Enzymatic Release of Phenolics from Fruit Skins – With Grape as the Main Model

Anis Arnous
Ph.D. Thesis
2009
Enzymatic Release of Phenolics from Fruit Skins – With Grape as the Main Model

Anis Arnous
Ph.D. Thesis
M.Sc. in Food Quality Management
The thesis is submitted in fulfilment with the requirements of the degree of PhD in Enzyme Technology
June, 2009
Preface

This work was carried out Mar. 2004 – Feb. 2006, and Aug. 2007 – June 2009. The work was commenced at BioCentrum, DTU, and was completed at the Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, DTU, after our group relocated in autumn 2006.

The work was accomplished under the supervision of Prof. Anne S. Meyer, Head of Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, DTU. To her I wish to express my gratitude for continues support during the whole course of this study, and for providing the excellent facilities for my work and for the encouragement, exceptional ideas, and tireless optimism that have kept me going.

The PhD project was funded 1/3 by the grant “FELFO project 2056-03-0026, Novel process strategies in fruit juice productions” and 2/3 from a PhD scholarship from DTU.

The thesis is submitted in fulfilment with the requirements of the degree of PhD in Enzyme Technology.

Anis Arnous

Tuesday, 30th of June, 2009
Abstract

Microbially derived pectinolytic enzyme preparations are widely used for pre-press fruit maceration in the industrial production of apple and berry juices, ciders, and wines to increase juice yields. The skins of both grapes (*Vitis vinifera* L.) and apples (*Malus domestica*), but notably of grapes, are known to be rich sources of phenolic compounds. Maximizing the release of phenolics from the skins is of significant importance for the product quality, and in the case of red wine, also for the colour, of the final products. In recent years, there has been an increasing interest in using enzymes more aggressively to enhance the release of coloured pigments and antioxidant phenolic compounds during the pre-press treatment in both juice and wine processing. Moreover innovative valorisation of the processing fruit skins is an important part of the development of sustainable production methods in large-scale food processes. Biocatalysis based techniques for upgrading of the low-value fruit skins is a safe way to accomplish a sustainable valorisation. More detailed knowledge about the location and bonding of the phenolics within the fruit skin polysaccharide matrix, and on the relationship between carbohydrate composition and phenolics release and yields, would, however, allow a more rational targeting of the enzymatic treatments and in turn also allow for improved fruit juice and wine processes. Hence, the main objective of the present PhD study was to examine and characterise the enzyme catalysed release of phenolic compounds from fruit skins using wine grape skins as the main model system.

Several hypotheses have been formulated and put into test to examine the interplay between enzymatically catalyzed cell wall degradation and retrieval of phenolics. Moreover, this knowledge will build a strong foundation for innovative upgrading of plant residues in general. Obtaining quantitative knowledge about the structural makeup, including the glycosidic bonds of the fruit skin polysaccharides, is the first step.

In the present work, a method for evaluation of grape and apple skin cell wall carbohydrates using acid hydrolysis was developed. As a first step, after acid hydrolysis, the monosaccharide profile of the skin cell wall was determined using anion exchange chromatography with electrochemical detection (HPAEC-PDA). With the use of a stepwise, iterative, calculation methodology, the monosaccharide profile was used to quantitatively predict the levels of different structural plant cell wall polysaccharide elements (e.g.
homogalacturonan, xyloglucan, rhamnogalacturonan-I, rhamnogalacturonan-II, mannan, cellulose) in apple and grape skins. For both the grape and the apple skin samples, the polysaccharides in the skin cell wall matrix appeared to be mainly made up of pectins (mainly homogalacturonan and rhamnogalacturonan-I). Furthermore, evaluation of the profile of the phenolics in grape and apple skins were done using reversed phase chromatography with UV-VIS diode array detection (RP-UV/Vis) after methanolic (60%) sequential chemical extraction. High levels of phenolics were found in the grape skin, particularly anthocyanins, while apple could be a potential source of catechins. A high level of Klason lignin was found by gravimetrical determination in grape (~45 %) and a lower level in apple (~35 %).

