This has significant implications for winemakers since the choice of enzyme combinations is crucial to deconstruct the layers of pectin-rich cells to get to the pectin-coated xyloglucan-rich skins cells which presumably need to be disrupted effectively to 'open-up' these cells to allow anthocyanins, tannins, hydroxy-cinnamates, and flavour and aroma compounds (e.g. monoterpenes) to leach into the fermentation matrix to support the production of wine of the desired quality. Given genetic differences between cultivars, factors such as skin thickness and pectin structure; and the known observation that some cultivars are easier to extract from (e.g. colour) than others; this study provides a basis and model for understanding the nature and complexity of the grape cells walls within grape berry tissues. This work, performed on Cabernet Sauvignon, provides an avenue for future

studies with the ultimate aim of tailor-making enzyme blends to achieve optimal quality wines from grapes from different vineyards, cultivars and ripeness levels.

Acknowledgments

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Authors' contribution

Y. Gao, J. Moore and M. Vivier designed the study, Y. Gao performed the experiments and collected the data. Y. Gao interpreted the results and drafted the manuscript. J. Moore and M. Vivier advised with data interpretation and manuscript preparation. J. Fangel and W. Willats performed the CoMPP analysis. All authors read and approved the final manuscript.

Chapter 4

Research results

Effect of intra-vineyard ripeness variation on the efficiency of commercial enzymes on berry cell wall deconstruction under winemaking conditions

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Effect of intra-vineyard ripeness variation on the efficiency of commercial enzymes on berry cell wall deconstruction under winemaking conditions

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Key words

Cell wall, intra-vineyard variation, grape development, fermentation, grape pomace, enzymes, Multivariate data analysis.

ABSTRACT

Intra-vineyard variation grape berry ripening occurs within bunches, between bunches on the same vine and between vines. Although it is assumed that such variation also occurs at the grape berry cell wall level, no study to data has investigated in any depth. Here we have used a intra-vineyard panel design to investigate pooled bunches from six vines (per panel) in the context of a winemaking scenario. The dissected vineyard was harvested by separate panels, where each panel was then subjected to a standard winemaking procedure with or without the addition of three different enzyme preparations for maceration. Adjacent untreated panels acted as the enzyme controls. Hence we combined two studies into one design. Cell wall material harvested from the treated and untreated panels were subjected to high throughput cell wall profiling tools combined with multivariate data analysis. The study showed that significant variation at the cell wall polymer level occurred across the vineyard amongst the different panels. Furthemore, all enzyme applications had a strong and clear effect in reducing this variation through de-pectination. What was most interesting is that while de-pectination occurred the levels of esterification were unaffected by the enzymes. This is a positive for wine quality as no methanol or acetates would have been produced from the de-pectination and not all natural grape berry variation is affected. This study provides clear evidence that enzymes can positively influence the consistency of winemaking without necessarily removing all variability provided from the vineyard. This study provides a foundation for further research into the relationship with grape berry cell wall architecture and enzyme formulations.

INTRODUCTION

Winemakers are annually challenged to obtain consistent wine quality (defined loosely as a recognized style and typicity associated with a specific cultivar. However, various factors (genotype and environmental) can both positively or negatively impact on the development and ripening processes in the vineyards which in turn result in a significant degree of variation (a lack of synchronicity of berry ripening being one example) in the chemical and biochemical composition of the final harvested berry pools (Hannah *et al*, 2013). Cabernet Sauvignon, as one of the most iconic wine grape cultivars has been studied with respect to the role of environmental factors (e.g. UV exposure, water availability) (Martinez-Luscher *et al*, 2014) and viticultural practices (Bobeica *et al*, 2015) that influence berry composition, evaluating the impacts on sugars, organic acids, flavonols, anthocyanins and tannins. Variability in chemistry and biochemistry is a phenomenon that is observed at the vine-to-vine, bunch-to-bunch and even berry-to-berry level within vineyards; this has been validated from several studies in a number of cultivars (Bramley, 2005, Pagay and Cheng, 2010, Zhang *et al*, 2015). In the standard winemaking scenario, it is also an important challenge to consistently release favorable compounds (e.g. tannins, anthocyanins etc.) from harvested berries into the final wine via maceration and fermentation. Berry skin cells contain many and high concentrations of these beneficial compound (Gomez *et al*, 1994, Ortega-Regules *et al*, 2008a) and need to be broken down to increase the final wine's structure, body, potential for ageing (mediated commonly by the quality of tannins obtained), as well as expressing the typicity (style and type commonly associated with a specific cultivar and winemaking process e.g. a Bordeaux blend) of the cultivars used to produce the final wine.

In the past couple of decades, various techniques have been tested to aid the maceration process, these include using different yeasts, manipulating the temperature (e.g. cold soak maceration), physical interruption (e.g. by punch downs or electrical pulse treatment) (Sacchi, 2005, Delsart *et al*, 2012, Cholet *et al*, 2014) in order to "open up" the skin cells. Enzymes are also major additives that have been applied in order to effectively degrade the skin cell wall polymers thereby improving maceration (Ortega-Heras *et al*, 2012) These enzymes are crude

extracts from the microorganisms (mainly fungi) which are considered as GRAS (generally recognized as safe) for human health. A number of experimental studies in the past has worked on the relationship (i.e. correlation) between the release of wine favorable compounds and use of maceration enzymes, and assessed the positive and negative impact on the wine quality (Revilla and Gonzalez-SanJose, 1998, Romero-Cascales *et al*, 2012, Fia *et al*, 2014). However, the direct effect of enzymes on the variation of harvested berry cell walls in the context of intra-vineyard variation and possible effect in relation to the fermentation and final wine has not been analysed in much depth.

The information generated from most previous studies is still exclusively reliant on the indirect (i.e. inferred) approach based on monosaccharide composition and concentration obtained from analyzing isolated and degraded polymers (Nunan *et al*, 1997, Arnous and Meyer, 2009). The utility of using glycan microarray technology (i.e. CoMPP, Comprehensive Microarray Polymer Profiling) with well characterized mAbs and CBMs (see Table 2.1 in chapter 2) targeting specific epitopes associated with specific polymers (e.g. mAb LM15 which targets the XXXG motif glucan backbone in unsubsituted xyloglucan), the opportunity to more directly evaluate enzyme actions on polysaccharides by virtue of changes in their epitope abundance and/or exposure (Moller *et al*, 2007, 2008, Zietsman *et al*, 2015a, 2015b), which allows a more direct evaluation of enzyme-mediated changes at the cell wall polymer level. By combining this high-throughput approach with classical cell wall profiling methods, Moore *et al.* (2014a) found conserved development profiles (but also some differences) for Cabernet Sauvignon (a wine grape cv.) and Crimson Seedless (a table grape cv.) at a comparative level for samples of (de-seeded) whole berries collected at green (berry touch), veraison and ripe stages. To test the relationship between ripening levels and enzyme mediated maceration on *Vitis vinifera* cv. Pinotage skin cell walls, Zietsman *et al* (2015a) performed a study over two vintages (with Brix at 22.7 and 26.5). This study demonstrated the importance of the harvest ripening levels which resulted in a greater degree of degradation (presumable due to endogenous enzymes and senescence) of more ripe berries lessening any impact that adding enzyme can make on berry cell walls in winemaking conditions at this late stage (Zietsman *et al,* 2015a). A further study on Pinotage by Zietsman *et al* (2015b) also demonstrated under optimized buffered laboratory conditions using

isolated skin cell walls that different enzyme combinations helped to (i) unravel the berry skin cell wall while others enhanced (ii) degradation (de-pectination) significantly; the former appears to 'loosen' up the pectin enhancing extraction. This fits well with the study reported in Chapter 3 (Gao *et al*, 2015) where whole berries of Cabernet Sauvignon were processed to wine and pomace. The berry pomace samples from the winemaking were then fractionated chemically and enzymatically, and a novel approach used by combining CoMPP analysis with classical fractionation, as well as GC-MS and FT—IR analyses, revealed evidence for two layered structures (an esterified pectin-rich inner layer and a hemicellulose-rich pectin-coated outer layer) being present (Chapter 3, Gao *et al,* 2015); the less strongly bound pectin-rich inner layer being probably 'loosened-up' (unraveled) by enzymes (Zietsman *et al,* 2015b). However, there is no study as far as we aware, that focused on investigating the intra-vineyard grape berry cell wall variability at harvest and the relationship with maceration enzymes used commercially in a typical winemaking process.

The aims of this study are to assess the relationship between intra-vineyard grape berry cell wall variation at harvest and use of commercial enzyme preparations. The study employs a chequered-board design (see Figure 1) dissecting the Cabernet Sauvignon vineyard (used in Chapter 3) into untreated and enzyme-treated panels (similar to that in Young *et al*, 2015) by analyzing the resulting berry cell wall datasets generated using cell wall profiling approaches (Moore *et al,* 2014a; Gao *et al,* 2015) coupled with multivariate analysis approaches applied to the data (Moore *et al*, 2014b, Zietsman *et al*, 2015a).

MATERIALS AND METHODS

Grape berry sampling layout. In this study, the grape samples (*Vitis vinifera* cv. Cabernet Sauvignon) were harvested in March, 2014 from the Welgevallen experimental vineyard of the Department of Viticulture and Oenology, Stellenbosch University, South Africa. The vineyard is situated (33°56'42"S, 18°51'44"E), composed of alluvial soils with light to medium texture and is arranged in a north-south row orientation. Vines are drip-irrigated and trained on a seven-wire vertical trellis system, each row was divides into panels using wooden poles and each panel contains 6 vines. The harvest was applied on 24 panels which are alternating

to each other (see layout in Fig. 1), the panels were carefully selected for producing representative samples where diseased/damaged vines were not present, and all the panels in this block had the similar sunlight exposure as well as being exposed to the same viticultural treatment during the 2013/2014 season. Panels were harvested separately at Brix 24 (advised by the viticulturists tending the vines using a classical viticultural random sampling for sugars throughout the experimental area) for three different maceration enzyme treatments with fermentation (see Fig. 1 and Table 1), and grape berries (approx. 15 kg) from each panel (6 vines) were pooled together and split into 3 biological replicates (5 kg each) for subsequent fermentations.

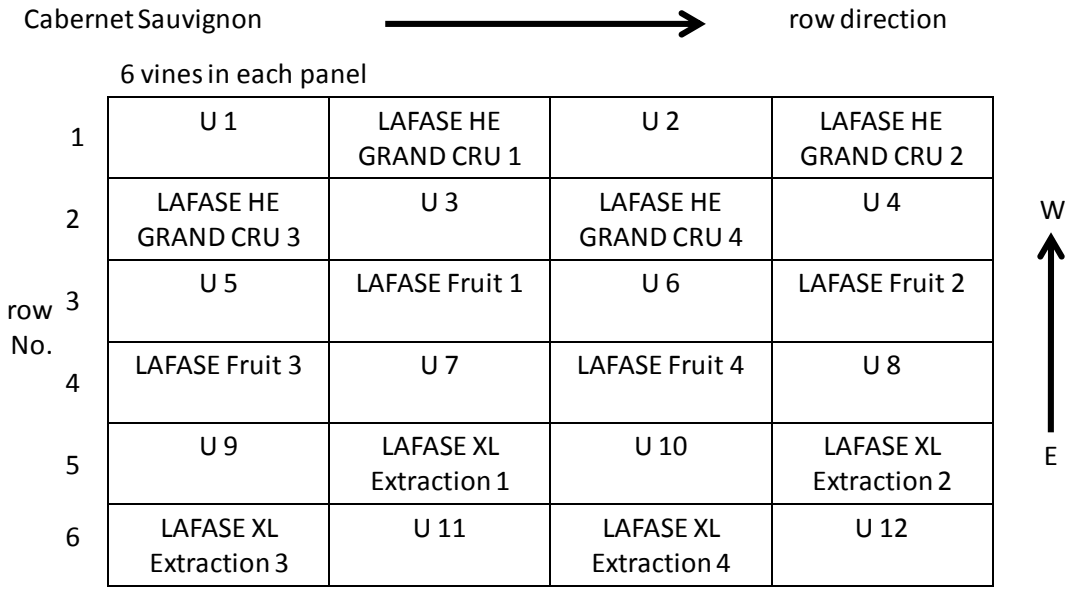

Figure 1. The harvest plan of Cabernet Sauvignon, each block represents a panel which consists of 6 vines, U refer to untreated fermentations.

Table 1. Information of commercial enzyme preparations (Laffort, France) used in this study

Commercial enzyme preparation	Properties	Application
Lafase XL Extraction	Liquid enzymatic preparation for red wine maceration and clarification	Extraction and clarification
Lafase HE Grand Cru	Pectolytic enzyme preparation, purified in CE for the production red wines that are rich in colouring matter and structured tannins, destined for ageing.	Maceration
Lafase Fruit	Purified pectolytic enzyme preparation for the production of fruity, colourful and round red wines.	Maceration

Vinification of Wine. After harvest, berry bunches were pooled from each panel destemmed and crushed and then split into into polypropylene buckets (5 kg each X 3 for each panel (as 3 biological repeats, 72 buckets in total), sodium metabisulfite was added (30 ppm of SO_2) after crush, and then the "must" was inoculated with pre-rehydrated yeast (*Sacchromyces cereviseae* VIN13, Anchor Yeast, South Africa) according to the manufacturer's instructions and recommendation for fermentation. Three commercial enzyme preparations (Table 1), named LAFASE HE GRAND CRU, LAFASE FRUIT and LAFASE XL Extraction (LAFFORT, France), were applied to the must, using the layout presented in Figure 1, according to the manufacturer's recommendation (5g/100kg grape for GRAND CRU and FRUIT, 4ml/100kg grape for XL). Fermentation was conducted at 25 ^{o}C, and the pomace of each bucket was punched down twice a day to maceration. At the end of fermentation, the wines were pressed using a basket style wine press to generate the free run wine and pomace samples for subsequent analyses.

General Oenological Parameters of wine. Before the inoculation with the yeast, the must from each panel (Fig. 1) was measured for sugar levels the Brix^{o} value (soluble solids) to assess the variability of ripening levels, by classical viticultural parameters, within the vineyard experimental block. After the fermentation, the enzyme-treated and untreated wines were sampled at ca. 50 ml units which were analysed using Fourier transform infrared (FT-IR) spectroscopy with a WineScan FT120 Basic (Foss Analytical, Hillerød, Denmark) instrument to assess standard oenological parameters for the consistency of the winemaking conducted. The oenological parameters that were tested for included: pH, volatile acidity, total acid, glucose + fructose, and ethanol levels and several other known wine compounds. The scanning program of the instrument was set to perform duplicate analyses per sample (i.e. two technical repeats).

Cell wall sample preparation from pomace. After the wine was pressed; composite sampling was performed (10 positions on the linearised press-cake of each fermentation) to get approx. 5 grams of fully representative pomace as outlined in the theory of sampling (Petersen *et al,* 2005), the pomace samples were then de-seeded and milled (30 Hz for 30s) using a Retsch MM400 mixer mill (Retsch, Haan, Germany) under liquid nitrogen. The resulting powder was processed according to Gao *et al,* 2015 (chapter 3). Briefly, the powder

was heated in 80% ethanol at 95°C for 20 min, and then centrifuged to discard the supernatant, the residue was washed using a series of organic solvents (i.e. methanol, chloroform and acetone) (as described in Moore *et al*, 2014b). At the end of washing steps, the alcohol-insoluble residue (AIR) was air-dried and resuspended in Milli-Q water (at similar volume of AIR), frozen at -80 °C overnight and then freeze-dried. For the cell wall analysis of fresh berries, the samples had been collected after the berry crush immediately kept at ice-cold temperatures with liquid nitrogen for processing as described earlier for treated fermented pomace.

Monosaccharide compositional analysis of pomace cell wall material. The AIR samples sourced from fresh de-seeded berries and fermented pomace were analysed for their monosaccharide composition according to the method described in chapter 3 (Gao *et al,* 2015). Briefly, the sample was hydrolyzed using 2 M TFA (2 h, 110 °C) to monosaccharides, which were then converted to their methoxy derivatives using methanol/methanol HCl (16 h, 80 °C), followed by the silylation with HMDS/TMCS/pyridine (3:1:9, Sylon HTP kit, Sigma-Aldrich, MO, USA). The separation and analysis of these sugar derivatives were performed using a gas chromatograph (Agilent 6890 N, Agilent Technologies, CA, USA) coupled to an Agilent 5975 MS mass spectrometry fitted with a polar (95% dimethylpolysiloxane) ZB-Semivolatiles Guardian GC column (30 m, 0.25 mm ID, 0.25 µm film thickness). The nine main cell wall monosaccharides were analysed: arabinose (Ara), fucose (Fuc), rhamnose (Rha), xylose (Xyl), mannose (Man), galacturonic acid (GalA), galactose (Gal), glucose (Glc) and glucuronic acid (GlcA).

Comprehensive microarray polymer profiling (CoMPP) analysis of pomace cell wall material. To assess the variation of whole berry cell wall samples on the polymer level, AIR (10 mg) sourced from grape pomace (with and without enzyme treatment) was sequentially extracted using CDTA (diamino-cyclo-hexane-tetra-acetic acid) and NaOH to obtain the pectin-rich and hemicellulose-rich fractions respectively (Moller *et al*, 2007). These fractions were then spotted onto nitrocellulose membranes and then probed with a number of monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs). The raw data of the signal values were recorded and used for the multivariate data analysis; whereas the mean readings were scaled and then presented as a microarray heatmap, the highest signal

was set as 100, and others were normalized according to the highest signal, a cutoff value of 5 was applied.

Multivariate and Univariate data analysis. The data generated from monosaccharide compositional analysis was evaluated for statistical significance using univariate statistical analysis software tools (Statistica 10, Statsoft Southern Africa Analytics, Sandton, South Africa). Multivariate analysis was performed using the SIMCA 14 software package (Umetrics AB, Umea, Sweden) to perform the PCA (principal component analysis) and OPLS (Orthogonal projections to latent structures) analyses.

RESULTS AND DISCUSSION

In this study, the vineyard of *Vitis vinifera* cv. Cabernet Sauvignon had been dissected into a number of blocks (panels, 6 vines from each panel), and the berries (ca. 15 kg) from each panel were split into three containers and fermented separately. A previously optimized and validated cell wall profiling approach (Moore *et al*, 2014b, Gao *et al*, 2015, Chapter 3) has been performed on AIR sourced from the fresh and fermented pomace to study the cell wall variation at polymer level which present in the berries sourced from the vineyard at harvest, as well as assessing the impact of using different enzyme preparations in the fermentation on the cell wall integrity. The ripening levels of berries in the vineyard and the general wine parameters after fermentations were also recorded.

After crushing the berries, the juice (3 biological replicates for each panel) was used to test for ripeness level which was expressed as Brix^{o} value a routine term used in wine industry for sugar levels. Fig. 2 showed the results of grape ripeness levels (mean number from 3 replicates plus standard deviation), the panels were colored according to the development stages, in the dissected vineyard block.

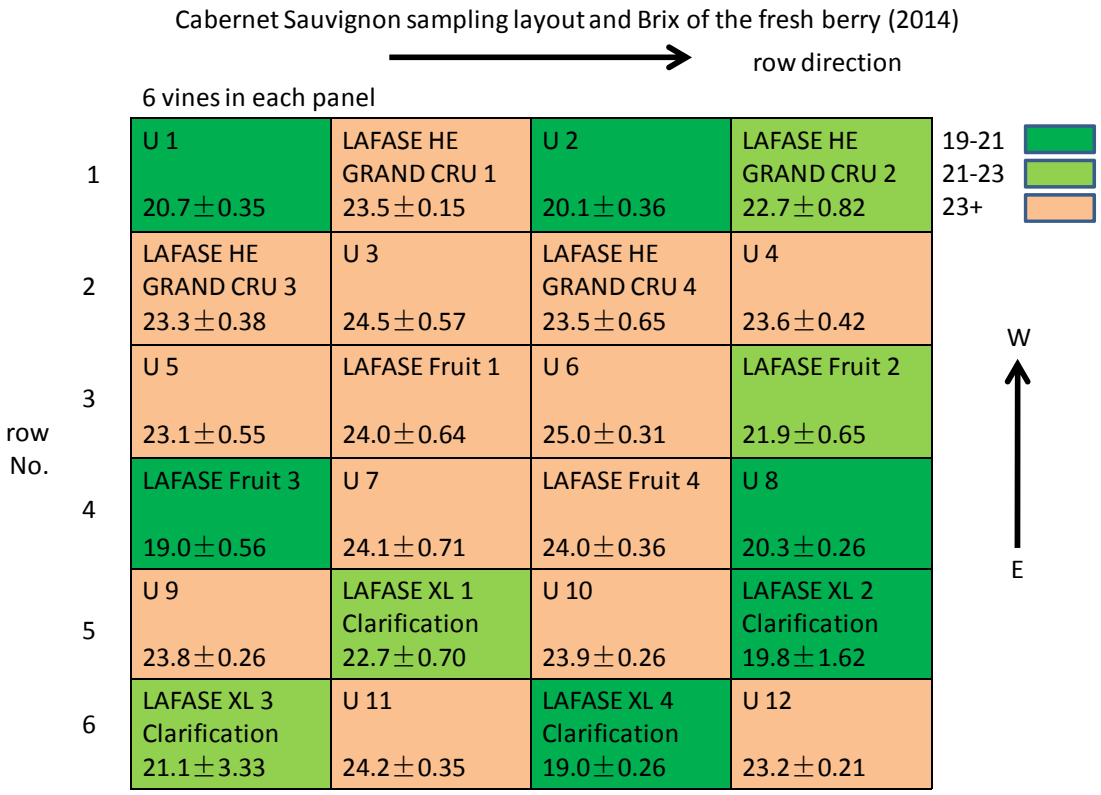

Figure 2. Variation of berry soluble solid (Brix^{o}) at different panel on the vineyard, the level of ripening was categorized into 3 stages depending on Brix value (mean values from 3 biological repeats), including stage 1 (19-21), stage 2 (21-23) and stage 3 (23+). The shades of green (dark green for 19-21 Brix, light green for 21-23 Brix, and Pink for 23 + Brix. are according to the ripeness stages.

From the Fig. 2, it is clear that a reasonably and statistically significant amount of variation in berry sugar levels (i.e. viticultural defined ripening) exist within the vineyard block classified as 24 degrees Brix according classical viticultural sampling methods., The differences observed can be as large as 5 degrees (Brix^{o}) between the panels even where some panels are closed to each other. A higher degree of resolution going down to 2-4 vines per panel would probably show even more variation. The panels selected in this study (in consultation with viticulturalists) have very similar vigor levels (controlled through drip irrigation) and have been exposed to uniform vineyard treatment practices throughout the season. However, clearly some other factors not identified, apparently, had impacted sugar accumulation of of the berry bunches in each panel; here it should be noted that berry bunches were manually harvested per panel, and visually inspected with those bunches showing evidence for example of shriveling and disease symptoms being discarded. It must

be borne in mind that the juice tested was sourced from the crushed berries from a single panel, so this Brix°, actually reflects the mean sugar level for each panel. The variation study was not performed on a vine, or single bunch level because we also wished to remain semi-industrially relevant for the testing of enzyme preparations (and we also have shown volumes less than 5 kg buckets produce highly irreproducible results in our system of skins, pulp, seeds and juice (data not shown)) and to focus on intra-vineyard six vine panel resolution. We can safely assume that such variation also exists at much higher panel resolutions, as a number of studies have already been performed to analyse the berries, bunches and vines at a higher resolution level in vineyard scenario's (Bramley, 2005, Papay and Cheng, 2010, Zhang *et al*, 2015, Young *et al,* 2015). However, standard viticultural and commercial winemaking practices involve selection of random berry bunches from the whole vineyard in order to test sugar levels in the juice obtained after pooling and crushing the berries. The Brix° value obtained from this analysis assumed to be a good reflection of the average from the entire vineyard. Due to this common assumption any variation occurring within panels, vines and bunches, are often overlooked and/or ignored.

No significant differences were observed on berry cell wall from monosaccharide compositional analysis

Gas-chromatography combined with mass spectrometry provides information on bulk cell wall chemistry by analyzing the sugar derivatives obtained from acid hydrolysed cell wall material. From the analysis of AIR sourced from fresh de-seeded berries, Fig. 3 shows the monosaccharide composition (w/w of sugar yield to AIR) of AIR sourced from fresh de-seeded berries, the data has been divided into 3 sub-figures for ease of interpretation. Firstly, it is clear that all the AIR contains an abundance of GalA (ca. 260 – 290 µg/mg), which is similar to the previous results (see Chapter 3, Gao *et al*, 2015) and confirmed the berry cell wall is rich in pectin (Saulnier and Thibault, 1987), some other pectin polymers (e.g. RGI, arabinan and galactan side chains) can also be inferred from the presence of Rha, Ara and Gal, as well as xyloglucans and mannans from the Xyl, Glc and Man present (see Fig. 3). It is interesting to note that marked variations were found in the monosaccharide composition, especially in the pectin component (e.g. GalA), even if these are not significantly different

between panels. The Brix values are also provided in each of the sub-figures (Fig. 3A-C). An interesting trend from the observation of the Brix values in relation to the monosaccharide data (Fig. 3) is that more ripe berries (i.e. higher Brix) has a slightly lower amount of pectin component and relatively higher amount of hemicellulose, although seemingly not statistically significant using classical univariate statistics. Compositional changes of berry cell walls in relation to berry development (or ripeness) are not always in complete agreement in the literature. In a study on Shiraz grape berries it was found that GalA levels increase from veraison (Brix^{o} 19) to over-ripe stage (Brix^{o} 27) (Vicens *et al*, 2009), but this analysis was performed on skin-only, which is similar to a recent study on Pinotage grape skins by Zietsman *et al*, 2015a). However, in contrast, a decreasing trend was found in other cultivars during the ripening, such as in skins of Monastrell and Merlot by Ortega-Regules *et al*, (2008b). Similarly a marked decrease of GalA mol% was observed in a comparative study of cell wall ripening of wine grapes (cv. Cabernet Sauvignon) and table grapes (cv. Crimson seedless) from green to ripe stage (Moore *et al*, 2014a). In the case of Moore *et al*. (2014a), however, the study was on whole berries, not just skins, and so this is probably due to a proportional changes in amounts of pectins to hemicellulose; and the changes observed were slight for GalA.

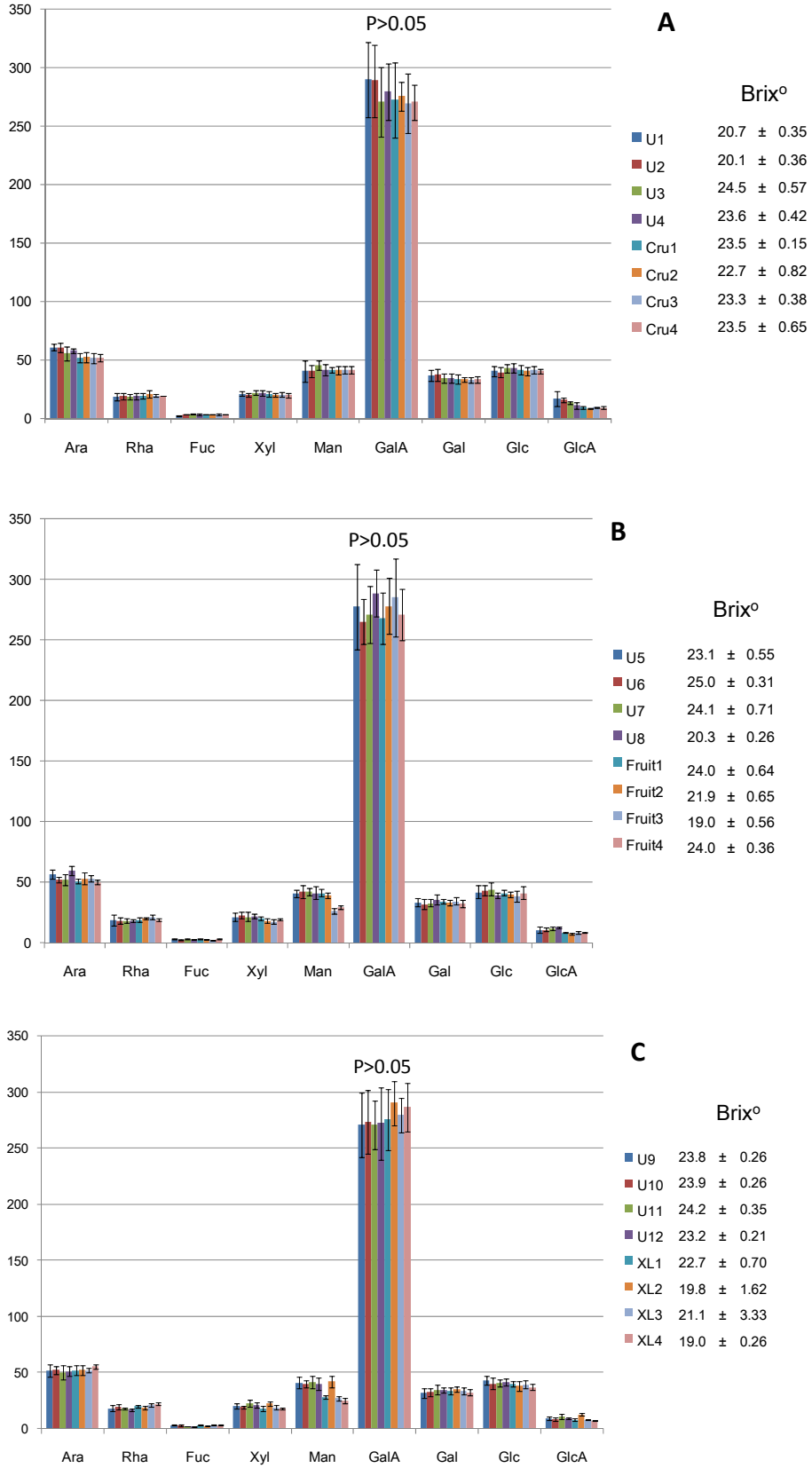

Figure 3. The monosaccharide composition of AIR sourced from fresh berry from different panels on the vineyard and the berry soluble solid (Brix) values.

Figure 3 (with captions). The monosaccharide composition of AIR sourced from fresh berry from different panels on the vineyard and the berry soluble solid (Brix) values. **A.** U1-4 (untreated), Cru1-4 (Lafase HE Grand Cru); **B.** U5-8 (untreated), Fruit 1-4 (Lafase Fruit); **C.** U9-12 (untreated), XL1-4 (Lafase XL Extraction). The composition is expressed in µg/mg (dry weight in AIR sample), Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats; different letters indicate a significant difference for a specific sugar, between the different stages (95% confidence level, ANOVA, P=0.05).

Fermentation process was validated using FT-IR spectroscopy

These pre-characterised ripe berries from each panel were fermented separately with or without selected commercial enzyme preparations (see list in Table 1). To ensure the winemaking was relatively uniform for the treatments and their controls a FT-IR spectroscopy analysis (using the FOSS Winescan instrument) of the wines were performed and pre-calibrated programme was applied to determine the standard oenological parameters (shown in supplementary Table 1). The general oenological parameters (supplementary Table 1) for all treatments conformed to a typical red wine, no faults or major deviations were evident. PCA models and plots of the data were also created to compare untreated and enzyme-treated pomace in visually informative manner (Supplementary Fig. 1). Interestingly, by performing multivariate data analysis on these parameters for all the wines, it was found that clear clustering could be repeatedly observed between the specific enzyme-treated wine and their untreated control wines (Figs. 4A, B, C). The interesting observation that the Cru, Fruit and XL enzymes presented separation plots from their controls, but that these were due to different variables is not clearly understood and we therefore lack possible reasons for these observations (Figs. 4A, B, C). Nevertheless, an interesting phenomenon observed is that the variation of the biological repeats is lower with the enzyme-treated wine (except for Cru) than their adjacent untreated wine, this could suggested that the additional enzyme can reduce the variation and improve consistency in the final wine based on these standard reference parameters. The one with lowest impact on variability is Cru, but it may be explained by the information given by the manufacturer (http://www.laffort.com/en/products/enzymes/87), which states that is that the Lafase HE

Grand Cru is an enzyme product suitable for longer maceration, however, in this study, the fermentation with all three enzymes were performed for the same periods (ca. 5 days), far less time than what Cru is intended for. Additional parameters (such as tannins, anthocyanins) are often measured for red wines but these were beyond the scope of the study which was firmly fixed on the direct affect these enzymes had on the berry cell walls in relation to the intra-vineyard variation in berry maturity.

Significant de-pectination of enzyme-treated pomace observed by GC-MS and CoMPP

After fermentation, the fermented pomace was de-seeded and processed to AIR, in order to be profiled using GC-MS and CoMPP to see the impact on the berry cell wall from the winemaking, with and without enzymes; as outlined previously for the fresh grapes. As in Fig. 3, the results were divided into 3 sub-figures (Fig 5A, B and C) for ease of interpretation, these include the enzyme-treated (4 panels) and their "neighboring" untreated (4 panels) fermentations in each of the sub-figures. Thus, Fig 5 contains A (untreated U 1-4 versus Lafase HE Grand Cru treated 1-4), B (untreated U 5-8 versus Lafase Fruit treated 1-4) and C (un-treated U 9-12 and Lafase XL Extraction 1-4).

By comparing the monosaccharide compositions obtained from the pomace AIR sourced from the enzyme-treated and untreated fermentations; it can be clearly seen that there is a statistically significant decrease of GalA in enzyme treated pomace compared to untreated pomace (Figs. 5A, B and C), which strongly indicates de-pectination had occurred and was facilitated by the addition of commercial enzyme preparations. Other observations include a decrease in Ara and Rha from enzyme treatments, these have also been found in the previous study (see chapter 3, Gao *et al*, 2015 and Zietsman *et al*, 2015), which could infer the degradation of RGI, arabinan side chains and AGPs. An increase for Xyl, Man and Glc was also found for all three enzyme-treated compared versus untreated samples, this also confirmed the de-pectination and exposure of hemicellulose (e,g, xyloglucans and mannans) observed in enzyme-treated wine fermentations (see Chapter 3) by Gao *et al*, (2015) and Zietsman *et al*, 2015a)

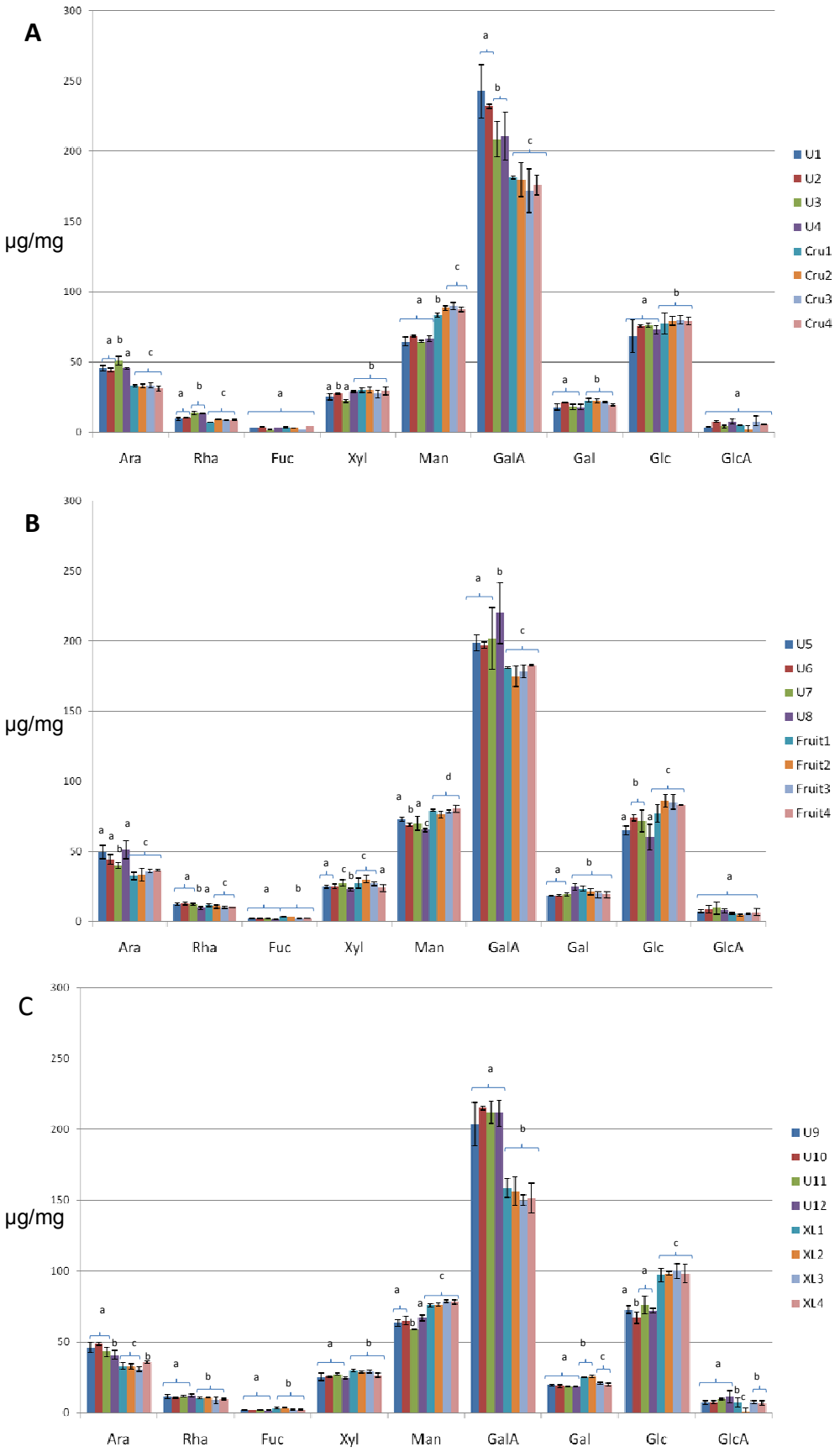

Figure 5. The monosaccharide composition of AIR sourced from fermented pressed berries(i.e. pomace) (Cabernet Sauvignon)

Figure 5 (with captions). The monosaccharide composition of AIR sourced from fermented pressed berries (i.e. pomace) (Cabernet Sauvignon) (.**A.** U1-4 (untreated), Cru1-4 (Lafase HE Grand Cru); **B.** U5-8 (untreated), Fruit 1-4 (Lafase Fruit); **C.** U9-12 (untreated), XL1-4 (Lafase XL Extraction). The composition is expressed in µg/mg (dry weight in AIR sample), Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats; different letters indicate a significant difference for a specific sugar, between the different stages (95% confidence level, ANOVA, P=0.05).

Apart from the efficient de-pectination that was observed due to the added enzymes, another phenomenon is also identifiable when we compare untreated pomace alone. That is the variations in concentrations of monosaccharides due solely to the fermentation process (Fig. 5). For example the concentration of GalA varied between ca. 240 µg/mg (U1) to 190 µg/mg (U6), similar e variation was also found for other monosaccharides, such as Ara, Xyl and Glc (Fig. 5). Interestingly, by looking at the Brix^{o} levels of berries in different panels (Figs. 2 and 3), the difference of ripening level can be correlated to these monosaccharide compositions of fermented pomace to some degrees. It can be seen that riper berries undergo more effective de-pectination compared to the less-ripe berries, without the addition of commercial enzyme preparations. This can be explained by the native endogenous enzymes (e.g. endo-polygalacturonases and pectin methyl esterases) produced by the grapevine in it's tissues including berries (Nunan *et al*, 2001), can also facilitate the breakdown of cell wall during their release during the crush and subsequent fermentation. However, most of them, from literature (Gil *et al*, 2015), do not appear to show their optimum activity in the winemaking conditions due to its complex and relatively harsh chemical environment, having a low pH, tannins, acids, and inhibitors (high sugars, polyphenols, organic acids and bisulfate salts). Zietsman *et al.* (2015a) performed an enzyme treatment winemaking study on berries harvested from two vintages which were at two different ripening levels on average. The authors found that in the control peeled skin samples from berries sourced from higher ripening levels (Brix^{o} 26.5) went through more effective cell wall degradation than the skin from less ripe berries (Brix^{o} 22.7) and stated that the native enzymes could be the factor for these differences in de-pectination.

The information acquired from monosaccharide compositional analysis provides information on bulk chemistry, however, it does not give direct access to the polymer epitope level. Here CoMPP, using mAbs and CBMs, allows for the direct detection of cell wall polymers (polysaccharides and proteins) by virtue of their epitopes AIR sourced from de-seed fermented pomace from all fermentations were extracted sequentially using CDTA and NaOH to obtain pectin-rich and hemicellulose-rich fractions respectively, and then probed with a number of mAbs and CBMs which have been validated previously on grape berries (Moore *et al*, 2014a, see Chapter 3 Gao *et al*, 2015). SIMCA Multivariate data analysis tool (Umetrics, Sweden) were used to create models of the data and prepare principal component analysis (PCA) plots (Fig. 6) to visualize the CoMPP data as there are so many more variables (antibodies) for each observation (sample) than the GC-MS datasets.

From the PCA plot (Fig. 6A), it is clear to see the untreated fermented samples (12 samples, 4 technical replicates each, n = 48 in total) and enzyme-treated samples (12 samples, 4 technical replicates each, n = 48 in total) are well grouped into two main clusters. An interesting phenomenon is that the untreated samples are more spread out (shown as arrow, PC1=51.2%) than the enzyme-treated samples, and the loading variable plot (Fig. 6B) showed that the CDTA extract from enzyme-treated samples is positively correlated to LM8, LM10 and LM21 (mainly hemicellulose), and negatively correlated to all of the pectin antibodies.

This confirmed that that significant de-pectination has been facilitated by addition of the enzymes (Fig. 5), and that these enzymes are very effective at reducing the berry cell wall intra-vineyard variation due to differences in ripeness (i.e. Brix levels).

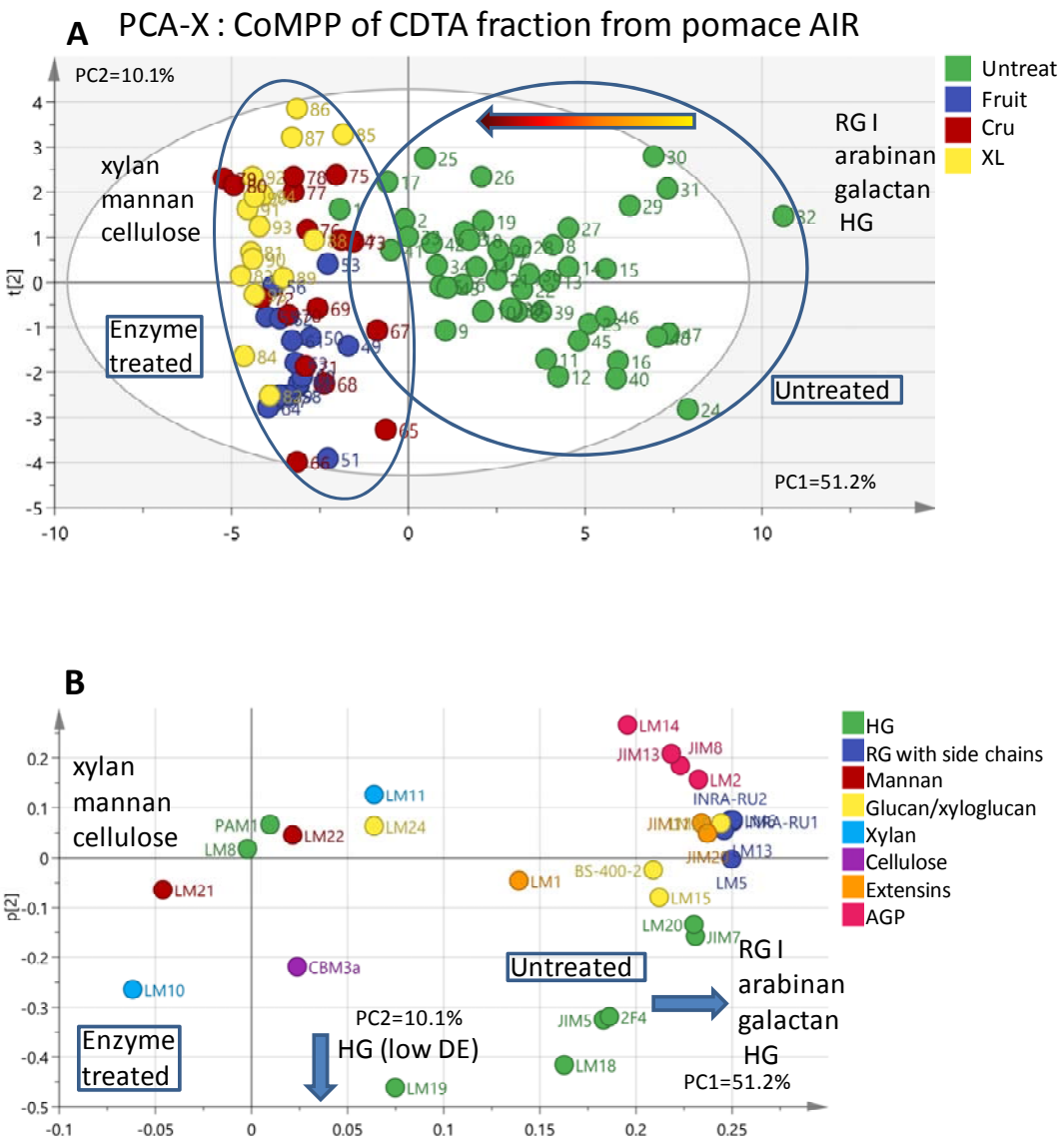

Figure 6. PCA score plot (**A**) and Loading plot (**B**) of the CDTA (pectin-rich) extract from AIR sourced from fermented berry pomace. Untreat – untreated fermentation, Fruit – Lafase Fruit, Cru – Lafase HE Grand Cru, XL – Lafase XL Extraction. The color is according to the treatment in **A,** and to the different groups of cell wall polymers in **B.**

Interestingly, in contrast PC2 explains 10.1% of variance in the plot, but here it can be seen that the variation has not been reduced by addition the enzyme (Fig. 6)., The loading plot shows that the main drivers of the separation were identified to be mainly HG with low degree of esterification (HG low DE, recognized by mAbs LM18, LM19, JIM5 and 2F4, downward pointing arrow in Fig. 6B). Contribution plots were also performed on untreated an enzyme-treated samples (data not shown), respectively, to look at the main contributors causing the separation on PC2, and resulted in the same, again to be HG with low DE. These

data therefore strongly suggest that esterification levels play the most important role in PC2 separation (Fig. 6B). Therefore the samples located at the bottom side of PCA plot (Fig. 6A) should contain more HG with low DE, and samples at upper side should contain more HG with higher levels of DE. Furthermore, this may also suggest the differential impact of those enzyme preparations, instead of reducing the variation on the esterification levels, the different compositions in the enzyme mixtures are able to both work on any available de-esterified HG (through endo-polygalacturonase action for example) or equally on available esterified HG (through pectin lyase action for example), but the mixtures do not actively de-esterify so probably contain little in the way of these enzymes. This hypothesis can be supported by the enzyme-treatments shown in Fig. 6A, e.g. where most of XL-treated samples are situated in the opposite direction to Fruit-treated samples, which would suggest that Fruit enzyme mix contains more HG with low DE, and could suggest that XL enzymes work more efficiently on de-esterified HGs.