Detailed studies, to examine the release of phenolics during enzymatic (pectinolytic and cellulolytic) degradation of the cell wall polysaccharides in the skins of Merlot and Cabernet Sauvignon wine grapes (*Vitis vinifera* L.), were reported. Anthocyanins and flavonols were released from the skins during the early phases of the enzymatic treatments. However, the anthocyanins were degraded during further enzymatic treatment due to temperature as the main factor; while flavonols underwent transformation from glycosylated (rutin) to deglycosylated (quercetin). Phenolic acids, including hydroxybenzoates and hydroxycinnamates, were released as a function of the liberation of monosaccharides, i.e. as a function of the enzyme catalyzed cell wall degradation of the skins. These data suggest that *p*-coumaric acid was also released during enzyme catalyzed degradation of acylated anthocyanins, probably as a result of cinnamate esterase activity. The data provided unexpected new clues to how the enzymatic treatment with multicomponent pectinolytic enzymes may promote a) discriminated release of phenolics from grape skins, and b) molecular changes in the phenolics, and provides a new base for upgrading of phenolics from wine grape skins for winemaking and grape pomace valorisation.

Finally an *in planta* *o*-hydroxylation activity in the grape skin was discovered. This endogenous activity results in catalytically conversion of *p*-coumaric acid to caffeic acid, and the kinetics of this activity is also reported. The discovery of this *in planta* *o*-hydroxylation activity may open the door for a novel type of endogenous enzyme based bioconversion strategy to improve the levels of the natural antioxidant caffeic acid in grape juices and wines.
Dansk Sammenfatning

I den industrielle produktion af æble- og bærjuice, cider, og vin tilsættes pektinolytiske enzymer fra skimmelsvampe med henblik på at opnå forflydning af frugtmassen inden saft-presningen og dermed øget saftudbytte. Frugtskallen eller -skrællen fra både druer (*Vitis vinifera* L.) og æbler (*Malus domestica*), er rige på fenoler. I de seneste år har der været en tiltagende interesse for at bruge enzymer mere offensivt for at forbedre frigivelsen af de phenoliske forbindelser, både farvede pigmenter og antioxidative fenoler, fra frugtskallen i forbindelse med den enzymatiske behandling før pressetrinnet i både saft- og vinprocesser.

Maksimering af frigivelsen af fenoler fra frugtskallen er af stor betydning for kvaliteten af det endelige produkt, og hvad angår rødvin, også for farven. Derudover er innovativ oparbejdning af de tiloversblevne presserester, herunder af skaldelene, nu blevet en afgørende parameter i for udvikling og opretholdelse af økonomisk konkurrencedygtige processer i vin- og frugtsaftindustrien. Teknikker baseret på biokatalyse vurderes at være særlig attraktive til oparbejdning af de tiloversblevne frugtskeller, idet der med biokatalyse kan designes precise og skånsomme processer. En forudsætning for udvikling af mere målrettede, rationelle enzymatiske metoder hertil kræver imidlertid viden om lokalisationen og de mulige bindinger der fastholder fenolerne i frugtskal-matricen - herunder relationen mellem kulhydratsammensætningen i frugtskallen og frigivelsen og udbyttet af de phenoliske komponenter. Sådan viden vil også forbedre grundlaget for udvikling af bedre saft- og vinprocesser. Formålet med nærværende PhD afhandling var derfor at undersøge og karakterisere enzymkatalyseret frigivelse af fenoler fra frugtskeller med drueskeller som det primære modelsystem. En forudsætning for udvikling af mere målrettede, rationelle enzymatiske metoder er også foruddragelse af de tiloversblevne frugtskeller.

hovedsageligt af pektin (primært homogalakturonan and rhamnogalakturonan-I). Den kvantitative profilering af fenolerne i drue- og æbleskal blev undersøgt vha. revers-fase HPLC med UV-VIS diodearray detektion (RP-UV/Vis) efter sekventiel kemisk ekstraktion af fenolerne med methanol (60 % vol/vol). Høje niveauer af fenoler, specielt af anthocyaniner, blev fundet i drueskal, mens æble blev fundet at kunne være en potentiel kilde til catechiner. I både drue- og æbleskal, blev der fundet høje niveauer af Klason-lignin, hhv. ~45 % vægt/vægt i drueskal og ~35 % i æbleskal.