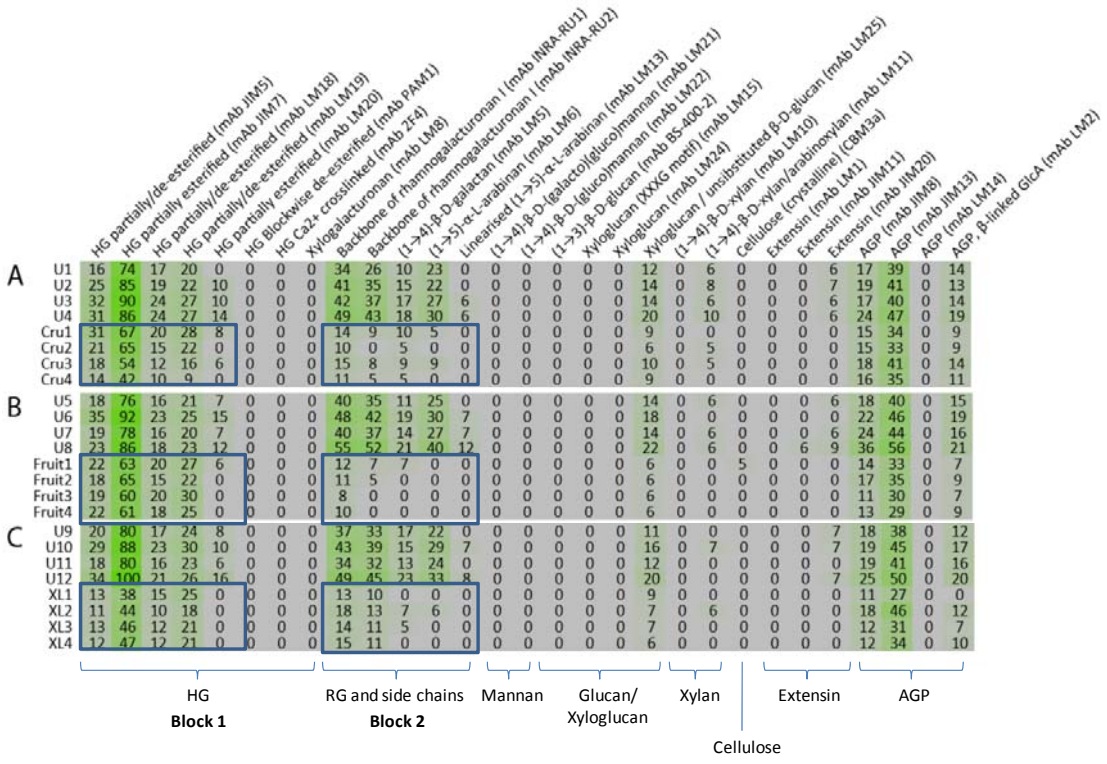

Figure 7. CoMPP (Comprehensive Microarray Polymer profiling) analysis of CDTA extract of cell wall samples. The heatmap showed the relative abundance of plant cell wall glycan associated epitopes present in AIR sourced from fermented berry pomace. **A.** U 1-4 and Cru 1-4; **B.** U 5-6 and Fruit 1-4; **C.** U 9-12 and XL 1-4. The highest signal was set as 100 and others were adjusted accordingly, the color intensity is correlated to the mean spot signal. A cut-off (<5) was applied to all heatmaps.

The heatmap (Fig. 7) was also generated to support the PCA plot data by representing the relative abundance of the epitopes in numerical form, the highest signal was set as 100 on the heatmap, and others were adjusted accordingly, a cut off (<5) was applied. As in the loading plot (Fig. 6B), variables were categorized into 8 groups and labeled on the heatmap. The CDTA fractions (heatmap in Figs. 7A, B, C) of fermented pomace mainly contains 4 main groups, including HG (JIM5, JIM7 and LM18, LM19, LM20), RG with its side chains (INRA-RU1, INRA-RU2 for RGI, LM5 for galactan, LM6 and LM13 for arabinan), Hemicelluloses (LM25 for xyloglucan), extensin (JIM20) and AGPs (JIM8, LM2 and LM14), these confirmed the previous study (Chapter 3) by research Gao *et al*, (2015) which validated the CoMPP approach in winemaking conditions. The high levels of HG confirmed the GalA data from GC-MS data (Figs. 5A, B, C). The ability of the added enzymes to effectively de-pectinate can also be observed in CoMPP heatmap where the abundance of HG epitopes in enzyme-treated samples (Cru, Fruit and XL) is markedly lower than those from untreated samples (U1-U12), and same phenomenon is observed for RGI epitopes, which supports the decrease in GalA and Rha in reported in Figs. 5A, B and C. AGP epitopes were also found to show markedly lower signals, specifically for JIM8, JIM13 and LM2, when enzymes were added which may correspond with the decline of Ara in the GC-MS data (Figs. 5A, B and C). The decrease of the Ara monomer in enzyme treated pomace can also be explained by the lower signals for mAbs LM6 and LM13 (branched and linearised arabinans respectively). There is also some decline observed for mAbLM25 (recognizes xyloglucan) in the enzyme-treated samples, despite the fact that CDTA extract contains mainly pectin. But it is known some xyloglucan associated with pectin will be co-extracted together and stay in this fraction, when the enzymes peel the pectin out of the cell wall, this associated xyloglucan will also be removed. This has been found, in a number of studies (e.g. Gao *et al*, 2015 and Zietsman *et al*, 2015), and may also support the idea of possible covalent bond between AGP, pectin and xyloglucan (Popper and Fry, 2008). A pectin, arabinoxylan and AGP cross-linked macromolecule has already been found recently in Arabidopsis leaves was found for example (Tan *et al*, 2013). Furthermore, the heatmap (Fig. 7) also supports the argument accounting for PC2 variation (due to changes in HG esterification levels) from the PCA and loading plots (Figs. 6A and B), by looking at the block 1 in HG part of the heatmap, it can be seen that the ratios of HG (high DE,

mAbs JIM7) to HG (low DE, mAbs JIM5) signal abundances are different among the different enzyme-treated samples, in XL, the ratio is approaching 4 (JIM7 to JIM5), but only approximately 3 in Cru and Fruit, these means the XL has worked on the de-esterified HG more effectively than other two treatments, and other two worked better on esterified HGs. Cross-comparing the PCA plot (Fig. 6) and the heatmap (Fig. 7) of CDTA fraction), we can summarize that the PC1 variation is mainly contributed by the RGI (block 2) and HG (block 1), and the PC2 variation is mainly contributed by the HG (block 1).

In Chapter 3, the berry pomace was shown to have two main fractions, an inner fraction which is dominated by the hemicellulose (mainly xyloglucan) and some strong associated pectin (HG and RG), coated by another pectin-dominated fraction (HG with high DE) (Gao *et al,* 2015), which probably has to be removed or unraveled to achieve a effective de-pectination. To test if the fermentation (with or without enzymes) causes the degradation of cell walls in hemicellulose dominant inner fraction, NaOH was used to extract the hemicellulose fraction from remaining pomace AIR after the CDTA extraction had been performed. Fig. 8 shows the PCA plot of the raw CoMPP data obtained from the NaOH extract samples of untreated and enzyme-treated pomace.

PCA analysis of the raw data generated from CoMPP analyses of NaOH fraction for these fermented pomace (with and without enzyme treatment), is shown in Fig. 8. The score plot (Fig. 8A), shows that the pomace samples have been clustered into very distinct groups (with little spread or variation as seen for the pectin samples). Both groups show the same data-cloud shape which appears driven by the combined influence of PC1 and PC2 together (Fig. 8A, B). PC1 explains by 41.8% variance and the main drivers causing the separation were identified to be mainly pectins (mAbs INRA-RU1, RU2, LM 18, 19) and cell wall proteins (mAbs JIM 8, 13 and LM2) on the same side as untreated pomace, whereasPC2 explained 19.3% of the variance with the main contributors being hemicellulose-related epitopes (glucan/xyloglucan, recognized by mAbs LM15, 25).

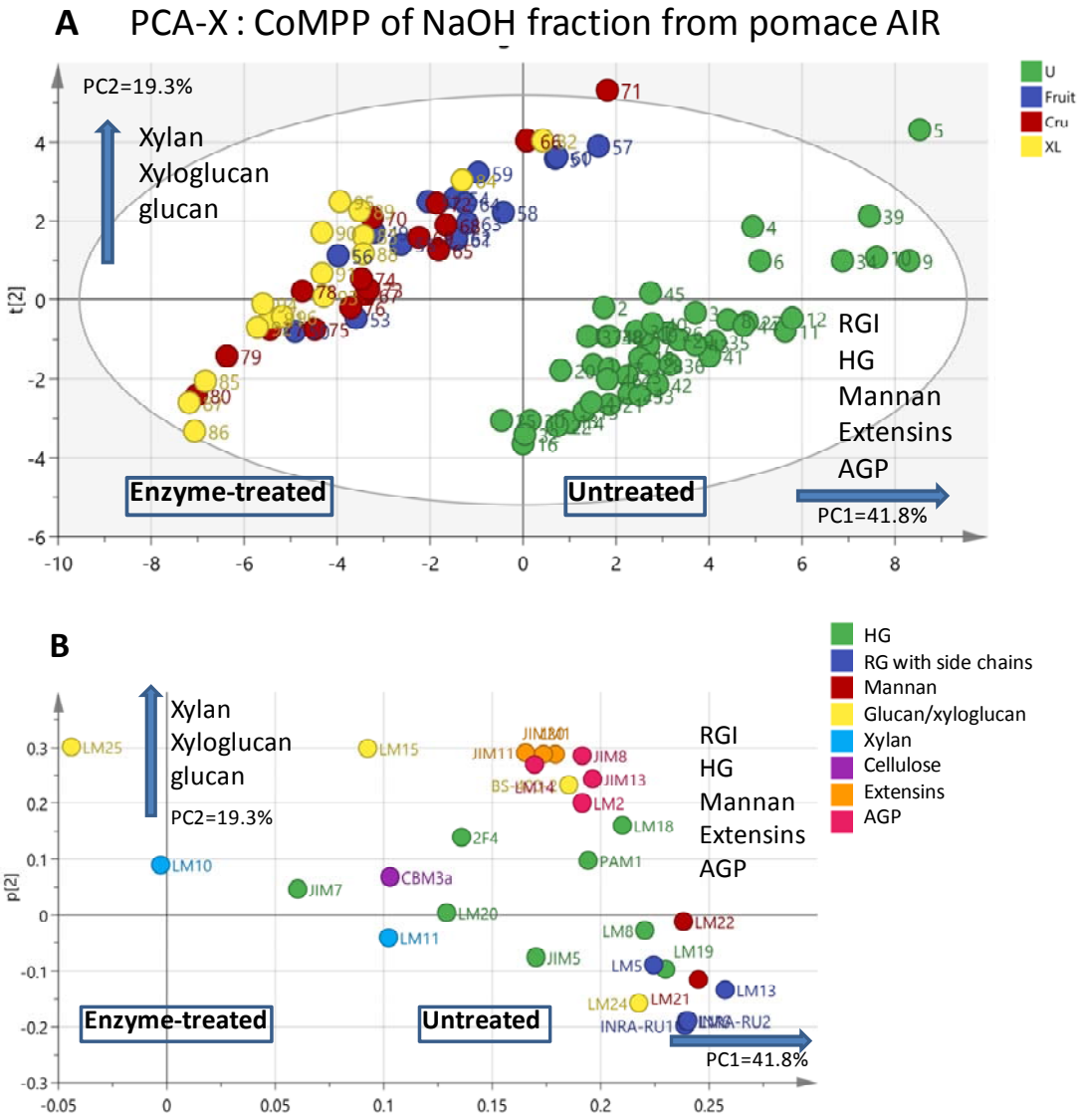

Figure 8. PCA score plot (**A**) and Loading plot (**B**) of the NaOH (hemicellulose-rich) extract from AIR sourced from fermented berry pomace. Untreat – untreated fermentation, Fruit – Lafase Fruit, Cru – Lafase HE Grand Cru, XL – Lafase XL Extraction. The color is according to the treatment in **A,** and to the different groups of cell wall polymers in **B.**

Interestingly, the variation of the epitope abundance of this fraction of untreated pomace is not as high as that which is found in the CDTA fraction (Fig. 6), which probably means that the main variation of total berry pomace cell wall data is mainly contributed by the pectin-rich fraction, and the structure of hemicellulose-rich fraction is fairly constant between grape cell walls within the vineyard scenario. This can be supported by a number of studies on cell wall changes in fruit ripening, which strongly show that the decline of galactans (Lackey *et al*, 1980, Vicente *et al*, 2007a, b, Gribaa *et al*, 2013, Moore *et al*, 2014a)

and the de-esterification of pectins (Fischer and Bennett, 1991, Lecas and Brillouet, 1994, Vicens *et al*, 2009) are the major players in grape berry cell wall changes. The major hemicellulose-related loading variables driving the variation in the NaOH fractions of treated from untreated pomace are mAbs LM15 and LM25 (i.e. xyloglucan epitopes).

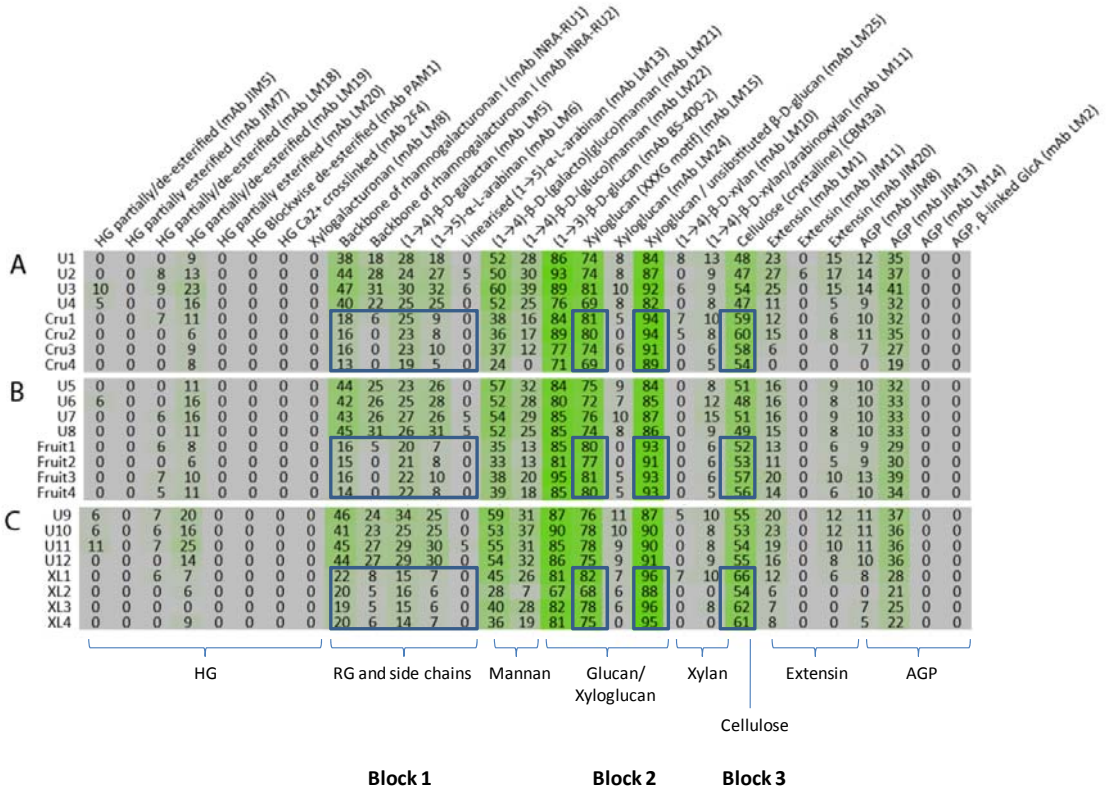

Figure 9. CoMPP (Comprehensive Microarray Polymer profiling) analysis of NaOH extract from cell wall samples. The heatmap showed the relative abundance of plant cell wall glycan associated epitopes present in AIR sourced from fermented berry pomace. **A.** U 1-4 and Cru 1-4; **B.** U 5-6 and Fruit 1-4; **C.** U 9-12 and XL 1-4. The highest signal was set as 100 and others were adjusted accordingly, the color intensity is correlated to the mean spot signal. A cut-off (<5) was applied.

The heatmap of NaOH extract of the treated and untreated samples (Fig. 9A, B and C) identified the epitopes associated with the main hemicellulosic polymers corresponding to xyloglucans (mAbs LM15, LM24 and LM25), glucans (mAbs BS-400-2), mannans (LM21 and LM22), crystalline cellulose (but this also recognizes xyloglucan) (e.g. CBM3a), and this generally confirms the exposure of these epitopes, particularly xyloglucan-cellulose and mannans. The pectic epitopes 'coating' this layer of xyloglucan-cellulose rich cells, as explained in Chapter 3 (Gao *et al,* 2015), were also detectable on the heatmap (Fig. 9).

These coating pectic epitopes were also detected and show up in the heatmap (Fig. 9), including as RGI (mAbs INRA-RU1 and INRA-RU2) and its side chains (mAbs LM5 for galactans, LM6 and LM13 for arabinans), as well as the low abundance of HG epitopes (mAbs JIM5, LM18 and JIM19), however, HG with high esterification degree were not identified, but this is unsurprising as the NaOH saponified the pectins during the extraction (as discussed in Chapter 3; Gao *et al*, 2015). These data confirmed the hemicellulose-rich fraction with strongly associated pectins; an explanation could be the presence of strong bonds between pectin and xyloglucan (Cumming *et al*, 2005, Popper and Fry, 2008), and/or the tight hydrogen bonding between the RGI side chains and cellulose microfibrils (Zykwinska *et al*, 2007). Apart from the wall polysaccharides, cell wall proteins were also detected in the NaOH fraction (e.g. AGPs recognized by mAbs JIM13 and LM14; and extensins recognized by mAbs LM1 and JIM20. Comparison of enzyme-treated versus untreated fermented pomace from CoMPP heatmap reveals that all the enzyme-treated have lower epitope abundance for HG, RGI and its side chains, while a slight increase in xyloglucan epitopes (e.g. mAb LM25) would support exposure of hemicellulose and cellulose by enzyme action. . The difference of epitope abundance between untreated and enzyme treated pomace NaOH extract supports the evidence of two fractions in berry pomace cell wall (see Chapter 3, Gao *et al*, 2015). The inner hemicellulose structure of pomace cell wall is strongly bound with tightly-associated pectin (mainly RGI with its side chains), and masked by a pectin-rich fraction (mainly highly esterified) (Gao *et al*, 2015). To penetrate into cell, the outer pectin layer has to be removed to some extent, in order to let the enzymes penetrate into hemicellulose structure. In this study, the CoMPP heatmap (Fig. 7) and PCA plot (Fig. 6) of CDTA (pectin-rich) fraction showed that the commercial enzyme preparations facilitated the de-pectination, thus, the inner hemicellulose fraction became more exposed after removal of pectin fraction. Eventually, the enzymes worked on and removed some of the tightly-bound pectin on hemicellulose and increase the epitope exposure of the associated polymers, such as xyloglucan and cellulose (mAbs LM15, LM25 and CBM3a). The mannans in the NaOH (recognized by mAbs LM21 and LM22) in this fraction also showed a marked decrease (Fig. 9) in enzyme treated fraction versus the untreated samples. Mannans have few known roles in plant physiology, such storage functions in the seeds (Reid *et al,* 2003), however, they only

account a small proportion in dicot primary cell wall, but relatively higher in secondary cell wall in coniferous plants (see Benova-Kakosova *et al*, 2006). The differential impact of the enzymes were again supported by the heatmap, PC1 is mainly driven by the RG1 variables (in block 1), while PC2 is mainly driven by glucan/xyloglucan epitopes (in block 2) and cellulose (in block 3). It can also be seen that XL treatment resulted in more exposure of hemicellulose and cellulose than the other treatments, but it did not work as effectively as the others on RGI, this could mean XL has less activity against the 'hairy" region of pectin (RGI and esterified HG, De Vries *et al*, 1982) compared to the other treatments.

CONCLUSION

Taken overall, this is the first study, as far as we aware of in the literature, that looks into the intra-vineyard variation, of the grape berry cell wall biochemistry structural composition at harvest, using a chequered-board experimental design approach using vine panels (Young *et al,* 2015). , We found even though a number of factors (such as row orientation, vigor, water treatment had been controlled for) and a seemingly visually invariant vineyard block had been chosen, there was still significant variation in sugar levels (up to a 5 degrees difference in Brix levels) at panel resolution in harvested grapes. This variation was inevitably brought into winemaking which was also performed at panel resolution in triplicate. Just observing the cell wall changes between untreated panels within the vineyard it was clear that the riper berries (where more cell wall degradation had taken place) had clear evidence of de-pectination from the fermentation itself; as had been previously observed (Zietsman *et al,* 2015a).

The effect of the enzyme addition seemed for the most part to increase the uniformity of the panel treated wines, except for Cru treatment, when standard oenological parameters were tested for. What was quite clear from the study is that the pectinases present in all three enzyme preparations worked effectively of significantly reducing the pectin variation in berries compared to untreated controls; hence a significant de-pectination occurred, bringing all berries to a uniform degree of de-pectination even with 5 degrees Brix differences between some panels. Although the enzymes preparations de-pectinated very well, they did

not reduce the variation in esterification levels of the grape pectins in all three treatments. This is very important as no methanol or acetates would have been released from the process which could have detrimentally affected wine quality. The alkali fraction showed that all enzymes equally opened up the hemicellulose component, exposing the inner layers of the grape tissue closest to the skin cells. This further supports the argument for two layers in the pomace fractions (see Chapter 3, Gao *et al,* 2015).

Thus, it is clear that enzymes are quite important for winemaking when significant variation exists in the vineyard, and winemakers may wish to harvest early to ensure consistency of the final wine; and to remedy lower tannins and pigments by longer maceration approaches; depending on the desire for high volume with consistent quality or being more risk adverse due to changing environmental influences around the harvest period, to rather get lower volumes of better uniformity and 'typicity' for their wines.

This study has taken us further in understanding the interaction of berry cell walls and enzymes during winemaking, showing the positive effects of commercial enzyme mixes on reducing variation and consistent quality from natural variation within vineyards. These enzymes are however still semi-purified mixtures from crude fungal extracts. In order to take this work further into providing scientific understanding of the relationship between grape berry cell wall structure and enzyme action under winemaking conditions; the use of highly pure enzyme preparations are needed to understand which enzymes in the mixtures contribute to the efficacy of the unraveling and de-pectination process *in vino*.

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Authors' contribution

Y. Gao, J. Moore and M. Vivier designed the study, Y. Gao performed the experiments and collected the data. Y. Gao interpreted the results and drafted the manuscript. J. Moore and M. Vivier advised with data interpretation and manuscript preparation. J. Fangel and W. Willats performed the CoMPP analysis. All authors read and approved the final manuscript.

Supplementary data

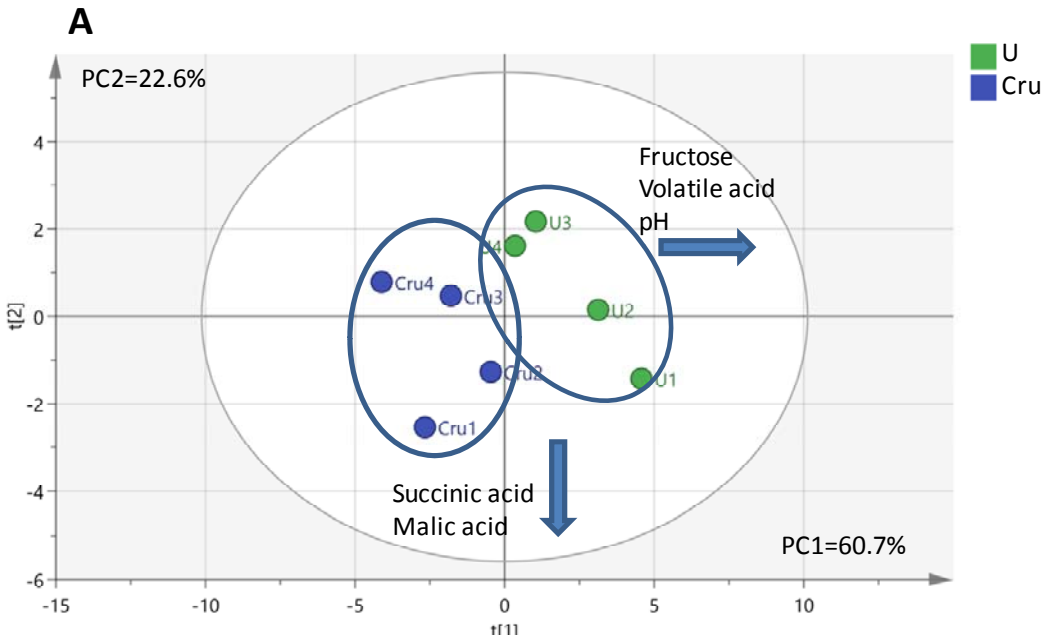
Table 1. General wine parameters of the final wine acquired from the fermentation in different condition. The values for each parameter are the mean values from two technique repeats performed by the FOSS Winescan.

Sample Id	pH	VolatileAcid	TotalAcid	MalicAcid	LacticAcid	Glucose	Fructose	Ethanol	Glycerol	Succinic Acid	Tartaric acid	Acetic Acid
U1	3.55	0.6	6.59	2.57	0.01	-0.6	1.09	12.57	9.17	2539.81	454.24	908.01
U2	3.5	0.55	6.66	2.56	0.01	-0.43	1.19	13.12	9.5	2536.71	499.94	816.55
U3	3.48	0.5	6.66	2.57	-0.01	-0.29	1.16	13.9	9.81	2453.9	655.42	713.35
U4	3.48	0.43	6.6	2.61	0.05	-0.02	1.12	13.98	9.56	2445.03	692.89	624
Cru1	3.42	0.23	6.81	2.8	0.13	-0.21	0.99	12.99	9.72	2503.62	884.85	401.49
Cru2	3.43	0.37	6.74	2.68	0.11	-0.41	1.16	13.16	9.47	2529.46	815.65	556.68
Cru3	3.44	0.31	6.72	2.69	0.11	-0.05	1.2	13.36	9.74	2452.65	833.97	476.97
Cru4	3.36	0.31	6.94	2.7	0.08	0.14	1.09	13.52	10.13	2436.41	986.76	477.83

Sample Id	pH	VolatileAcid	TotalAcid	MalicAcid	LacticAcid	Glucose	Fructose	Ethanol	Glycerol	Succinic Acid	Tartaric acid	Acetic Acid
U9	3.42	0.47	6.8	2.64	0.09	-0.08	0.95	12.84	9.57	2598.66	690.15	697.15
U10	3.36	0.41	6.85	2.73	0.1	-0.21	1.02	13.03	9.47	2554.7	854.16	617.51
U11	3.32	0.32	6.74	2.81	0.1	0.27	3.95	13.33	9.73	2426.83	997.47	482.62
U12	3.38	0.35	6.6	2.76	0.11	-0.04	3.42	13.38	9.42	2505.23	827.45	544.96
XL1	3.33	0.24	7.07	2.9	0.15	-0.03	1.16	13.62	10.04	2562.39	1011.05	389.88
XL2	3.31	0.19	7.05	2.96	0.2	0.06	2.41	13.31	9.88	2532.37	1074.16	356.96
XL3	3.31	0.13	6.94	3.21	0.12	0.03	7.73	13.36	9.95	2496.97	997.52	305.96
XL4	3.34	0.19	6.97	3.09	0.07	0.1	5.63	13.29	10.12	2507.48	994.85	373.67

Sample Id	pH	Volatile Acid	TotalAcid	MalicAcid	LacticAcid	Glucose	Fructose	Ethanol	Glycerol	Succinic Acid	Tartaric acid	Acetic Acid
U5	3.43	0.53	6.89	2.49	0	-0.14	1.09	13.66	9.92	2653.17	607.88	803.9
U6	3.39	0.55	6.88	2.46	0.07	0.03	1.18	13.73	9.83	2561	713.99	792.65
U7	3.38	0.44	6.72	2.68	0.11	0.02	0.89	12.99	9.27	2511.43	845.22	640.89
U8	3.41	0.33	6.72	2.92	0.2	-0.12	0.88	12.93	9.25	2528.77	827.86	509.85
Fruit1	3.31	0.39	6.81	2.58	0.09	-0.13	1.09	12.73	9.05	2532.93	916.09	579.16
Fruit2	3.33	0.26	6.76	2.75	0.07	-0.01	1.12	13.22	9.36	2469.67	1079.5	390.5
Fruit3	3.34	0.23	6.85	2.81	0.11	0.14	1.22	13.36	9.6	2502.92	1006.66	368.52
Fruit4	3.33	0.29	6.91	2.9	0.13	0.04	1.21	13	9.62	2511.47	991.04	441.61

PCA-X: General Oenological Parameters



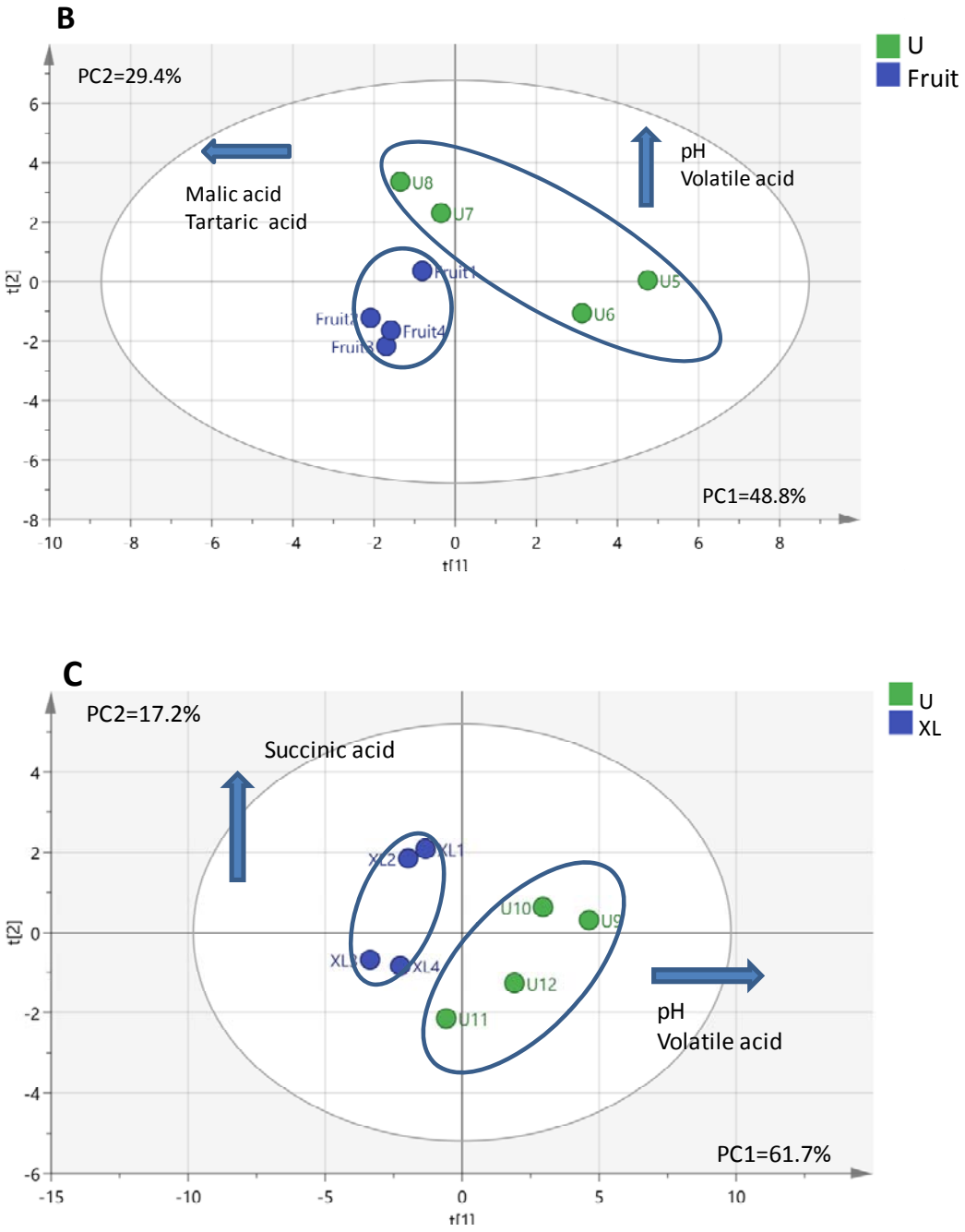


Figure 1. PCA score plots of oenological parameters from final wine of different fermentation (untreated and enzyme-treated). **A.** U1-4, Cru1-4; **B.** U5-8, Fruit1-4; **C.** U9-12, XL1-4. The value for each variable (wine parameter) is mean value generated from two technique repeats performed by the FOSS Winescan.

Chapter 5

Research results

Using combinations of recombinant pure pectinases to elucidate the deconstruction of the polysaccharide-rich grape cell wall during winemaking

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Carbohydrate Polymers

Using combinations of recombinant pectinases to elucidate the deconstruction of the polysaccharide-rich grape cell wall during winemaking

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Key words

Pure recombinant enzymes, fermentation, grape pomace, multivariate data analysis, grape berry cell wall structural model.

Abstract

The effectiveness of enzyme-mediated maceration processes in red winemaking relies on a clear picture of the target (berry cell wall structure) to achieve the optimum combination of specific enzymes to be used. However, we lack the information on both essential factors of the reaction (i.e. specific activities in commercial enzyme preparation and the cell wall structure of berry tissue). In this study, the different combinations of pure recombinant enzymes and the recently validated high throughput cell wall profiling tools were applied to extend our knowledge on the grape berry cell wall polymeric deconstruction during the winemaking following a combinatorial enzyme treatment design. The multivariate data analysis on the glycan microarray (CoMPP) and gas chromatography (GC) datat revealed that the pectin lyase performed as effectively as commercial enzyme preparations in de-pectination of berry cell walls, and the combination of endo-polygalacturonase and pectin methyl esterase did not degrade the pectin as we predicted, but rather unraveled it. The combinations that contained other enzymes were shown to degrade side chains, but not de-pectinate and de-polymerise, also provided useful and new information on the complexity of the grape berry cell wall architecture. By adding the information acquired from this study to previous berry cell wall studies, a hypothetical model describing cell wall structure of different tissue types of grape berry was established. This model can aid us in a number of future studies apart from winemaking, such as fruit development and ripening and plant pathogen interactions of grapes. Most importantly it provides testable hypotheses for future studies on grape berry deconstruction using wine enzymes tailored for specific applications in winemaking.

1. Introduction

Grape berries contain a number of nutritious (health beneficial) and flavour enhancing (aroma components etc.) compounds which are shown to be mainly localized in the vacuole(s) of berry skin cells (Bindon *et al*, 2014). These compounds include anthocyanins, proanthocyanins (i.e. grape tannins), stilbenes (e.g. resveratrol), aromatic terpenes etc. (González-Barreiro *et al*, 2015); and are considered the the main chemical constituents strongly influence the overall perceived quality parameters (e.g. typicity) by contributing to the colour, flavour, body, mouth-feel, storage and ageing potential, etc. of the wine produced (Garrido and Borges, 2013, González-Barreiro *et al*, 2015). Release of these favourable compounds relies heavily on the efficiency and the control of the berry cell wall deconstruction process (Gao *et al*, 2015; Zietsman *et al*, 2015a). The maceration process during the fermentation is tailored by the winemaker to achieve optimal extraction of these metabolites and macromolecules from the pooled harvested berries into the alcoholic fermentation (AF) during of the conversion must into wine (Ortega-Heras *et al*, 2012, Cholet *et al*, 2014). The maceration process, mainly in red winemaking, involves fermenting berry skins (i.e. cap) with must (i.e. pomace and juice) being punched down several times a day during the AF (Ortega-Heras *et al*, 2012, Cholet *et al*, 2014). In addition, a number of techniques, involving the manipulation of temperature, and/or the physical (e.g. pulsed electric treatment) treatment, have been applied to enhance the maceration output (i.e. by measuring indirect factors such as colour) (Cholet *et al*, 2014). However, these techniques are often inconsistent, although providing benefits some of the time, several side-effects do exist in the maceration process, such as the unintended production of high volatile acidity and off flavours which spoils the wine leading to significant economic losses (Revilla *et al*, 1998, Sacchi *et al*, 2005, Fia *et al*, 2014).

Commercial enzyme preparations have been added during the maceration process to aid the cell wall degradation and release of favourable compounds for many years (Ortega-Heras *et al*, 2012, Romero-Cascales *et al*, 2012). However, the general scientific understanding how these enzymes, which are produced from wood-rot fungi, act on grape berries (a very different biochemical and chemical matrix) is far from clear (Gao *et al*, 2015; Zietsman *et al*, 2015a). There is much unknown about the enzyme action, inferred from studies on other

species and tissues (not grapes), which may not apply. We for example do not have sufficient information on target grape cell wall polymers and epitopes for enzymes to act on, although this has been remedied partially with studies (see chapter 3, Gao *et al*, 2015; Zietsman *et al*, 2015a, 2015b). Recent research has shown the effects can vary by using different commercial enzyme preparations on the same berry lots; and that the general ripening level of the harvested grapes had a direct effect of efficacy of the enzyme preparations, even where the main enzyme was purported to be (endo-polygalacturonase; ePGase) (Zietsman *et al*, 2015a)., We have also observed using a spatial experimental design intra- vineyard variation of harvested grape lots in combination relationships with AFs using three commercial enzyme preparations (unpublished data, see Chapter 4) showing the complex but significant variation reduction achieved using enzyme additions. However, crude semi-purified enzyme preparations may still have unwanted side-activities which could negatively impact the wine processing and final quality (Fia *et al*, 2014). Hence, more scientific knowledge of berry cell wall architecture can help the researchers design more customisable enzyme preparations; possibly even at the cultivar level, providing tailor-made solutions for winemakers, to achieve optimal maceration, but also importantly advancing our fundamental understanding of grape berry plant cell wall structure-function relationships at a polymer architectural level.

Generally, the plant cell wall is commonly associated with a static barrier which acts to protect the enclosed cell from outer stresses and contributes the plant's hydrostatic skeleton (Brett and Waldron, 1996), however, more evidence shows the dynamic nature of a changing plant cell wall responding to biotic and abiotic stressors, as well as developmental cues (Albersheim, 2009). Even though the grape berry cell wall has been intermittently studied for few decades using various classical carbohydrate biochemistry techniques, such as gas chromatography, spectroscopy, and size exclusion chromatography, with the general cell wall polymer inferred from these investigations (Saulnier and Thibault, 1987, Nunan *et al*, 1998, Nunan *et al*, 2001, Doco *et al*, 2003), as well as attempts to cell wall composition with polyphenol extraction during winemaking (Arnous and Meyer, 2010, Bindon *et al*, 2014, Hernández-Hierro *et al*, 2014). There is generally a limited of understanding of the more intricate fundamental architectural nature of the grape cell wall. Several general plant cell wall cross-linking models of cell wall have been proposed through data collected on various

plants species including *Arabidopsis thaliana* (Somerville 2004 ;Coenen *et al*, 2007, Popper and Fry, 2008, Park and Cosgrove, 2012), however, these models are constantly undergoing re-evaluation as new data is generating new hypotheses concerning our pre-conceived ideas about plant cell wall architectures (<xxt double mutant Chevalier and Keegstra, 2010>, Vincken *et al*, 2003, Zykwinska *et al*, 2007). It is important to consider that cell wall structure and composition varies among the species (Carpita and Gibeaut, 1993) and within different plant organs and tissues of the same species (Somerville *et al*, 2004). As limited studies have been performed on grape cell walls, as opposed to other species, it was very important to obtain more information on cell wall architecture in this species in the context of maceration and winemaking. Cell wall profiling approaches has been validated on grape leaves, grape berries and winemaking studies to directly probe changes in cell wall polymer organization and architecture (Moore *et al*, 2014a, 2014b, Gao *et al*, 2015, Zietsman *et al*, 2015a, 2015b), the information provided from these studies not only confirmed the datasets acquired using classical techniques, but through the addition of CoMPP technology has generated a significant amount of new knowledge on subtle changes at the polymer epitope level. However these profiling and fractionation methods alone have not brought us to a fuller understanding of the role of carbohydrate active enzymes (and their combinations) in disrupting and deconstructing grape cell wall architecture during the winemaking process. For this we need a more detailed combinatorial experimental design and study of enzyme action on grape cell walls.

In chapter 3 of this thesis (published Gao *et al*, 2015) we validated the use of chemical fractionation combined with CoMPP to deconstruct the wine polysaccharides and bulk pomace released during a standard red wine fermentation (using a clarification enzyme) made from Cabernet Sauvignon grapes. In chapter 4 we evaluated the effect of commercial (crude semi-purified mixtures of carbohydrate active enzymes) on the inter-vineyard variation (using a checkered board design) of grapes harvested from the Cabernet Sauvignon experimental farm and showed a significant reduction in cell wall variance through the action of these enzymes, Now we wish to probe more deeply into the architectural structure of a grape matrix undergoing cell wall deconstruction. The special focus of this study is the novel application of various combinations of pure recombinant pectinase enzymes, with a

commercial enzyme preparation (maceration) as a control in the winemaking experiments. The choice of different enzyme combinations was chosen based on our current knowledge of grape berry cell wall polymeric composition and architecture. The aim of this study, was to use the combinatorial data generated from using the high-throughput cell wall profiling tools to understand the successive cell wall enzyme targets during winemaking and in so doing to build a model berry cell wall architecture from which hypotheses can be generated and subsequently tested.