Table of Contents

Preface ........................................................................................................................................... v
Abstract ....................................................................................................................................... vii
Dansk Sammenfatning .............................................................................................................. ix
Table of Contents ......................................................................................................................... xi
List of Figures ............................................................................................................................ xiii
List of Tables .............................................................................................................................. xiv
List of publications ...................................................................................................................... xv
Acknowledgements .................................................................................................................... xvi
Hypotheses .................................................................................................................................. 18
Aim .............................................................................................................................................. 19
Chapter 1 Background about phenolics in grape berry: Origin, classification, and functionality as dietary antioxidants ......................................................................................................................... 20
1.1 Nutritional implications of phenolic compounds .............................................................. 20
1.2 Grape as an important source of phenolics ................................................................. 21
1.3 Phenolics in red wine grape: Their origin and classification ............................................ 23
    1.3.1 Nonflavonoids (low molecular weight) .............................................................. 24
        1.3.1.1 Hydroxybenzoic acids .............................................................................. 24
        1.3.1.2 Hydroxycinnamic acids ........................................................................... 24
    1.3.2 Flavonoids ............................................................................................................. 25
        1.3.2.1 Anthocyanidins/Anthocyanins .............................................................. 26
        1.3.2.2 Flavanols (flavan-3-ol) .............................................................................. 27
        1.3.2.3 Flavonols ..................................................................................................... 28
        1.3.2.4 Tannins ......................................................................................................... 28
    1.4 Red wine grape phenolics as dietary antioxidants .......................................................... 30
        1.4.1 Antioxidant activity in vitro of different phenolics in red wine grape .......... 30
            1.4.1.1 Total phenolic compounds .............................................................. 30
            1.4.1.2 Cinnamates and hydroxybenzoates ................................................... 30
            1.4.1.3 Flavonoids ................................................................................................... 31
            1.4.1.4 Anthocyanidins ...................................................................................... 31
            1.4.1.5 Flavanols (flavan-3-ol) ............................................................................ 31
            1.4.1.6 Flavonols ................................................................................................ 32
Chapter 2 Enzyme technology to upgrade grape skins by improving release of phenolics ...... 33
2.1 Composition of polysaccharides in the primary cell wall of dicots ......................... 33
2.2 Commercial enzymes used in wine and juice making .............................................. 34
List of Figures