2. Material and methods

2.1 Vinification and Maceration

Grape berries (*Vitis vinifera* cv. Cabernet Sauvignon) were harvested from the Welgevallen experimental vineyard (33°56'42"S, 18°51'44"E, Department of Viticulture and Oenology), Stellenbosch University, South Africa. The vineyard is arranged in a north-south orientation and trained on a seven-wire vertical trellis system, with drip-irrigation system. The Brix° level for the harvest was 24 (sugar content approx. 275 g/l), ascertaine during standard viticulture practise (random selection from vineyard, see chapter 4). The harvested berries were pooled over the whole vineyard, in order to emulate a typical commercial harvest and winemaking process, and then split into separate buckets (5 kg each), and then crushed (with de-stemmed) separately before individual fermentations. Sodium bisulfate (SO_2) was added (30 ppm) into each bucket after crushing to prevent the growth of spoilage microorganisms, again standard winemaking practise. *Sacchromyces cereviseae* commercial strain VIN13 (Anchor Yeast, Cape Town, South Africa) at 0.2 g/l (rehydrated and prepared following the manufacturer's directions) was inoculated into each bucket. To each of the buckets were added different combinations of recombinant enzyme(s) (sourced from Novozymes, Denmark); buckets were inoculated in triplicate for statistical reproducibility. These recombinant enzyme additions were (1) the enzyme-free negative control, (2) EPG (Endo-polygalacturonase), (3) EPM (Endo-polygalacturonase plus Pectin Methyl esterase), (4) PL (Pectin Lyase), (5) ARA (Endo-polygalacturonase, pectin methyl esterase and Arabinanase), (6) GAL (Endo-polygalacturonase, pectin methyl esterase and Galactanase) and (7) Grand cru a commercial preparation from Laffort (Bordeaux, France) (which acted as our positive

control). Information on mode of action of the enzymes was listed in Table 1. As stated all recombinant enzymes are from Novozymes (Denmark) and the dosage of enzyme added was according to the the manufacturer's instructions (i.e. effectively overdosed). The wine was fermented at 25°C for approx. 10 days until the sugar level approached zero (<5 g/l), and then pressed to separate the fermented skins and pulp (pomace) from the free-run wine. The pomace samples were selected to be representative by a composite sampling approach from each bucket following the Theory of Sampling (Petersen *et al,* 2005), while the wine was stored at -4 °C until further analysis.

Table 1. The enzyme combinations used in this study ("Minus" mark refers to no, "Plus" mark refers to Yes). Untreated (U) with no additional enzymes is used as negative control, Lafase HE Grand cru (Cru) is used as positive control.

Treatment	Endo-polygalacturonase (EPG) EC 3.2.1.15	Pectin methyl esterase EC 3.1.1.11	Pectin lyase EC 4.2.2.10	Endo-arabinanase EC 3.2.1.99	Endo-galactanase EC 3.2.1.89
U	–	–	–	–	–
EPG	+	–	–	–	–
EPM	+	+	–	–	–
PL	–	–	+	–	–
ARA	+	+	–	+	–
GAL	+	+	–	–	+
Cru	Unknown protein content. Description from manufacturer: Pectolytic enzyme preparation, purified in CE for the production red wines that are rich in colouring matter and structured tannins, destined for ageing(Laffort, France).				

2.2 **Cell wall preparation from experimental pomace**

The pomace samples from each fermentation were de-seeded, and then milled in liquid nitrogen using a Retsch Mixer Mill (30 round/minute, 30 seconds, Retsch, Haan, Germany). The resulting powder was incubated in 80 % v/v ethanol at 95 °C for 15 min to deactivate any endogenous enzymes, thereafter the residual milled ethanol precipitated pellets were washed by a series of organic solvents (methanol, chloroform, acetone, as outlined in the methods of chapter 3), following solvent treatment the pelleted material was resuspended in dH_2O and freeze-dried to yield an alcohol insoluble residue (AIR) powder for subsequent analysis.

2.3 **Monosaccharide composition analysis using gas chromatography**

To analyse and compare the bulk chemical degradation of the cell walls; AIR sourced from pomace of each fermentation was analysed using gas chromatography combined with mass spectrometer (GC-MS) to determine their cell wall monosaccharide composition, the method is clearly described in chapter 3 of this thesis. Briefly, AIR samples were hydrolysed using trifluoroacetic acid (TFA, 2M) at 110 ^{o}C for 2 hours, and then the liberated monosaccharides were transformed to their methoxy forms using 1M methanolic HCl after incubation at 80 ^{o}C for 16 hours. Silylation reagent (HMDS+TMCS+Pyridine, 3:1:9, Supelco, USA) was applied for 20 min at 80 ^{o}C to generate the silylated sugar derivatives which were then dissolved in analytical reagent grade cyclohexane. GC-MS (Hewlett Packard 5890series II) was used to analyse nine monosaccharides: arabinose (Ara), rhamnose (Rha), fucose (Fuc) xylose (Xyl), mannose (Man), galacturonic acid (GalA), galactose (Gal), glucose (Glc) and glucuronic acid (GlcA), each analysis consisted of 3 technical replicates.

2.4 **Infra-Red (IR) spectroscopy of wines for determining oenological parameters**

A calibrated spectroscopic method was used on all experimental wines to confirm the consistency of all ferments performed in the experimental plan. In order to analyse the main enological parameters, the wines (50 ml in triplicate from each fermentation) were analysed using Fourier transform infrared (FT-IR) spectroscopy using a WineScan FT120 Basic instrument (Foss Analytical, Hillerød, Denmark). The oenological parameters tested were: pH, volatile acidity, total acid, glucose + fructose, and ethanol levels. The scanning was performed in duplicate per sample (with two technical repeats).

2.5 **Comprehensive microarray polymer profiling (CoMPP) of cell wall samples**

To analyse and compare the degradation of the cell wall polymers by virtue of changes in their epitope abundance, AIR sourced from fermented pomace from each of the fermentations was sequentially extracted first with CDTA (diamino-cyclo-hexane-tetra-acetic acid) and then with NaOH (as described in Moller *et al*, 2007); to obtain a pectin-rich fraction and a hemicellulose-rich fraction respectively. Extracted liquid fractions, after centrifugation,

were printed on nitrocellulose membranes and then probed with a series of monoclonal antibodies (mAbs) and carbohydrate binding module (CBMs) (see chapter 2, literature review). The raw data generated were used for multivariate data analysis, and were also separately normalized for creating the microarray heatmaps, the highest signal was set as 100, and others were be adjusted accordingly, a cut-off at 5 was applied.

2.6 **Univariate and multivariate data analysis**

The Statistica progarmme (Statsoft, Sandton, South Africa) was used for perform the univariate data analysis on the monosaccharide composition data, in order to ascertain statistically significant changes between the treatments (using ANOVA, p=0.05). Multivariate data analysis was performed on CoMPP raw data imported directly into the SIMCA 14 software package (Umetrics AB, Umea, Sweden). SIMCA 14 (Umetrics AB, Umea, Sweden) was used for principal component analysis (PCA) and generating the OPLS correlation matrix model for verification of treatments (Zietsman *et al*. 2015a). Orthogonal projections to latent structures (OPLS) models were used to focus on the differences between each enzyme treatment effect versus its control (a series of OPLS models were created and using correlation loadings were represented as PCA plots.

Results and Discussion

To provide more insight into the manner in which grape berry cell wall polymers may associated with each (covalently and non-covalently) architecture and how this (these) structure(s) are unraveled (Zietsman *et al*, 2015b) and degraded (Chapter 2, Gao *et al*, 2015) by carbohydrate enzyme mixtures we performed in this combinatorial enzyme design experiment. High-throughput cell wall profiling tools with multivariate tools allowed us to dissect how each enzyme combination acted during the ferment. A number of factors were taken into consideration for the design; such as the fact that

Most commercial enzyme blends claim that the endo-polygalacturonase (EPG) is the core enzyme, thus a fermentation treatment with only pure recombinant EPG was included. Furthermore, considering that berry pectin is highly methyl esterified (Gao *et al*, 2015), and also the presence of Rhamnogalacturonan I and its side chains (linearised and branched

arabinans, galactans) were detected (Gao *et al*, 2015), other combinations were also applied which included pectin methylesterases, pectin lyases, arabinanases and galactanases; for the coding system EPG, PME, PL, ARA, GAL in different enzyme "cocktails" (see materials methods section 2.1.)

Infrared spectroscopic data confirmed consistency of fermentations amongst the enzyme treatments and controls

To ensure that standard oenological parameters of the fermentations were similar between treatments and controls before commencing the process of cell wall analysis, the final wines from the seven treatments scanned spectroscopically using a FOSS winescan. These quality control checks included a number of general wine parameters and these data are shown in supplementary Table 1. Generally, the wine parameters showed evidence of a consistent fermentation without any faults evident, such excessive volatile acidity, meaning the ferments reflected well a standard red winemaking process albeit on an experimental scale. This ensured confidence that these fermentations and the pomace were suitable for comparative analysis via high-throughput cell wall profiling tools. Interestingly, the multivariate data analysis of the spectroscopic data grouped these treatments into several clusters (Supplementary Figs. 1A and 1B.) based on variables such as pH and sugar etc.., the significance of this is unclear.

Significant de-pectination observed by PL and Grand Cru as determined by monosaccharide composition analysis

Gas chromatography is a useful quantitative method to determine the cell wall monosaccharide composition of the plant samples, the information on specific cell wall polysaccharide levels is generally inferred from these bulk chemistry data. In this study, AIR samples sourced from fermented de-seed pomace was hydrolysed, and the released monosaccharides (nine main cell wall sugars) from each sample were derivatized and

analysed using gas chromatography. Fig. 1 showed the concentration of nine main monosaccharides in AIR (mg/g in dry mass) from the 7 fermentations (3 biological repeats), in general, all the AIR reveals an abundance of GalA (ca. 180-250 mg/g), with the presence of Ara (ca. 40-50 mg/g), Gal (ca. 30-40 mg/g) and Rha (ca. 15-20 mg/g) representing the main pectin constituents. In addition; Xyl (ca. 25-40 mg/g), Man (ca. 60-80 mg/g) and Glc (ca. 40-60 mg/g) concentrations reflected hemicellulosic polymers, such as xyloglucan and mannans. These compositions are similar to the recent studies on fermented berry cell walls (Gao *et al*, 2015 (chapter 3), and Pinotage skin in Zietsman *et al*, 2015a), confirming earlier data showing that the berry cell wall is rich in pectin (Saulnier and Thibault, 1987).

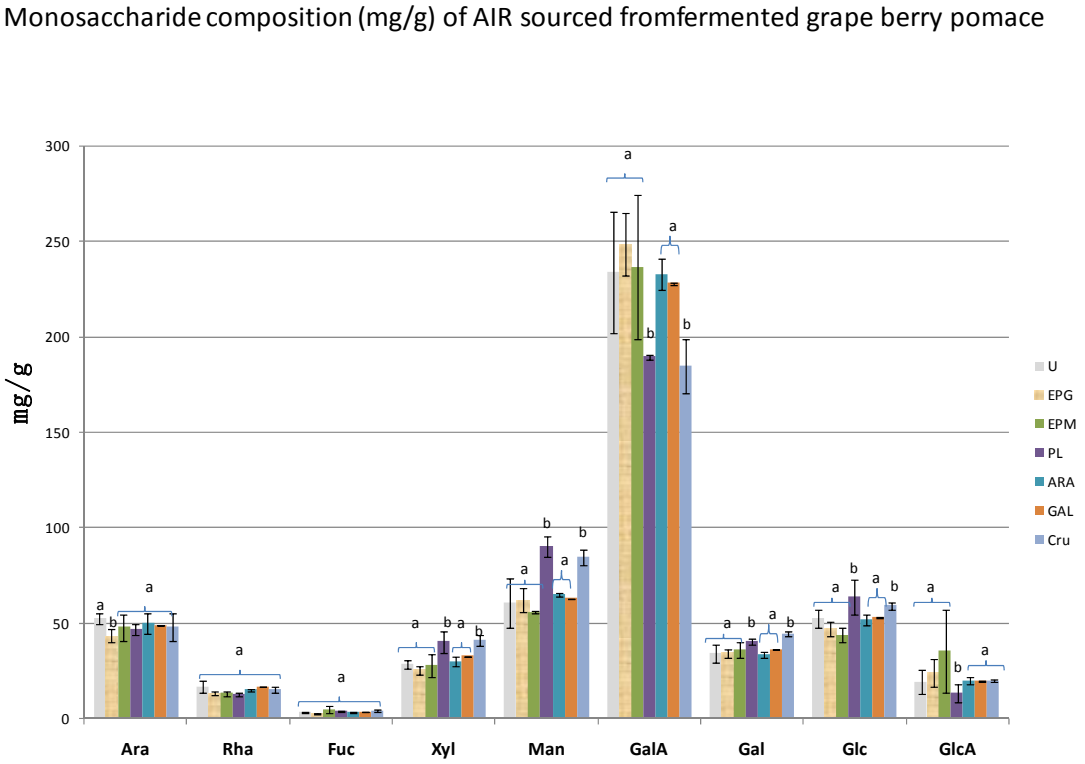


Figure 1. The monosaccharide compositions of AIR sourced from fermented pomace of seven fermentation treatments (Legend codes: Untreated represented as (U), Endo-polygalacturonase as (EPG), EPG + Pectin Methyl Esterase as (EPM), Pectin Lyase as (PL), EPG+PME+Arabinanase as (ARA),EPG+PME+Galactanase as (GAL), Laffort HE Grand Cru as (Cru)). The composition is expressed in mg/g (dry weight of AIR samples), the monosaccharide abbreviations are: Ara: arabinose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; GalA: galacturonic acid; Man: mannose; Gal: galactose; Glc: glucose; GlcA: glucuronic acid. Error bars represent the standard derivation of the mean value of three biological repeats; different letters indicate a statistically significant difference between treatments for a specific monosaccharide, (95% confidence level, ANOVA, p=0.05).

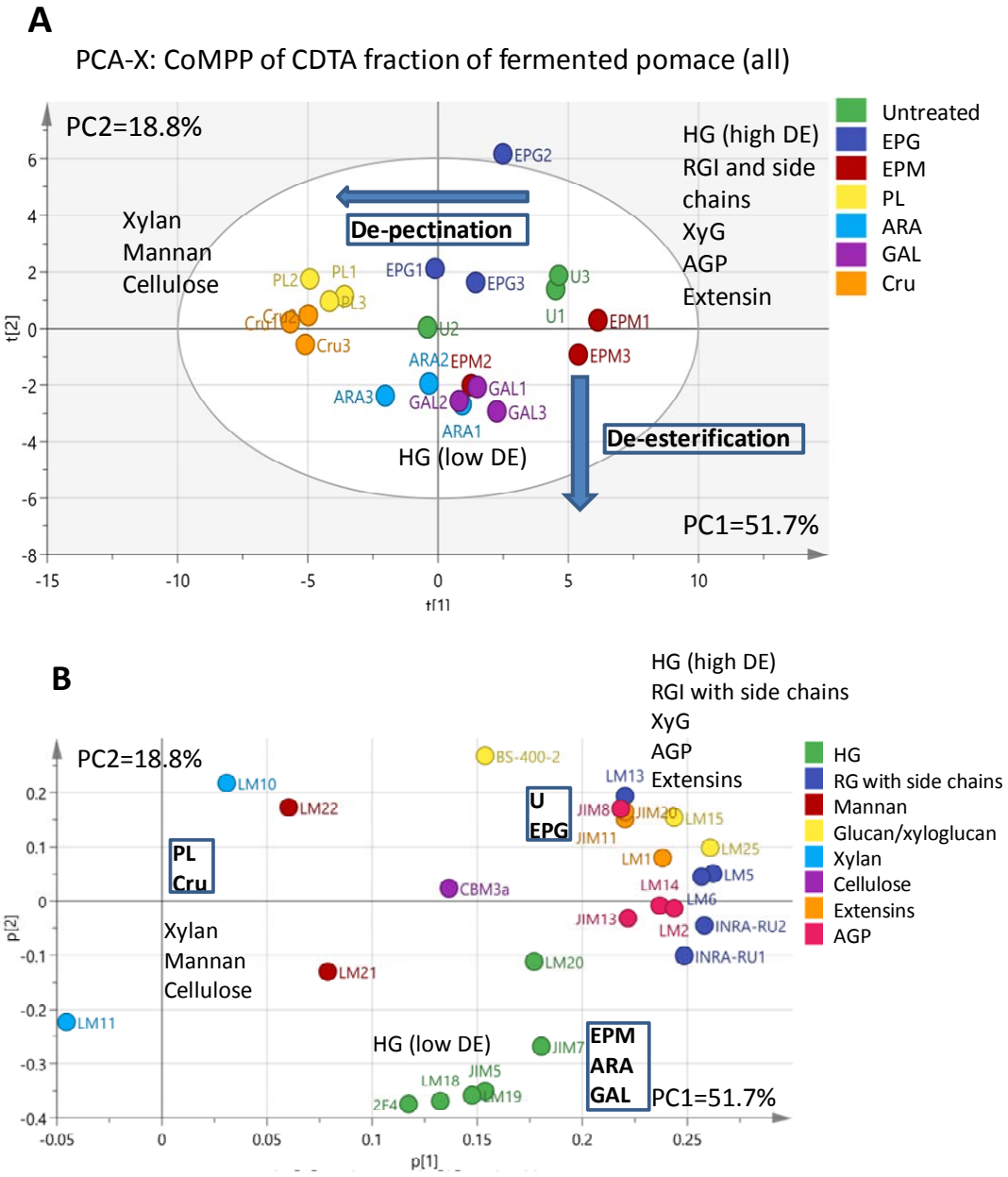
By comparing the GC data, the statistical significant decrease of GalA in PL and Grand cru treated pomace is clearly observable (Fig. 1), which strongly infers that the de-pectination occurred more effectively by applying these two enzyme preparations, the increase of concentration of hemicellulose substitutes (Xyl, Man and Glc) also confirmed the de-pectination as this would have caused more exposure of xyloglucan, cellulose and mannans (see Chapter 3, Gao *et al*, 2015). In contrast, the other enzyme combinations did not show a clear and statistical significant impact on the pomace cell wall structure based on their monosaccharide levels. A number of previous studies have stated that the grape berry cell wall has high degree of esterification (more than 50%) (Nunan *et al*, 2001, Vicens *et al*, 2009, Gao *et al*, 2015), the GalA can be either esterified with a methyl group or acetyl group. These side chains can prevent certain enzymes, such as endo-polygalacturonases (EPG) and pectate lyases (Bonnin *et al*, 2014) from cleaving the pectin main chain (Van Alebeek *et al*, 2002). This may explain why the cell wall structure did not undergo the effective degradation by applying only EPG in the winemaking process. Pectin methyl esterases were added in 3 combinations to help with de-esterification of methyl group, then to open up the "hairy" pectin main chain for the EPG to penetrate and act on presumably de-esterified polymers. However, from these monosaccharide composition data, the concentration of GalA did not decrease significantly as we would have predicted; other two combinations (ARA and GAL) with PME showed similar trends indicating no significant decrease in GalA levels. GC analysis of monosaccharide composition is only able to show the information of the bulk chemistry, but cannot show more structure information like the architectural complexity of AIR samples (see Chapter 3, Gao *et al*, 2005). To acquire more detailed information on the actions of these enzymes at polymer epitope level, CoMPP using the cell wall glycan mAbs and CBMs, was therefore performed.

De-pectination and de-esterification is revealed by CoMPP and multivariate data analysis

CoMPP employs a number of well characterised monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) (Fig. 2C) which target specific epitopes associated to different cell wall polymer types (e.g. HG, RGI, AGPs, extensins, etc.) (Moller *et al*, 2007). By

combining this technique with enzyme treatment we aimed to enhance our understanding of how carbohydrate active enzymes (i.e. pectinase) are able to degrade and unravel the multi-layer structure of grape pectin-rich cell wall, albeit indirectly as it is an *in vitro* analysis. The AIR sourced from the experimental fermentations was sequentially extracted using CDTA and NaOH, to generate a pectin-rich fraction and a hemicellulose-rich fraction respectively (see methods). Each fraction experimental sets were printed on a series of nitrocellulose membranes and then probed individually with 27 mAbs and CBMs (see chapter 2). The raw data generated from CoMPP were analysed using multivariate data analysis (SIMCA) for creating the PCA models (scores and loadings) and were processed to generate the associated heatmaps (Fig. 2A-C). In Fig. 2A, it is clear that different treatments clustered into 3 main groups. PL and Cru sit very close to each other and the major variables (loading plot in Fig. 2B) which correlates with this main component separation (PC1=51.7%) from the other treatments are mainly an elevated hemicellulose polymer abundance (such as xylan, recognized by LM10) and a diminished abundance of pectin epitopes, which is further supported by the monosaccharide compositional analysis dataset (i.e. statistically significant decrease of GalA, Fig. 1), suggesting effective de-pectination by these enzymes. PC2 explains 18.8% of the variation in the scores plot (Fig. 2B) and separates EPM, ARA and GAL treatments from the U and EPG samples. The corresponding loading plot (Fig. 2B) suggests loading variables mAb LM18 and mAb JIM5 (reflecting HG with low degree of DE) act as the main contributor to separation of these samples, which would infer, de-esterification in the samples containing PME (i.e. EPM, ARA and GAL). U and EPG cluster together in the score plot (Fig. 2A) with the corresponding loading variables, indicating an abundance of HG (high DE), RGI, XyG, AGP and extensin epitopes (Fig. 2B)showing that the EPG treatment impacted very little on berry cell wall de-pectination and de-esterification. The raw data was also scaled to generate a heatmap (Fig. 2C) on the relative epitope abundance of the mAbs and CBMs in the different treatments relative to the controls. From the signal abundance, the heatmap confirmed the GC dataset (Fig. 1) showing de-pectination occurred (HG and RGI) while xyloglucan and cellulose epitopes increased in abundance (see chapter 3; Gao *et al,* 2015, Zietsman *et al*, 2015a). The comparison of the abundance of different treatments supports the PCA score plots Fig. 2A) and loading plot (Fig. 2B) for the PC1 component, it is

clear that PL and Grand cru worked more effectively at de-pectination (decrease of HG polymers and RGI epitopes) polymers, again confirming the GC data (Fig. 1). PC2 variation is also possible to be observed on the heatmap, the epitope abundances of HG (low DE) polymers in EPM, ARA and GAL are higher compared to U, these include JIM5, LM18, 19 and 2F4, which resulted in a relatively higher ratio of HG (low DE) to HG (high DE), driving the separation on PC2 axis (Fig. 2A, B).



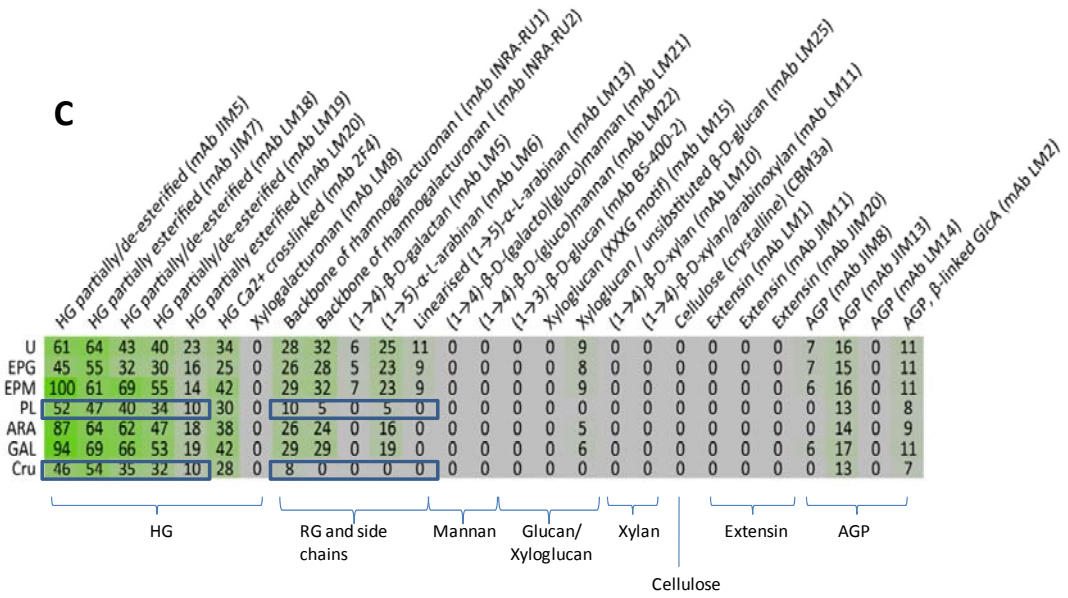


Figure 2. CoMPP results showing a PCA score (**A**) plot and loading (**B**) plot of the CDTA extract (pectin-rich) of seven treated pomace. (**C**) CoMPP heatmap of abundance of antibodies on pomace CDTA extract, the values of variables are average of three biological replicates. A cut-off (<5) was applied. The colors of plots are according to the treatments in (**A**) and to the polymer category in (**B**).

The analysis of the CDTA dataset of the six enzyme treatments and the untreated control are presented as PCA scores (Fig. 2A) and loading (Fig. 2B) plots; and a corresponding heatmap (Fig. 2C), for subsequent interpretation. The scores plot (Fig. 2B) shows PC1 and PC2 explaining 52% and 19% of the variance in the CoMPP data respectively. Clear clustering is evident in the scores plot where U and EPG are positioned at the centre to upper-right of the plot; whereas EPM, ARA and GAL are situated below the PC-1 axis in the direction of the PC2 distinct from U and EPG. Whereas PL and Cru are situated far to the right of the scores plot separating clearly along the PC1 axis distinct from U, EPG, EPM, GAL and ARA samples. Inspection of the corresponding loading plot (and contribution plots; not shown); show that U and EPG are situated in close proximity to the untreated state showing high relative abundance of epitopes associated with untreated samples such as is found in the control U (see Figure 2B). The samples EPM, ARA and GAL are situated along the PC2 axis, below the PC1 horizontal, axis with denotes a higher abundance of pectin epitopes, such JIM5, JIM7, LM19 and interestingly the 2F4 epitope. PL and Cru are very well separated from the other

samples along the PC1 axis and this appears driven strongly by a clear deficiency in most pectin epitopes, most notably RG1 (INRA-RU1, INRA-RU2), galactans (LM5) and arabinans (LM6) (which are side chains of RG1); hence clearly these two enzymes are very effective at removing RG1 and HG thereby de-pectinating. This is indicated in the scores plot annotation (Fig.2A) where PC1 is described as the main de-pectination direction; whereas PC2 is considered de-esterification and unravelling of the pectin components, exposing more epitopes. Inspection of the heatmap (Fig2C) reveals more or less similar information in that PL and Cru (boxed in Fig.2C) show significant de-pectination with HG (JIM5 etc.) but particularly RG1 side chains (INRA-RU1 etc.) showing marked removal by these enzymes. Similarly EPM, ARA and GAL show a seemingly higher abundance of HG epitopes than U and EPG in the heatmap (Fig.2C) supporting a role for unraveling of pectin but not complete de-pectination.

To further simplify the interpretations the models were repeated but with the inclusion of only the PL and Cru treatments versus the U controls (see Fig. 3).Fig. 3A shows a very distinct separation of PL and Cru from the U samples and this appears primarily due to the PC1 component which explains 68.5% of the variance in the data. The primary loading variables driving this separation from U appears to be xylans, mannans and cellulose (LM10, 21 and CBM3a) (see Fig. 3B). Interestingly, PL separates from Cru in the PC2 direction (see Fig. 3A) accounting for 11.8% of the variance, suggesting difference in degradation between these two enzyme preparations on in the wine matrix. These differences in the PC2 direction can be explained by Fig. 3B (the loading plots), which shows that the separation of PL from Cru and U (Fig. 3A) due mainly to mAbs for HG with low DE, such as LM18, 19, 2F4, JIM5 (Fig. 3B). This is supported by evidence that PL is more effectively on HG with high DE, but shows markedly lower activity on HG with low DE (Prade *et al*, 1999). Thus, appears to be not entirely similar to PL in its action in grape maceration of cell walls (Fig. 3A).

Inspection of the corresponding heatmap (Fig. 3C) supports the PCA scores (Fig. 3A) and loading (Fig.3B) plots. PL and Cru show a significant decrease in abundance of all the HG and RG1 side chain epitopes compared to U samples (see Fig. 3C boxed), and the differences in the PC2 direction between PL and Cru appear to be driven due to PL showing a higher

abundance of HG epitopes with low DE (LM18, 19, 2F4, JIM5) than Cru (Fig. 3C). It is also important to notice that the epitope signal of RGI abundance also showed a significant decrease (Fig. 3C), however, PL does not have a similar the activity to RG lyase which can degrade the RGI backbone (Prade *et al,* 1999). The supplier Novozymes has confirmed this as Pectin Lyase (PL) and not RG lyase. A possible explanation of these data may due to the cleavage of the HG main chains on either side of the alternating RGI polymer (block-wise model) resulting in the release of RGI from the cell wall structure, as well as the side chains that bind (associated with) to them. Two models have addressed the cross-linking structure of RGI and HG, one is the "smooth and hairy region" model in which HG alternates block-wise with RGI and XGA (xylogalacturonan) to form the backbone of pectin (Schols and Voragen, 1996), another one is the "RGI backbone" model which assumes that the HG, XGA, arabinans and galactans would occur as side chains of RGI backbone (Vincken *et al*, 2003), in this study, the berry cell wall model data presented is better explained using 'smooth and hairy region' as HG degradation on either side of a RGI block facilitates the release of RGI.

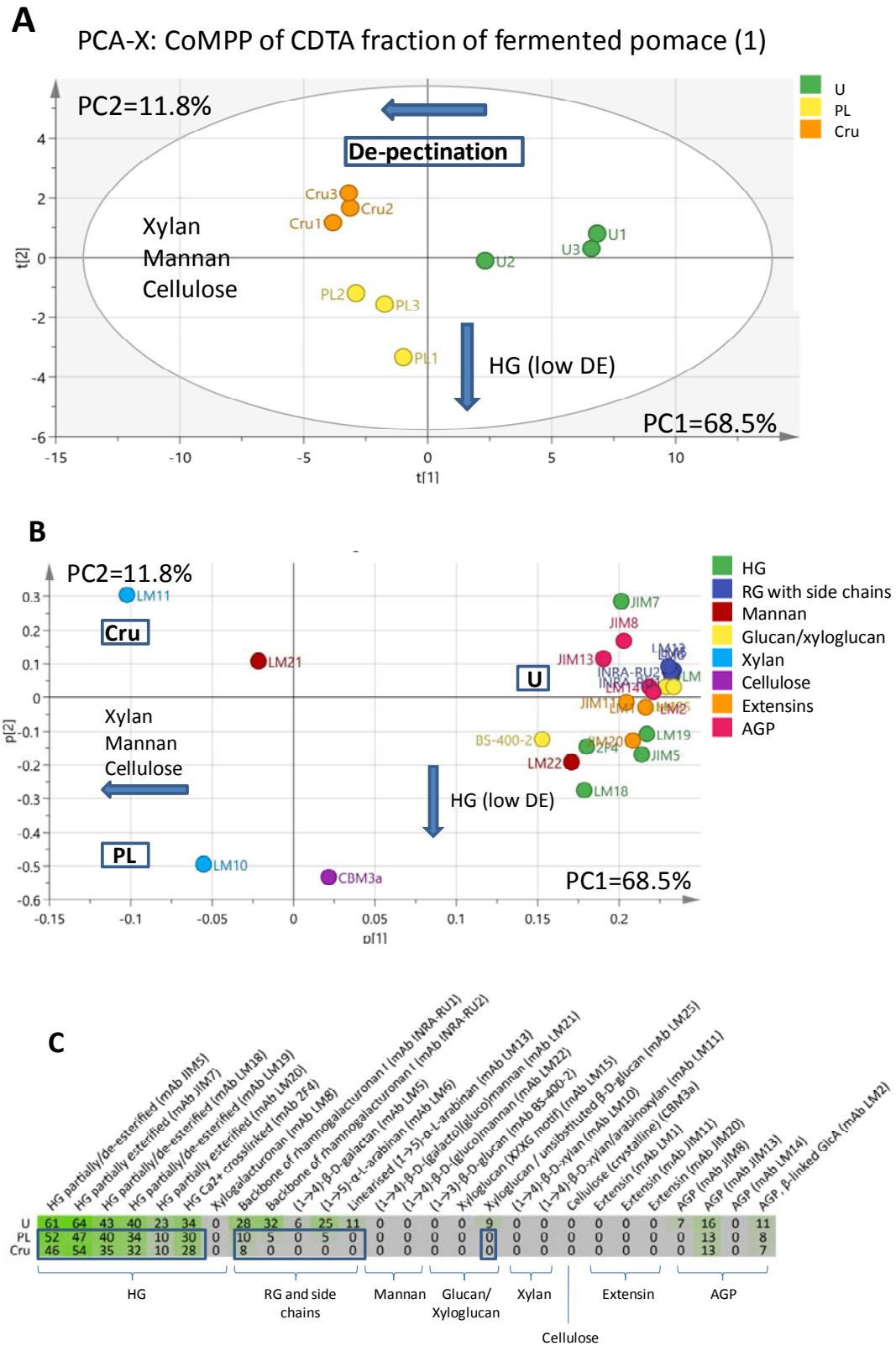


Figure 3. CoMPP PCA score (**A**) plot and loading (**B**) plot of the CDTA extract (pectin-rich) of three treated pomace (U, PL and Cru). (**C**) CoMPP heatmap of abundance of antibodies on pomace CDTA extracts, the values of variables are average of three biological replicates. A cut-off (<5) was applied. The colors of plots are according to the treatments in (**A**) and to the polymer category in (**B**).

We next focused on the treatments (i.e. EPG, EPM, GAL and ARA) that did not show the same strong PC1 direction (i.e. PL and Cru) from U samples in the main pectin CoMPP PCA model (Fig. 2). We remodeled the data but this time omitting PL and Cru from the analysis to generate a PCA score plot (Fig. 4A). Visual inspection of the score plot (Fig. 4A) would seem to indicate that separation along the PC1 (41.3%) is the main variance in the data, but that this does not correlate with treatment effects; but probably rather sample variability (see later). The treatment affects were found along the PC2 component (23.2%) which showed EPM, ARA and GAL (circled in Fig. 4A) separating from U and EPG; interestingly, EPG seemed to show an opposite effect to U and the other treatments. High level of inter-sample variation is present in this remodeled data, especially with respect to U, EPG and EPM (Fig. 4A). Nevertheless, it supports the results reported in chapter 4 (Fig. 6A) which shows that the untreated pomace displays markedly high inter-sample variability explained due to the inter-vineyard differences in ripening berries. The corresponding loading plot (Fig. 4B) seems to show that inter-sample variation is due to a distinct separation of HG epitopes (right-hand side) from epitopes associated with hemicellulose (cellulose, xyloglucans), RG1, side chains and proteins (extensins and AGPs) (left-hand side) along the PC1 axis. Whereas PC2 variation is mainly driven by epitope exposure for mAbs HG (low DE) which are enhanced in EPM and distinctly for ARA and GAL samples (Fig.4B)., Here we suggest that the separation along the PC2 axis (Fig. 4B) is mainly due to the de-esterification processes associated with the inclusion of PMEases (EPM, ARA, GAL), albeit the two repeats of U overlap with EPM samples this just beat later ripeness stages with higher ratios of HG epitope abundance (hence low DE). In Chapter 4 it was shown that the commercial enzyme preparations reduced the inter-vineyard sample variance compared to the untreated samples by a strong de-pectination action, however, in that study it was not found to occur for the EPG only treatments. This could suggest that the EPG enzyme is not able to macerate and degrade the pectin of berry pomace due to its high level of HG esterification state (see Chapter 4). These confirmed the action of ARA and GAL as they contain the activities to cleave these side chains, the release of side chains may increase the exposure of RGI main chain for other enzymes (EPG, PME) in the combination to penetrate. However, same as EPM, for some reason the combination of EPG and PME did not show the expected activity we have

predicted, even some side chains of RGI has been released, the RGI main chain was not cleaved well as PL. The general patterns of the results presented in the PCA scores (Fig. 4A) and loading (Fig. 4B) plots can be confirmed by corresponding CoMPP heatmap (Fig. 4C), the treatments containing PMEase (EPM, ARA and GAL, first blocked off) have more exposure of HG (low DE) (mAbs JIM5, LM18, 19, 2F4) compared to the U and EPG. Similar results were found in a study of Pinotage skin cell wall fermented with different enzymes (Zietsman *et al*, 2015a). It was suggested in that study that these enzymes unravel the cell wall than rather degrading it., This would support our data on the multilayer structure of pectin fraction (see Chapter 3, Gao *et al*, 2015), which support the idea that de-esterification plus EPG action can probably only loosen the structure, but without effective chain cleavage action such as performed by PL (and Cru) (Fig. 3). The PC1 variation (RGI and side chain) can also be supported by the heatmap (See RGI block in Fig. 4C), where ARA and GAL treatments showed lower epitope abundance for mAbs INRA-RU2 compared to other treatments, as well as side chains (mAbs LM5, 6 and 13). However, the difference for mAbINRA-RU2 is small, even though it is a main contributor for PC1 variation in Fig. 4A, this therefore may suggest the limitation of the CoMPP heatmap presentation format (Fig. 4C), as it shows scaled values by compressing the dynamic range whereas PCA plots use raw data for modeling. Thus, when the heatmap (Fig. 4C) shows very high abundances for some mAbs (e.g. JIM 5 in this case), the subtle differences in epitope abundance for other mAbs at the other end of the scale is not visible. Thus, studying only heatmap data may result in a loss of information; hence the use of multivariate data analysis is essential for a more complete interpretation of the datasets.

Interestingly, in addition to the the marked decrease in epitope abundance for mAbs LM6 and 13; the signal for mAbs LM5 (galactans) decreased to 0 on heatmap (Fig. 4C) after ARA treatment, however, this combination does not have galactanase activity, this probably due to the cut-off of the heatmap being set at 5 and below. Thus, “0” here on the heatmap does not mean absolutely no signal has been detected, but that it is probably just above noise. Another reason for these observations may be that the cross-linking (covalent or non-covalent associations) between galactans and arabinans exist in this fraction, thus, galactans were released with the release of arabinan by work of arabinanase; or that AGP

epitopes were removed due to the cleavage of a galactose anchor with mAb LM6 recognising arabinose motifs on both arabinans and AGPs (Moore *et al,* 2006), The decrease of xyloglucan (XyG, recognized by mAbs LM25) can also support this assumption, as this putative arabinan-galactan cross-linking has been addressed to be the connecting chain for XyG and RGI (Popper and Fry, 2008), in previous studies (Moore *et al*, 2014, Gao *et al*, 2015), we have also found some XyG present in the pectin-rich fraction, thus, it is highly likely that XyGs and galactan have both been released from this pectin-rich fraction with arabinan in ARA treated fermentations. The signal for arabinan recognized by mAbs LM6 did not decrease as strongly as for PL and Cru (Figs. 2 and 3). However this is not surprising as that arabinanase in ARA when acting on linearised arabinan will stop cleavage branch points of arabinan (mAbs LM6). Alternatively, the signals of branched and linear arabinans (mAbs LM6 and 13) decreased indicating degradation even though there is no arabinanase activity in GAL treatment, and this may due to the some linearised arabinan motifs are present with galactan structures as arabinogalactans (AG)(Moore *et al,* 2006).

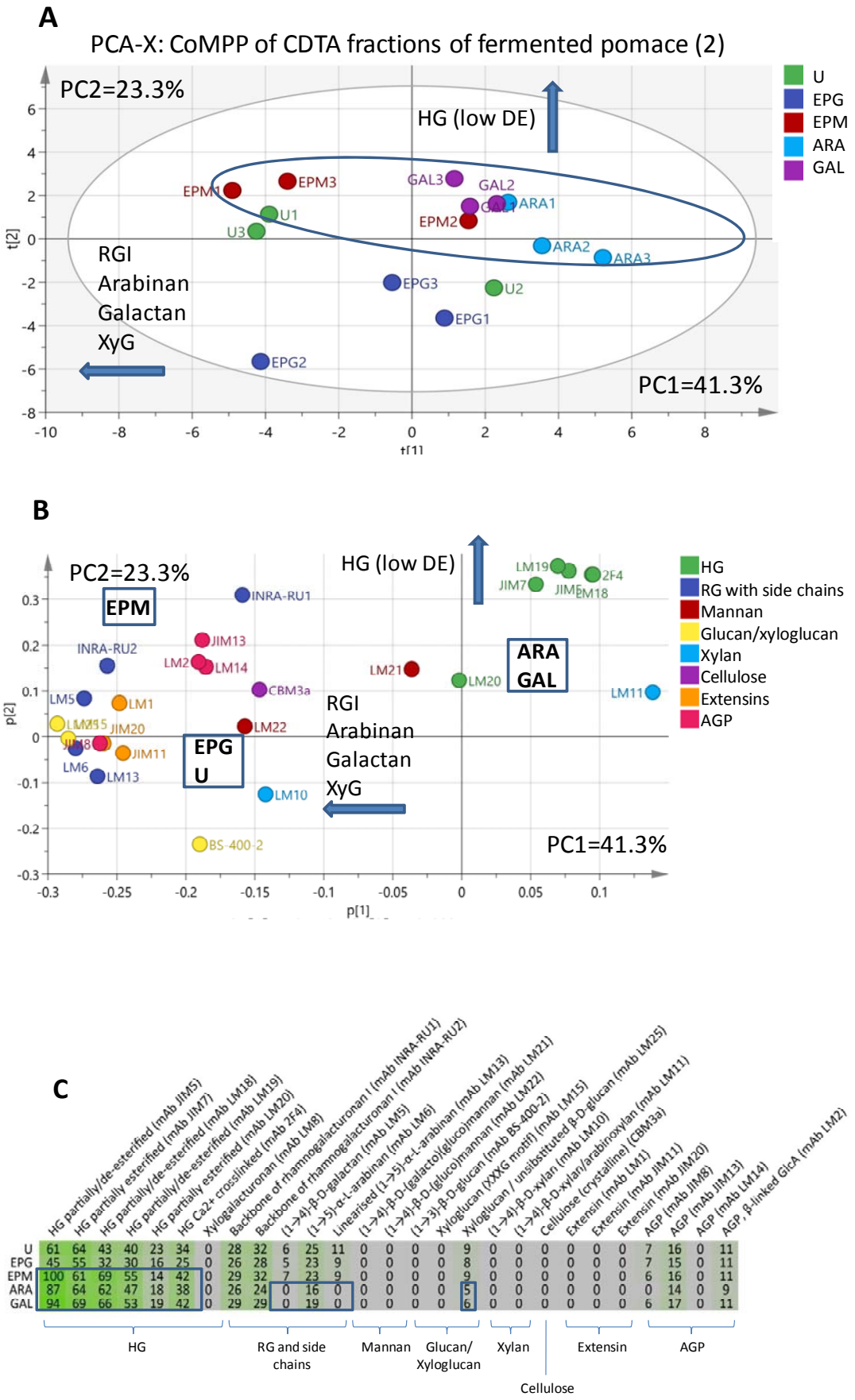


Figure 4. PCA score (**A**), loading plot (**B**) and heatmap (**C**) of CoMPP results of five treatments.

Figure 4 (caption). A. The PCA score plot of CoMPP results of five treatments (U, EPG, EPM, ARA and GAL). **B**. The heatmap of abundance of monoclonal antibodies and carbohydrate binding modules of CDTA (Pectin-rich) fraction of treated pomace after fermentation, the values for each variable (antibody) are the average of three technical replicate. A cut-off (<5) was applied. The colors of plots are according to the treatments in (**A**) and to the polymer category in (**B**).

To break down the grape berry cell wall during wine fermentations, the enzymes need to get through the pectin-rich fraction to reach the hemicellulose-rich fraction which mainly contains xyloglucan and some strongly associated pectin (high esterified HG and RGI) as a coating layer (Gao *et al*, 2015). In order to study this layer, NaOH was used for the extraction of the remaining hemicellulose fraction after the CDTA extraction for pectin (presented in Figs. 2-4), following the enzyme treatments; and these fractions were subjected to CoMPP analysis using the same set of mAbs and CBMs. Fig. 5A shows the NaOH PCA score plot with the six enzyme treatments and untreated control. A distinct separation can be observed between PL and Cru versus the other treatments (Fig. 5A), where PC1 explains 41.7% of this variation. The corresponding loading variables (Fig. 5B) correlating with this strong separation were identified as XyG epitopes (LM15 and 25). However unlike in Fig. 2A the samples U, EPM, EPG, ARA and GAL did not show much separation from each other (PC2 accounting for only 17.2%), presenting a more simple dataset for interpretation. This is confirmed in the heatmap (Fig. 5C) showing the strong influence of PL and Cru (blocked off) on the RG1 and side chain epitopes (i.e. mAbs INRA-RU1, INRA-RU2, LM5, LM6, LM13), but not the hemicellulose epitopes (e.g. mAb LM15, CBM3a etc.). This again confirmed the suggested two fractions/layers in grape pomace cell walls (see Chapter 3, Gao *et al*, 2015). Thus, PL and Cru penetrated more effectively and caused significant degradation of the highly esterified pectin-rich fraction (Fig. 5) that coats the xyloglucan-cellulose rich cell layers (see chapter 3; Gao *et al*, 2015).