Figure 1.1 Global production of main fruit crops including grapes (—), apples (—), oranges (—), pears (—), and cherries (—) (FAO-UN, 2009). ................................................................. 22
Figure 1.2 Main hydroxybenzoic acid derivatives reported in red wine grape. ..................... 24
Figure 1.3 Main hydroxycinnamic acids and their derivatives reported in red wine grape. ...... 25
Figure 1.4 Flavonoid base structure and standard numbering system........................................ 26
Figure 1.5 Structure of main anthocyanins in red wine grape.................................................. 26
Figure 1.6 Hydrolysis of a typical grape anthocyanin, malvidin-3-β-(6-p-coumaronyl)-D-glucose, yielding the aglycone malvidin (anthocyanidin), glucose, and p-coumaric acid. Modified from (Zoecklein et al., 1990). .......................................................... 27
Figure 1.7 Representative structures of flavanols (flavan-3-ols). .............................................. 28
Figure 1.8 Flavonols (aglycone-form) found in red wine grape................................................ 28
Figure 1.9 Representative structure of a) Hydrolysable tannins; b) Condensed tannins (proanthocyanidin), (Escarpa and Gonzalez, 2001). ................................................................. 29
Figure 3.1 Schematic diagram of the grape skin preparation steps ........................................... 53
Figure 3.2 Separation of neutral and acidic monocarbohydrates on the anion exchange column PA20 ® Dionex according to Arnous & Meyer (2008). ......................................................... 54
Figure 4.1 Full factorial setup development by examining rationally the influence of reaction parameters including enzyme to substrate ratio (1, 5.5, or 10%), time (2, 4, or 6 h), and temperature (25, 40, or 55 °C) and relating the finding to surface response as the final step. 74
Figure 4.2 Steps and conditions for enzyme catalyzed cell wall degradation of the grape skin. 75
Figure 4.3 HLPC chromatogram recorded at 365 nm showing the transformation of glycosylated flavanol (rutin) to deglycosylated flavanol (quercetin) during the enzymatic treatment with Pectinex® BE Colour enzymatic preparation : a) after 2 hours, b) after 6 hours. ................................................................................................................................. 76
Figure 5.1 Reversed phase HPLC chromatogram of rapid separation of phenolics. Caffeic acid is produced from hydroxylation of added p-coumaric acid by catalytic activity in the grape skin. ................................................................................................. 89
List of Tables

Table 1.1 Mortality from coronary heart disease among 35-64 year old males (Renaud and Delorgeril, 1992). ........................................................................................................................ 20
Table 1.2 Total phenol levels in grape berries (Zoecklein et al., 1990). ................................................. 22
Table 1.3 Classification of the main phenolic classes in red grape wine as a function of molecular weight. ........................................................................................................................................... 23
Table 2.1 Mode of action for the pectinase enzymes with their substrate specificity (Pilnik and Voragen, 1991; Burns J.K., 1991; Grassin and Fauquembergue, 1996). ................................................................. 36
Table 2.2 The cell wall degrading enzymes cellulases and hemicellulases. Mode of action and their substrate specificity are given (Grassin and Fauquembergue, 1996; Ward and Mooyoung, 1989). ........................................................................................................................................ 37
List of publications

The thesis is based on the following manuscripts:

I. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release.
   Pinelo, Manuel, Anis Arnous, and Anne S. Meyer.

II. Comparison of methods for compositional characterization of grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins.
    Arnous, Anis and Anne S. Meyer.
    *Food and Bioproducts Processing*, 86 (2008), pp. 79-86.

III. Quantitative prediction of cell wall polysaccharide composition in grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins from acid hydrolysis monosaccharide profiles.
    Arnous, Anis and Anne S. Meyer.
    *Journal of Agricultural and Food Chemistry*, 57 (2009), pp. 3611-3619.

IV. Discriminated release of phenolic substances from red wine grape skins (*Vitis vinifera* L.) by multi component enzyme treatment.
    Arnous, Anis and Anne S. Meyer.

V. Grape skins (*Vitis vinifera* L.) catalyze the *in vitro* enzymatic hydroxylation of *p*-coumaric acid to caffeic acid.
    Arnous, Anis, and Anne S. Meyer
Acknowledgements

I would like to express my sincere thanks to all my colleagues at the BioCentrum and later Centre for BioProcess Engineering at DTU, for creating inspiring conditions for work.

Special thanks to Katrine Landbo for sharing with me the joy/stress of the daily work. My thanks are also due to my friend and college, Manuel Pinelo, who is also co-author of one of the published articles.

I want to thank my parents for strong support all these years I have been away, and thanks to my whole family, especially my bothers Amr and Hosam for distance technical support with computer software and hardware.

Finally, I want to thank my loving angel, Ida, for her true love and support during all these years, for linguistic help, and for her never-ending encouragement especially at the last stage of my work.
Hypotheses

Phenolics present in fruit have been shown to have a beneficial role in human health, as phenolics have the ability to inhibit the oxidation of polyunsaturated fatty acid groups in low-density lipoprotein (LDL). Hence, in grape wine and in juice production it is of interest to improve the release of phenolics.