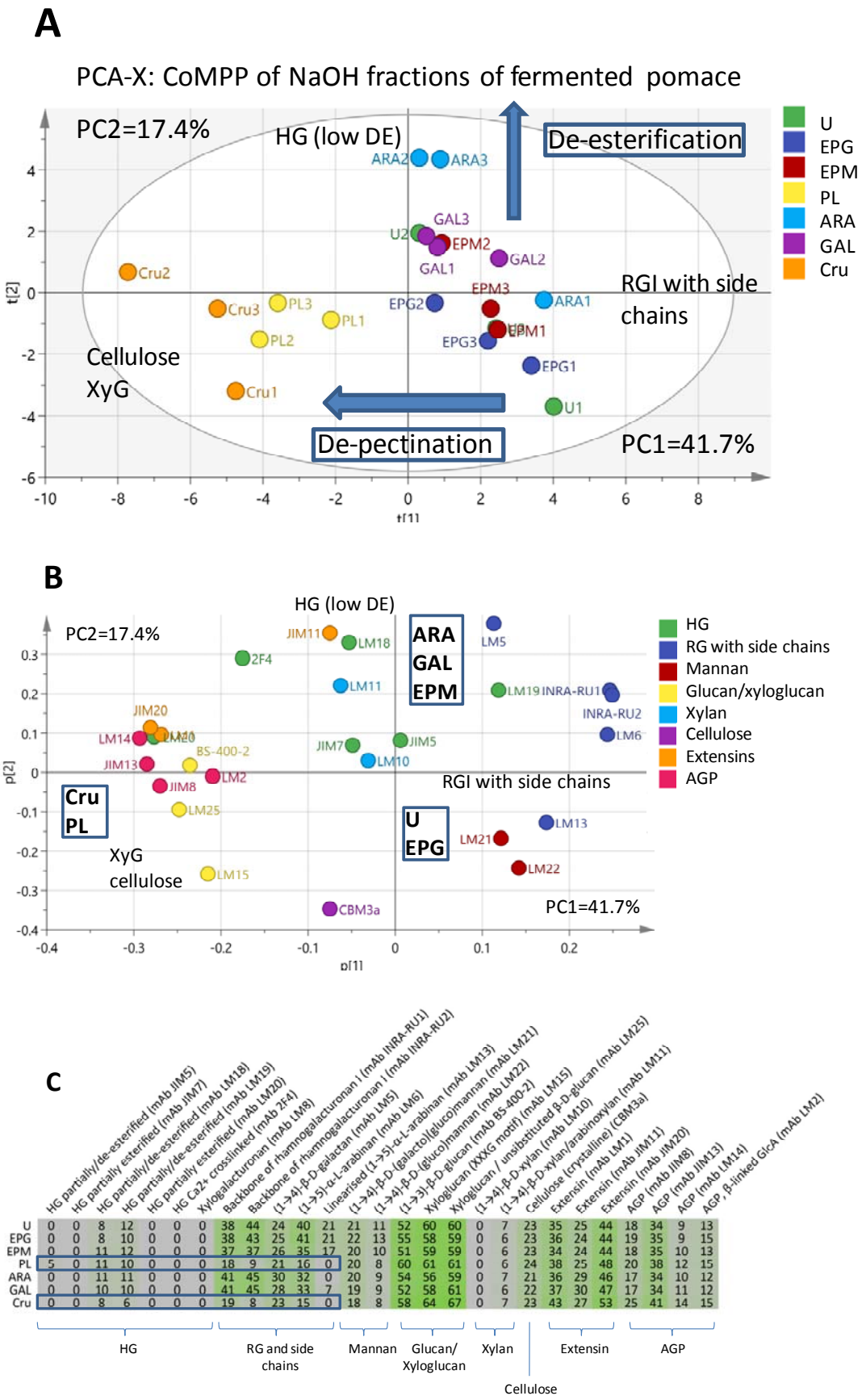


Figure 5. CoMPP results showing a PCA score (A); loading (B) plot and heatmap of the NaOH extract.

Figure 5 (caption). CoMPP results showing a PCA score (**A**) plot and loading (**B**) plot of the NaOH extract (Hemicellulose-rich) of seven treated pomace. (**C**) CoMPP heatmap of abundance of antibodies on pomace NaOH extract, the values of variables are average of three biological replicates. The colors of plots are according to the treatments in (**A**) and to the polymer category in (**B**).

The HG in this coating layer (Fig. 5C) was mainly recognized to be in the de-esterified form, due to the treatment with NaOH, a saponification reagent (Gao *et al*, 2015). Furthermore, the abundance of the ratio of RGI to HG (Fig. 5C) indicated that RGI dominates this pectin layer, and is highly likely to rather fit the "smooth and hairy regions" model (Schols and Voragen, 1996) of pectin than the "RGI backbone" model (Vincken *et al,* 2003). This layer is assumed to have a higher abundance of RGI with its side chains (arabinans and galactans), and connected with shorter chains of esterified HG at both ends.

It is also interesting to notice that the relative abundances of the cell wall proteins (extensins and AGPs) epitopes in this study are relatively higher than the NaOH fractions analysed in Chapter 4 (Fig. 9), which could be explained by masking effects (Marcus *et al,* 2008) due to sticky residual pectin on the hemicellulose fraction. Furthermore by comparing the abundance of hemicellulose (XyG epitopes mAbs LM15 and 25) and cellulose (CBM3a) from these two studies, the abundances of these two polymers appear relatively higher than in study reported in Chapter 4. This may due to more exposure of hemicellulose and cellulose epitopes, suggesting that the pomace cell walls (pectin fractions) of Chapter 4 were more extensively de-polymerization. As the datasets concerning Brix levels (from Fig. 2, chapter 4) showed that many grapes in that study were over 24, thus, the maceration process could be facilitated by the grape berries endogenous enzymes due to over-ripeness tissue self-maceration. Similar results were shown in Zietsman *et al.* (2015a) when the fermented skins of over-ripe berries and ripe berries were compared.

By comparing these enzyme treated fermentations, our hypothesis of two fractions of grape berry pomace cell walls appear well supported. A number of points indicate this; Fig 5C showed only PL and Cru performed effective de-pectination (Figs. 1, 2C and 3C) while other treatments did not provide a significant impacts. Interestingly, even though there is a

marked decrease of epitopes associated with RGI and its side chain arabinans (mAbs LM 6 and 13), the galactan abundance is fairly constant with PL or Cru treatments (Fig. 5C). One reason may be due to the potential binding of galactan to hemicellulose may prevent removal be pectinases given it deeply embedded in xyloglucan-cellulose framework (Popper and Fry, 2008). The removal of pectin also increased the exposure of hemicellulose, including cellulose epitopes, again probably due to epitope masking effects (Marcus *et al*, 2008), evidenced by the increase in signals for mAbs BS-400-2, LM15 and LM25 observed after PL and Cru treatment.

A proposal for a hypothetical evidence-based grape berry cell wall model

Based on the datasets generated from the recombinant enzyme-aided combinatorial fermentation experiments (this chapter), and the data presented in the earlier research chapters (chapter 3 and 4), a hypothesis for a grape berry cell wall can be proposed. From these data and literature references, a simplified diagrammatic model was designed to incorporate the major findings to date on grape berry cell wall structure (Fig. 6). The model is annotated using numbers (and a corresponding Table 2) to indicate tissue and cell wall polymer associations, from this study and previous research literature, for ease of inspection and clarification.

To summarize the major important points, the diagrammatic grape berry cell wall model shows that the pomace contains 2 main fractions these being pectin-rich and hemicellulose-rich, respectively. The pectin-rich fraction also contain low amounts of RGI which has more branched arabinans as side chains, however, other side chains (i.e. galactans) are also present. The cell wall proteins, such as AGPs, also associated with the pectin-rich fraction. The hemicellulose fraction is strongly associated with a RGI-dominant pectin layer, and coated with a multilayer highly-esterified HG-dominant pectin-rich fraction.

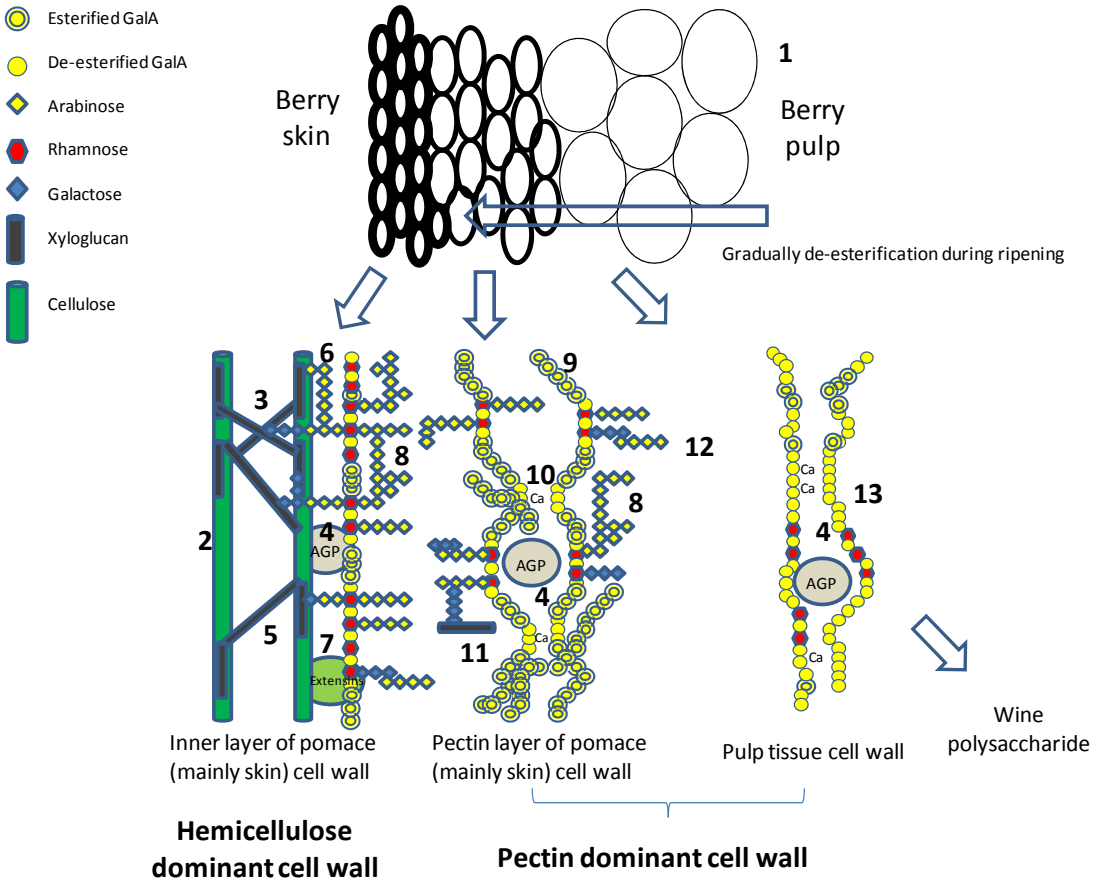


Figure 6. A simplified diagrammatic model of the grape berry cell wall. The numbers in the figure reflect the important studies on cell wall tissue and cell structure (in grapes and other species/systems) and are described and referenced in the Table 1.

Table 2. The annotation list, with associated descriptions and supporting references, for the simplified diagrammatic model of the grape berry cell wall tissue and cell structure

No.	Description	Reference
1	Tissues differences in Berry	Nunan et al, 1998
2	Tethering model of Hemicellulose -dominant fraction	Fry, 1989; Hayashi, 1989, Park and Cosgrove, 2012, this study
3	Parallel form of cellulose and associated xyloglucan	Guerriero et al, 2010, Brett and Waldron, 1996, Cumming et al, 2005, Popper and Fry, 2008
4	AGP is associated with all the fraction of cell wall	Moore et al, 2014a, Gao et al, 2015, Zietman et al, 2015, this study
5	Extensin is mainly associated with pomace cell wall	Jackson et al, 2001, Ribeiro et al, 2006, this study
6	Possible crosslink between arabinan and cellulose	Zykwinska et al, 2007
7	RGI-dombinant pectin layer coating hemicellulose fraction	Gao et al, 2015 and this study
8	Branched arabinan dominant the side chains of RGI	Huisman et al, 2001, Willats et al, 1998, this study
9	High esterification level of HG in pectin-rich fraction	Barnavon et al, 2001, Gao et al, 2015, this study
10	ca^{2+} crosslink in pectin-rich fraction, masked by esterified HG	Brett and Waldron, 1996, this study
11	Low amount of xyloglucan is asscoiated with pectin	Popper and Fry, 2008, Moore et al, 2014a, Gao et al, 2015
12	Unuaual linkage "Ara-Gal-Rha" in pectin-rich fraction	this study
13	De-esterified HG, RGI and AGP in pulp cell wall and wine polysaccharide	Gao et al, 2015

The model presented (Fig. 6) can well explain the mode of actions of the enzymes used in this study, it is also important to note that the cell wall is different at the tissue level, thus, the model we suggest is based mainly on pomace studies (see chapter 3, Gao *et al*, 2015)

and might not necessarily be an exact a fit for ripe berry pulp cell walls. The pomace data reflects the thick skins of wine grapes with associated tightly bound pulp layers. As grapes ripen the pulp layer probably starts to depolymerize after veraison (Creasy and Creasy, 2009), resulting in larger cells with thinner more fragile cell walls the more mature development stages (Nunan *et al*, 1998) leading to ripening. during the winemaking process, when the berries are crushed the pulp cell walls appear to be easily broken down and solublised into the wine, thus generating the complex wine polysaccharide composition we reported on in Chapter 3 (Gao *et al*, 2015), mainly composed of de-esterified HGs and RGI polymers, with the presence of AGPs; and unexpectedly some XyGs. The study on berry PMEase and esterification levels (Barnavon *et al*, 2001) claimed that the esterification level of HG in berries are decreased to 20%, which are not supported in our study on pomace. However, that study was on a whole berry basis, and is thus not directly comparable. Furthermore, we suspect the de-esterification process start from pulp tissue, and progressively and gradually develop into skin tissue in ripening period (shown as arrow in Fig. 6). The diagrammatic model of ripe grape berry cell walls (tissue and cells) provides a useful basis for understanding berry deconstruction and the action of maceration enzymes on wine grapes; particularly during red winemaking

Conclusion

Cell wall of grape berry, and its derived oligosaccharide and protein in wine, are important factors influencing wine maceration and clarification. However, even though the commercial enzyme preparations have been applied by industry for a few decades, the gap of our knowledge on berry cell wall structure is still present. It is no doubt that more information of how those polymer crosslink together to form the cell wall architecture has a number of implications, such as understanding better berry development, plant-pathogen interaction, as well as optimization of the activity of commercial enzyme preparation to overcome the inconsistency issues hampering currently the wine industry. In this study, we have applied the pure (recombinant) enzymes (mainly pectinases) to the winemaking process (Cabernet sauvignon), coupled with validated high throughput cell wall profiling tools, in order to reconstruct the berry cell wall structure from different degradation result with different

enzyme combinations. Glycan microarray generated a number of interesting data by probing the specific monoclonal antibodies and carbohydrate binding modules on the degraded pomace.

Firstly, it confirmed the high degree of esterification in pomace cell walls, this esterified HG, alternated with RGI, form the backbone of pectin and act as the outer layer for preventing the enzyme (e.g. endo-polygalacturonase) to penetrate, and can only be degraded after de-esterification occurred, or through the actions of pectin lyase. The pectin-rich outer layer is suggested to have a complex and multilayer structure, with a number of other polymers associated or embedded in it, such as Ca^{2+} crosslinked HG, AGP, and small amounts of xyloglucan. Secondly, several masking effects have also been observed, which would influence the efficiency of enzymes; as an example: branched Arabinan may block the EPG or PL to reach the pectin chain. Thirdly, based on the actions of arabinanase and galactanase, the berry cell wall may not only contain "Gal-Ara-Rha" as type I arabinogalactan, but also contain some unusual links such as "Ara-Gal-Rha" on its RGI chain. This study also confirmed that the inner hemicellulose-rich layer is coated with a thin layer of RGI dominated pectin with lower composition of esterified HG.

The model presented in this study gave a simplified look at how berry cell wall polymers bind to each other. In additionbe, we propose a second model with lower esterified HG that would more likely describe the cell wall structure of pulp cell. The second model is based on the first model, but contains thinner HG layers with lower degree of methyl esterification. This suggested feature can be supported by many studies on berry development, as pulp cells are expanding rapidly during ripening when de-esterification is believed to occur. This model can also be supported by the study on wine fermentation polysaccharides (Gao *et al*, 2015), since these polysaccharides in wine mainly originate from crushed pulp cells.

However, the models generated here is still exploratory in nature and a number of drawbacks in our experimental systems are limiting it, e.g. the semi-quantitative status of CoMPP, the lack of certain key antibodies (such as RGII), and the efficientcy of the recombinant enzymes used in the study. Nevertheless, the structural information generated from this study have potential impacts in a number of fields, Firstly, it provides a clearer

target for designing maceration and clarification enzyme preparations, in order to achieve the most efficient cell wall deconstruction, with less unwanted activity, Secondly, it provides baseline reference datasets for future berry developmental studies that can focus on several specific polymers involved. Thirdly, this improved understanding of cell wall structure is very useful when considering pathogen interactions with grape, particularly the mode(s) of infection and the crucial enzymes needed to penetrate into thick berry cell walls. The approach taken in this study would also be useful to expand to other grapevine cultivars, as the cell wall can be differ among the cultivars. It will be particularly interesting to compare the differences in cell wall structure during development and also correlate this to the transcriptional control underlying these differences.

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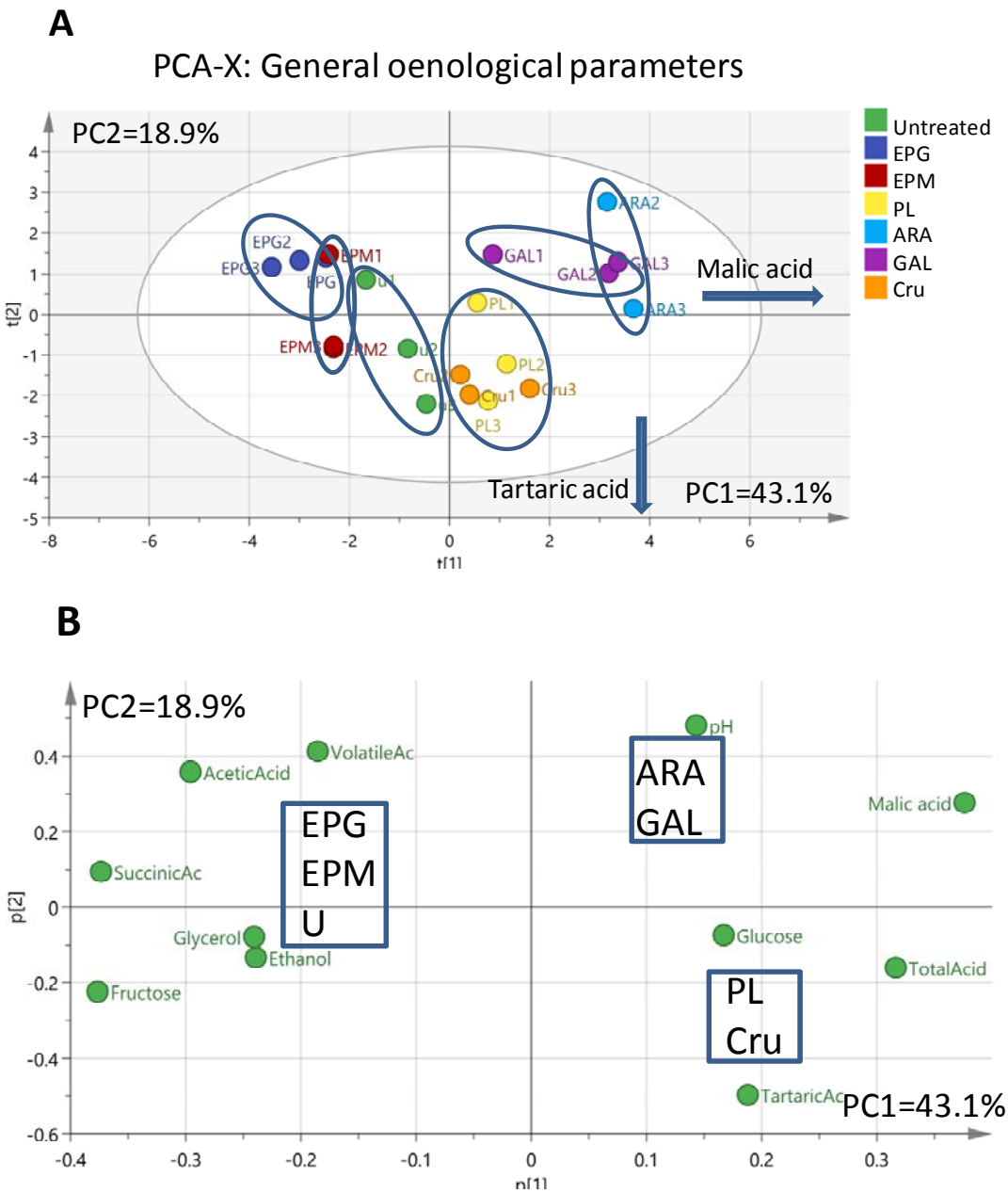
Authors' contribution

Y. Gao, J. Moore and M. Vivier designed the study, Y. Gao performed the experiments and collected the data. Y. Gao interpreted the results and drafted the manuscript. J. Moore and M. Vivier advised with data interpretation and manuscript preparation. J. Fangel and W. Willats performed the CoMPP analysis. All authors read and approved the final manuscript.

Supplementary data

Table 1. General wine parameters generated from FOSS Winescan on wine from seven fermentations, each value came from the average of 2 technique repeats.

Sample	pH	Volatile Acid	Total Acid	Malic Acid	Glucose	Fructose	Ethanol	Glycerol	Acetic Acid	Tartaric Acid	Succinic Acid
u1	3.41	0.29	7.4	2.81	-0.42	1.08	13.03	10.61	628.07	1825.53	1801.88
u2	3.39	0.2	7.4	3	0.03	1.05	13.57	10.87	460.57	2004.87	1741.13
u3	3.34	0.26	7.55	2.95	0.49	1.06	13.88	10.94	507.9	2578.21	1778.04
EPG1	3.38	0.39	7.36	2.66	-0.1	0.97	13.65	10.78	827.85	1906.42	1751.13
EPG2	3.4	0.41	7.54	2.71	-0.02	1.16	13.67	11.21	887.36	1896.24	1821.13
EPG3	3.43	0.43	7.5	2.66	-0.01	1.22	13.89	11.4	865.5	2003.2	1855.59
EPM1	3.42	0.41	7.34	2.58	-0.26	1.08	13.23	10.63	851.76	2119.02	1709.15
EPM2	3.41	0.28	7.41	2.71	0.02	1.18	13.93	11.09	682.42	2341.91	1781.67
EPM3	3.44	0.28	7.42	2.76	0.09	1.28	14.05	11.26	646.38	2312.09	1754.09
PL1	3.39	0.33	7.47	2.78	-0.15	0.96	12.61	10.08	648.67	2307.19	1472.2
PL2	3.33	0.29	7.66	2.99	-0.01	0.94	12.84	10.35	600.29	2561.73	1514.75
PL3	3.32	0.26	7.78	3.06	0.12	1.01	13.41	10.82	555.84	2576.27	1567.21
ARA1	3.58	0.6	7.88	4.29	-0.23	0.84	11.96	10.13	1135.34	1697.11	1285.83
ARA2	3.49	0.37	7.79	4.1	0	0.77	12.76	10.49	755.71	2063.42	1416.94
ARA3	3.49	0.31	7.72	4.06	0.64	0.89	13.1	10.78	469.13	2619.96	1459.45
GAL1	3.53	0.34	7.49	3.65	0.45	0.98	14.02	10.92	606.42	2149.04	1554.62
GAL2	3.5	0.24	7.6	3.94	0.17	0.7	13.33	10.74	454.26	2139.58	1450.5
GAL3	3.52	0.23	7.59	4.1	-0.03	0.74	13.17	10.57	485.19	2142.78	1401.16
Cru1	3.39	0.2	7.63	3.18	-0.23	1.1	13.76	11	498.34	2468.19	1418.12
Cru2	3.34	0.26	7.68	3.22	-0.25	1.08	13.52	10.89	581.33	2309.13	1384.11
Cru3	3.31	0.31	7.68	3.18	0.06	0.98	13.05	10.6	540.18	2665.21	1296.25



Supplementary Figure 1. FOSS winescan results from final wine of seven treatments showing a PCA score plot (**A**) and loading plot (**B**). Colors are set according to the treatments in (**A**).

Chapter 6

General discussion and conclusions

General discussion and conclusion

This study investigated how the cell wall of the red grape cultivar Cabernet Sauvignon (*Vitis vinifera*) changed and deconstructed (i.e. degraded and unravelled) at the polymer level during fermentation, using recently developed and validated high throughput cell wall profiling approaches (Moller *et al*, 2007, Moore *et al*, 2014a, 2014b; Zietsman *et al*, 2015a, 2015b) in combination with multivariate data analysis approaches (Zietsman *et al*, 2015a). The composition, deconstruction and the distribution of the main cell wall polymers were followed (i.e. tracked, Moore and Divol, 2011), starting from harvested grapes during maceration and into the final wine. The results obtained confirmed many of the previous studies but also took our understanding forward by providing more insights into the subtle structural and architectural changes (i.e. unravelling) that occurred during the process of berry cell wall maceration and fermentation; while also detecting the presence of polymers (by virtue of their epitopes) which were not expected to be present in certain samples (e.g. HGs and XyGs in wine, Chapter 3; Gao *et al*, 2015).

6.1 Validation of high throughput cell wall profiling approach on the grape berry pomace during the winemaking

In this study, pomace samples (berry skin with residual pulp) were for the first time analysed using the relatively recently developed high throughput cell wall approach, which included the glycan microarrays (i.e. CoMPP), and more classical methods (i.e. gas chromatography and FTIR), in combination with with classical fractionation techniques (Chapter 3; Gao *et al*, 2015). Zietsman *et al.* (2015a and 2015b), also studied grape pomace with CoMPP and GC analyses, but dit not perform fractionation analyses, clearly showing that this will provide additional essential information. Besides the analysis of pomace, this approach was also performed on the concentrated wine polysaccharides for the first time. Furthermore, the pomace was also fractionated using chemical and enzymatical approaches to dissect the cell wall structures (Moore *et al*, 2014b). Part of the main findings indicated that the berry pomace is particularly rich in pectin (well established by e.g. Saulnier and Thibault, 1987, Arnous and Meyer, 2009), but unusual polymers were also detected such as xyloglucan epitopes; not expected in a pectin-rich fraction extract. Secondly, from the datasets obtained from the fractionation work, we have now suggested, based on strong

evidence, that the pomace cell wall material contains two main fractions; (1) a pectin-rich outer layer (dominated by HG with high degree of esterification), we assumed this layer is mainly the residual pulp attached to the skin, and (2) a hemicellulose-rich inner layer as the second major fraction. Interestingly, we also identified that this hemicellulose-rich fraction is coated, or strongly associated with a highly methyl-esterified pectin (dominated by RGI), which may prevent the skin from being degradation by the pectinase-rich enzymes during winemaking. Thirdly, the profiling work on the enriched wine polysaccharides also indentified the present of HGs, RGI and AGPs, albeit in low concentration. Since the samples we worked on were pre-concentrated, this is suggested to be derived from the content of pulp primary cell walls, as these polymers mainly come from the berry crushing and are probably already depolymerised at 24 Brix (i.e. the harvest stage). The CoMPP results also showed that the HGs and RGI polymers in the wine fractions are mainly in de-esterified forms.

The results from the combined profiling and fractionation studies can be confidently confirmed as each of the datasets derived from a particular technique generally supported each of the other analyses on the same samples, although providing slightly different information. This, in our opinion, strongly validates the application of high-throughput cell wall profiling approaches to characterise changes occurring in the standard winemaking process, similarly to that shown for other plant tissues previously, such as tobacco leaves (Nguema-Ona *et al*, 2012) and grapevine leaves (Moore *et al*, 2014b). This validation work is very important for further studies, by establishing baseline reference datasets, as the experimental winemaking process followed in this study is very close to real winemaking processes (albeit not at an industrial scale). The data from the pomace cell wall profiles (identifying two main fractions) has important implications for the winemaker, potentially helping practioners understand the complexity of the substrate they work with, but also to provide insights into using tailor-made enzyme (cocktails) preparations to achieve more effective cell wall degradation for obtaining more free-run juice (must), colour pigments, tannins and other beneficial compounds in the final wine.

6.2 The role of intra-vineyard variation of harvested grapes (and their related cell wall differences) and the influence of the commercial enzyme preparations during the corresponding winemaking

Intra-vineyard variability is an important topic which has been addressed in a number of recent studies including (i) Hannah *et al*. (2013) looked at berry and wine composition in the context of climate change for wine grape growing countries (i.e. sugars, organic acids, phenolics, etc.); (ii) Zhang *et al*. (2015) studied rotundone variability in Shiraz vineyards; and (iii) in Young *et al*. (2015) which studied grapevine plasticity (Sauvignon Blanc in this case) in response to altered bunch zone treatment (i.e. leaf removal); and identified plastic (responsive) and non-plastic (non-responsive) metabolites to the exposure treatment; in an intra-vineyard experimental design. The checkered-board vineyard experimental design used in our study (Chapter 4) was based on that used in Young *et al*. (2015); and was derived from the Theory-of-Sampling (described in Peterson *et al*. (2005) placed in a biological context. This study (Chapter 4) was unique in combining the chequered board design (Young *et al*, 2015) for pooled harvesting of untreated and enzyme-treated denoted vineyard panels (a panel consisted of six vines); with measurements of basic grape berry parameters (i.e. Brix, soluble solids etc..) with the validated high throughput cell wall profiling approach (without fractionation) (from Chapter 3; Gao *et al*, 2015). After winemaking three datasets were obtained (i) basic winemaking parameters (i.e. Brix, pH, soluble solids etc.); (ii) knowledge of the treatments received (i.e. no enzyme, XL fruit etc.); and (iii) the corresponding cell wall datasets from treated and untreated panels (see Chapter 3). The results from Chapter 4 showed the clear variability of cell wall compositions from grapes within each pooled panel of the experimental vineyard, and it was noted that the different development levels of the pooled grape cell walls on average appeared to generally correspond with level of maturity determined via viticultural criteria (i.e. soluble solid content (Brix value) of berries. This correlative relationship with ripening (maturity) stage of berries with cell wall composition confirms what was described by Nunan *et al*, (1998) and more recently by Moore *et al*, 2014a at a general level although it is known that grape marc cell walls vary due to cultivar studied (Apolinar-Valiente *et al*, 2015). The cell wall profiles (Chapter 4) obtained from fermented pomace suggested that the variability present in berry maturity (i.e. ripeness) had been brought into the corresponding winemaking, resulting in relatively high amount of variation in the degree of cell wall degradation (mainly in the pectin-rich fraction) and the esterification levels of grape pectin samples. Interestingly, this variation of the pectin

degradation was significantly (markedly) reduced by the use of of all three of the commercial enzyme preparations. The corresponding exposure of hemicellulose epitopes in enzyme-treated pomace further confirmed the effectiveness of enzymes. This study also showed the importance of using a multivariate data analysis approach with the cell wall profiling datasets, which revealed that the variation of esterification of pomace increased by using the enzyme preparations; this could not be observed through visual inspection of the relevant CoMPP heatmaps. This study (Chapter 4) also show the benefits of a sound experimental design to identify specific treatment effects (e.g. enzymes) and the usefulness of a validated cell wall screening approach to assess enzyme efficacy in reducing grape cell wall variability in a winemaking scenario. This study confirmed the importance of using maceration enzymes in the winemaking, which clearly minimised the inevitable variability in degree of cell wall degradation (i.e. de-pectination) that correlated with the different maturity (ripeness) stages of the berries collected from different vineyard panels. This variability was also found in the final wine oenological parameters determined using a standard wine spectroscopic approach (i.e. calibrated FOSS winescan outputs), and showed that enzyme treatments reduced the variability in these wine parameters in the final wine obtained. However, it was difficult to make further headway in understanding the grape berry at a cell wall structural and architectural level by using only commercial enzyme preparations. Nevertheless, this study (Chapter 4) again confirmed the value using of the cell wall profiling tools to study enzyme treatments in a vineyard setting, but also provided a enzyme treatment validated “positive control” for maceration enzymes in winemaking conditions for the third and final experimental chapter of the thesis (Chapter 5).

6.3 The use of recombinant enzymes to elucidate aspects of grape berry cell wall architecture in a winemaking scenario

In Chapter 5 it was demonstrated the benefit of using pure recombinant enzymes, as single enzymes or in combinations, under winemaking conditions to deconstruct grape berries cell walls (i.e. maceration) in order to understand cell wall architecture. The great value of this study comes from the application of CoMPP methodology, by application of monoclonal antibodies and CBMs which identify carbohydrate polymers by virtue of the epitopes recognised, which provides an extremely valuable way to study the complex multi-layered structure of the grape cell wall, in combination with the application of various

recombinant enzyme mixtures, allowing for a more comprehensive understanding of grape cell wall structure.

Different effects on the cell wall degradation were observed from using different enzyme combinations. Firstly, the untreated samples (negative control) showed very weak effects on both pectin and hemicellulose fractions, which confirmed our expectations, and furthermore, endo-polygalacturonase (EPG) showed a similar weak impact as found for the negative control. In our view this supports the high esterification levels of pectin in the pomace, confirmed the previous findings reported in Chapter 3 (Gao *et al*, 2015). The high degree of pectin esterification was also confirmed by the other three treatments (termed EPM, ARA and GAL) which contain pectin methyl esterase (PME). Curiously, the combination of EPG and PME did not break down the pectin layer as we expected, but only unravelled the pectin structure (similar to results obtained from Zietsman *et al*, 2015a). A number of reasons for this were considered in the chapter, the most plausible is in considering the multilayer structure of HG (high esterification) backbones which make the synergistic action of EPGs and PMEs difficult, as well as the potential blocking effect from RGI side chains (i.e. branched arabinan). The use of arabinanase (ARA) and galactanase (GAL) in the PME preparations also helped to identify some unusual aspects of arabinogalactan and side chain properties in grape pomace cell walls which need further investigation. Lastly, it was observed that pectin lyase (PL) containing preparations worked as effectively as the commercial enzyme preparation (Lafase HE Grand Cru), by breaking through the highly methyl esterified RGI layers in the pectin and hemicellulose (albeit partially for the latter) layers of the berry pomace to de-pectinate (but not necessarily de-esterify) the layered cell wall tissues during winemaking. This suggested that pectin lyase is a core and essential enzyme in commercial pectinase preparations for winemaking.

The most interesting aspect of the work is to combine the information of the cell wall layers (Chapter 3, Gao *et al*, 2015) with the information gathered on recombinant enzymes (Chapter 5) and literature references. With this approach it was possible to generate a hypothetical grape berry cell wall model, in the context of winemaking (see Chapter 5). This model was describe to have three independent layered components in pre-crushed grape berries: (i) the skins plus the residual pulp (ii) the highly esterified more structured pulp layers, and (iii) the less esterified more depolymerised pulp. This model can be used to help design new enzyme formulations that are more effective for the specific winemaking objective (i.e. more effective and quick extraction; or slower maceration etc.) and thus aid the winemaker in his/her choices of enzyme preparations.

6.4 Limit of the current study and the future research avenues

To summarise, this study validated the high throughput cell wall profiling approach (in combination with fractionation methods) in a red winemaking scenario, which allowed for an extensive investigation of the grape berry cell wall architecture, and answered a number of questions of cell wall at the polymer level, such as the cell wall composition of the berry, the fermented pomace and the concentrated wine polysaccharides. While also investigating the correlations between the cell wall maturity (ripeness), Brix values and the use of enzyme treatments (commercial and recombinant), and which allowed for the development of a hypothetical grape berry cell wall model at the stage of winemaking.

CoMPP, as a relatively new technique, was used as a core tool in this study, and provided insightful datasets that went beyond what is obtainable through bulk chemistry information (i.e. monosaccharide composition analysis). Gas chromatography for monosaccharide compositional analysis has been used for decades as a classical cell wall method. However, it has shown limitations when compared to CoMPP, as the list of the number of new monoclonal antibodies targeting more specific epitopes continues to grow and subtle changes can be detected versus bulk chemistry. However it should be noted that these techniques in combination are an extremely insightful way to study cell wall architectural changes (in this context winemaking) as CoMPP in general provides a fast and effective way to profile and compare the cell wall samples, and has helped cell wall research to advance in other fields (e.g. Fangel *et al*, 2012, Nguema-Ona *et al*, 2013, Zhang *et al*, 2013) as well as in winemaking research (e.g. this study and Zietsman et al. 2015a). However, CoMPP still has some disadvantages. Firstly, it is a semi-quantitative method, the difference in sensitivity level (i.e. the difference in avidities of mAbs to epitopes from different polymers and polymer classes) of each specific cell wall polymer to antibodies may influence the results. This was accounted for in our study, through the use of other techniques (e.g. GC, FTIR) which helped to support the CoMPP results and put them in context. Secondly, the normalised and scaled heatmap visualisations may also mask information as these data represents the relative abundance and remove the dynamic scaling. This limitation can be somewhat overcome by applying multivariate data analyses on the CoMPP raw datasets, and in doing so we have identified important information from the PCA plots which was not easily identifiable in the CoMPP heatmaps (discussed in Chapters 4 and 5). Thirdly, the cell wall profiles acquired from CoMPP datasets can only suggest the possible association

between the polymers, but cannot prove the existence of actual linkages, however, this may lead in the direction for the application of additional techniques, such as NMR, liquid chromatography-mass spectrometry (LC-MS), hydrophilic interaction liquid chromatography (HILIC) with MS-MS, to provide detailed studies, asking specific questions which are now raised from the broad cell wall profiling approaches.

Another important consideration is that the cell wall analysis we performed is still on *in vitro* basis. The isolation and enrichment process may influence the cell wall samples by changing the actual structure or masking effect by other used chemicals. Thus, the information generated may not be fully true to the cell wall in the plant. The combined work of microscopy with monoclonal antibodies can be a next step of cell wall study on the berry and other plants. It will be very interesting if we can apply enzymes to this cell wall imaging work as well, thus, the location of the specific polymer can be identified, which would be very useful to understand the fundamental structure of the cell wall. However, a number of challenges are needed to be overcome, firstly, the preparation of the berry samples for microscopy work is not easy, especially in the ripe stages as many cells have started to de-polymerise at this stage. This was shown in microscopy images from Nunan *et al* (1998). Thus, the optimisation of the preparation method for berry cell wall is crucial before further experiments. Secondly, the selection of the mAbs is most important and it is advisable to generate a cell wall profile, including CoMPP, before the detailed microscopy work can be performed (Marcus *et al*, 2008).

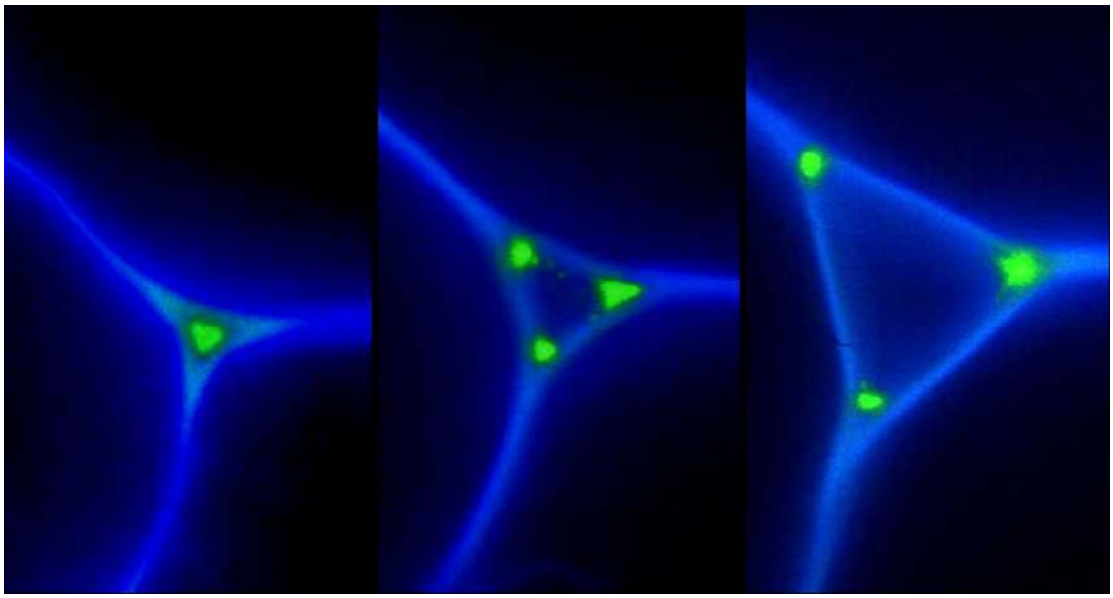


Figure 1 A. Three intercellular spaces in pea stem parenchyma (TS), showing space formation, labelled with Calcofluor White for cellulose (blue) and monoclonal antibody LM7 for non-blockwise-deesterified homogalacturonan (green). Hand-cut sections. From Knox cell wall lab website (http://www.personal.leeds.ac.uk/~bmbjpk/)

Furthermore, the optimisation of microscopy work combined with monoclonal mAbs will elevate us to the next level of understanding of grapevine berry cell walls. We would be able to generate a better view of the cell wall polymers distribution throughout the different tissue types (Fig. 2), understand more comprehensively the changes of berry cell walls during grapevine development. The localization of beneficial compounds can also be identified in detail by applying the chosen cell wall degrading enzyme with the microscopy with immunocytochemical work using cell wall mAbs and CBMs. Such compounds (e.g. pigments, tannins) may not be distributed evenly in one tissue type; thus the enzyme degradation and metabolite release could perhaps be observed in “real time”.

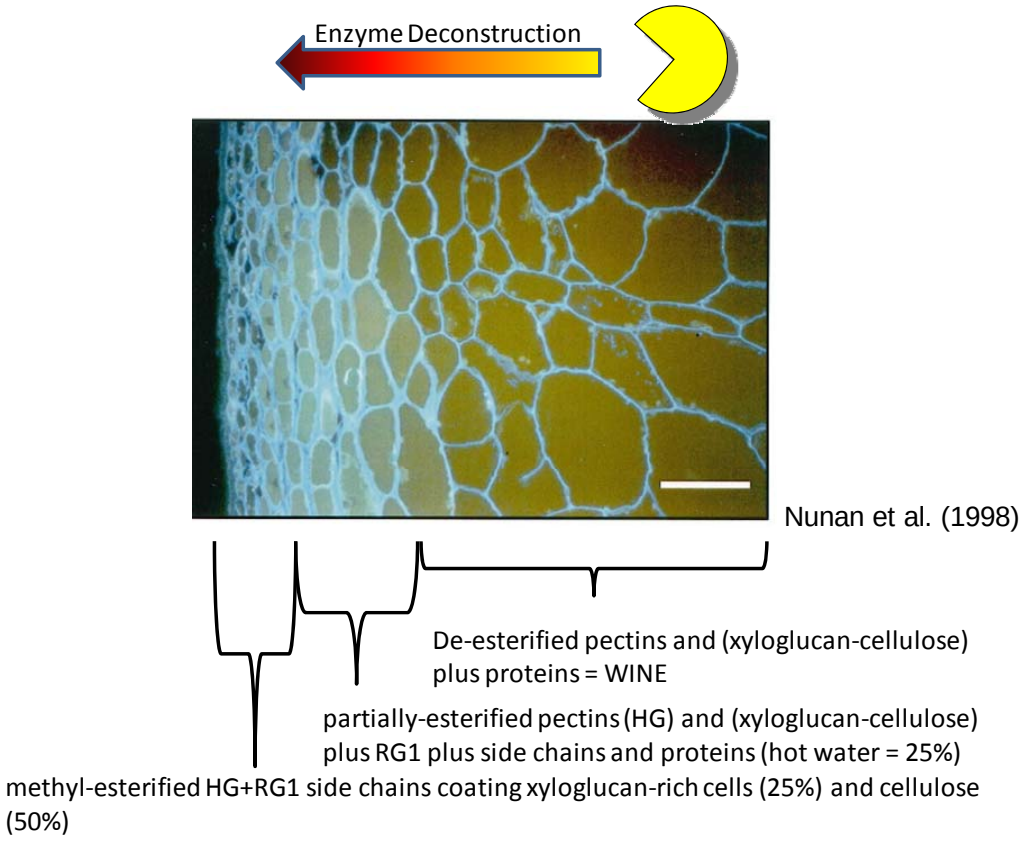


Figure 2. The hypothetical cell wall composition and structure (based on this study, especially Chapter 3, Gao *et al*. (2015)) of different tissue types of grape berry, the microscopy image is from Nunan *et al*, 1998.

This study also brings new insights for future wine research. As most of the previous research was on the monosaccharide level (Gorshkova *et al*, 1996, Nunan *et al*, 1997, Arnous & Meyer, 2009), the validated high throughput cell wall profiling approach now can provide the polymer-level view of the relationship of the cell wall polysaccharides with not only the extraction of these beneficial compounds, but also their impact on those compounds in the wine (such as stability, ageing). Thus, by having the relationships between many factors (e.g. cell wall models, use of enzymes, release of compounds, stability of compounds), the multivariate data analysis can be used more functionally to look for the interrelationships and cross-correlations (OPLS, O2PLS, etc.). Hence, multivariate models with grape cell wall information, oenological parameters and compound release data (e.g. polyphenols) would permit a more comprehensive view of the winemaking system and the importance of carbohydrate polymer degradation/disruption enzymes in facilitating these processes.

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