For phenolics located in the fruit skin in general and for grape skin in particular there is a lack of knowledge on the relation between the grape skin cell wall degradation and the release of the phenolics. This knowledge is essential in order to evaluate what role controlled enzymatic degradation could play.

The overall hypothesis underlying this PhD work was that a more detailed understanding of the interplay between enzyme catalyzed cell wall degradation and phenolics release could provide for design of targeted enzyme treatments in fruit juice and wine processing. An improved knowledge could possibly result in innovative upgrading of press residues by improving the release of phenolics.

Several hypotheses were tested in this PhD project:

It is possible to deduce the building blocks of fruit skin cell wall polysaccharides using monocarbohydrates profile analysis.

There is a relation between the degradation of the grape skin cell wall polysaccharides and the release of different phenolic classes.

Some phenolics classes are bound to the grape skin cell wall polysaccharides.

Some phenolics classes are in free form and not bound to grape skin cell wall polysaccharides.

It is possible to selectively extract and retain the phenolics which have the most potent antioxidant activity.

It is possible to enzymatically modify the phenolics to optimize their health potentials.
Aim

The aim of this PhD project was to explore in detail the enzyme assisted extraction of phenolics from fruits skin with grape as the main model. As a part of this, the aim was also to provide knowledge on how the phenolics are bound in the skin polysaccharide-lignin matrix by:

- Elucidating grape skin cell wall polysaccharide composition.
- Unravel how the phenolics are bound in the grape skin.
- Unravel the relation between the enzymatic hydrolyses of monosaccharides and the release of phenolic compounds from the cell wall of grape skin.
Background about phenolics in grape berries: Origin, classification, and functionality as dietary antioxidants

1.1 Nutritional implications of phenolic compounds

Epidemiological studies have shown that a high intake of fresh fruits and vegetables rich in phenolics is associated with a lower risk of mortality due to coronary heart disease (CHD) fatality. However, for the French population, the association of saturated fatty acid intake with mortality from coronary heart disease CHD does not apply. The reported coronary mortality per 10,000 people in the United States is 182, and in the United Kingdom even 380, whereas the overall mortality of French is 102, and in the Toulouse region it is as low as 78 (Table 1.1).

<table>
<thead>
<tr>
<th>Country</th>
<th>Mortality per 10⁴</th>
<th>Plasma Cholesterol (mg/dL)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>33</td>
<td>224</td>
</tr>
<tr>
<td>Toulouse (France)</td>
<td>78</td>
<td>216</td>
</tr>
<tr>
<td>France (General)</td>
<td>102</td>
<td>209</td>
</tr>
<tr>
<td>Stanford (USA)</td>
<td>182</td>
<td>240</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>380</td>
<td>240</td>
</tr>
</tbody>
</table>

*milligrams/decilitre: the traditional unit for measuring blood glucose

This discrepancy is referred to as the “French Paradox”. The French people consume similar amounts of saturated fats and have similar risk factors and comparable plasma cholesterol to the population in the United States. The one dietary factor that showed a negative correlation with CHD was the consumption of wine. An association between a reduction in CHD and alcohol consumption has been indicated. However, it is the consumption of red wines, which has proved to be the most beneficial (Renaud and Delorgeril, 1992). Compared to other alcoholic beverages, the main difference is the...
Chapter 1

Constitution of the phenolics in red wine, which is presumed to have a certain kind of biological activity, that might reduce the free radical-mediated oxidation of low-density lipoprotein (LDL) and so decrease artherogenicity (Caccetta et al., 2000).

The overall explanation for the beneficial role is that phenolics inhibit the oxidation of polyunsaturated fatty acid groups in LDL by intercepting the free radical propagation of LDL oxidation (Meyer et al., 1997). Other mechanisms have been proposed as possible explanation for the beneficial effects of red wine in the prevention of CHD, such as inhibition of platelet aggregation (Durak et al., 1999), and endothelium-dependent relaxation of blood vessels (Burns et al., 2000).

1.2 Grape as an important source of phenolics

In general, fruits are clearly an excellent source of phenolics (Naczk and Shahidi, 2006). Grape (Vitis vinifera L.) is among the three most produced fruits globally beside apple and orange. The annual production for 2007 was more than 60 million tonnes (Fig. 1.1). Future projection indicates an increase in the global grape production, particularly because non traditional geographical grape producers like South America and Africa are increasing their area for growing grape (FAO-UN, 2009).

Grape is one of the richest fruits in phenolics, particularly in the skin, and the seeds (Table 1.2). Estimates of total phenolics in red wines from different geographical regions vary from ~ 800 to 4000 mg·L⁻¹ (Haslam, 1998).
Figure 1.1 Global production of main fruit crops including grapes (—), apples (—), oranges (—), pears (—), and cherries (—) (FAO-UN, 2009).

Table 1.2 Total phenol levels in grape berries (Zoecklein et al., 1990).

<table>
<thead>
<tr>
<th>Component of red grapes</th>
<th>Phenol level, GAE* (mg•kg⁻¹ berries)</th>
<th>Phenol compound distribution in grape berries.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td>3525</td>
<td>Seeds 64.7%</td>
</tr>
<tr>
<td>Pulp</td>
<td>41</td>
<td>Pulp 0.8%</td>
</tr>
<tr>
<td>Juice</td>
<td>206</td>
<td>Juice 0.5%</td>
</tr>
<tr>
<td>Skin</td>
<td>1859</td>
<td>Skin 34.1%</td>
</tr>
<tr>
<td>Total</td>
<td>5631</td>
<td></td>
</tr>
</tbody>
</table>

*Gallic acid equivalent.

Phenolic compounds are extracted from different parts of the grape; the grape-skin, the seeds, and the stem in a water alcohol solution during the course of alcoholic fermentation (Zoecklein et al., 1990; Kennedy, 2008). But even with prolonged alcohol extraction of the skin during maceration, phenolic extraction rarely exceeds 50% of the total amount originally present in the grape (Haslam, 1998).
Chapter 1

As a result of the partial extraction of phenolics the full utilization of grape phenolics antioxidant activity is restricted to the soluble compounds in the juice. Grape skin represents a large portion of the grape pomace that is disposed after processing. It is possible that a significant proportion of the actual phenolic antioxidant activity of the berries is unaccounted for. Phenolics have a significant importance for red wine processing and quality, and there is a large potential for upgrading of phenolics from wine pomace. Despite this, there is a lack of precise knowledge and targeted technologies that enable the discriminated extraction of functional phenolics during processing of grapes that could also help valorisation of grape skins pomace.

1.3 Phenolics in red wine grape: Their origin and classification

Red grape wine contains a large array of phenolic compounds. One of the simplest and most useful classifications, for the most important phenolic compounds in food, was suggested by Escarpa and Gonzalez (2001). Based on this classification, red grape phenolics were listed into three groups according to their molecular weight (Table 1.3).

Table 1.3 Classification of the main phenolic classes in red grape wine as a function of molecular weight.

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Structure</th>
<th>Phenolic class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low(^1)</td>
<td>((C_6 – C_1))</td>
<td>Hydroxybenzoic acids</td>
</tr>
<tr>
<td></td>
<td>((C_6 – C_3))</td>
<td>Hydroxycinnamic acids</td>
</tr>
<tr>
<td>Intermediate(^1)</td>
<td>((C_6 – C_1 – C_3))</td>
<td>Flavonoids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High(^2)</td>
<td>((C_6 – C_1)_n)</td>
<td>Hydrolysable tannins</td>
</tr>
<tr>
<td></td>
<td>((C_6 – C_3 – C_4)_n)</td>
<td>Proanthocyanidins</td>
</tr>
<tr>
<td></td>
<td>(Condensed tannins)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Easily extractable phenolics or soluble phenolic fraction.

\(^2\) Non extractable phenolics or insoluble fraction.
Chapter 1

1.3.1 Nonflavonoids (low molecular weight)

The nonflavonoid group includes mainly two families: hydroxybenzoic and hydroxycinnamic acids. Nonflavonoids (low molecular weight) arise principally from the pulp of the berries as well as the seeds and skins extracted to juice during fermentation (Zoecklein et al., 1990).

1.3.1.1 Hydroxybenzoic acids

Hydroxybenzoic acids possess the simplest structure within the congregation of phenolic compounds which have the C₆–C₁ structure (Fig. 1.2). They are mainly located in the skin of grape. Gallic acid is the simplest phenolic acid and, together with vanillic acid, they are considered to be representatives for this group.

Gallic acid

\[ \text{Gallic acid} \quad R^1 = H, R^2 = R^3 = R^4 = \text{OH} \]

Vanillic acid

\[ \text{Vanillic acid} \quad R^1 = H, R^2 = \text{H}_3\text{CO}, R^3 = \text{OH}, R^4 = \text{H} \]

Syringic acid

\[ \text{Syringic acid} \quad R^1 = H, R^2 = R^3 = \text{H}_3\text{CO}, R^3 = \text{OH} \]

Protocatechuic acid

\[ \text{Protocatechuic acid} \quad R^1 = R^4 = H, R^2 = R^3 = \text{OH} \]

p-Hydroxybenzoic acid

\[ \text{p-Hydroxybenzoic acid} \quad R^1 = R^2 = R^4 = H, R^3 = \text{OH} \]

Figure 1.2 Main hydroxybenzoic acid derivatives reported in red wine grape.

Gallic acid and its dimer ellagic acid are important constituents of hydrolysable tannins. Gallic acid is also found in grape seeds combined with (-)-epicatechin to form epicatechin-3-α-gallate, and it can contribute to the formation of condensed tannins in grapes.

The technological processes used for wine production have a marked influence on hydroxybenzoic acid contents. During vilification, the extraction of these compounds depends not only on contact time with skin, but also on the ethanol content, which can increase the extraction (Gil-Munoz et al., 1999). Gallic acid content in California Cabernet Sauvignon red wines ranged between 65 mg·L⁻¹ and 126 mg·L⁻¹ (Burns et al., 2000; Ritchey and Waterhouse, 1999).

1.3.1.2 Hydroxycinnamic acids

Along with their derivatives, hydroxycinnamic acids are located primarily in the pulp of the grape. Hydroxycinnamic acids are represented by caffeic acid, p-coumaric acid and to less extent ferulic acid. They can be found in grape in the form esterified with tartaric acid.
Acid (Fig. 1.3) (Zoecklein et al., 1990). Hydroxycinnamic acids are mainly present in the pulp. In the skins they are present in lower amounts. The hydroxycinnamic acids can be bound to the glucose moiety of anthocyanins in red grape cultivars, and this is important for anthocyanin colour stability by intramolecular co-pigmentation.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Structural Formula</th>
<th>Derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>R¹ = H, R² = OH</td>
<td>Coutaric acid R = H</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>R¹ = R² = H</td>
<td>Caftaric acid R = OH</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>R¹ = OCH₃, R² = H</td>
<td>Fertaric acid R = OCH₃</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>R¹ = R² = OCH₃</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1.3 Main hydroxycinnamic acids and their derivatives reported in red wine grape.

Vast variation in the phenolic content of different red wines can be attributed to the grape varieties, as demonstrated by Mazza et al. (1999). A survey on the phenolic content of different varieties of wine in California showed that caffeic content could reach 12.8 mg·L⁻¹ for Cabernet Sauvignon aged red wine (Frankel et al., 1995).

Cafetaric acid (caffeoyl tartaric acid) could reach 14.6 mg·L⁻¹ and coutaric acid (coumaroyl tartaric acid) approx. 21.5 mg·L⁻¹ for some red wine samples (Burns et al., 2000). Ferulic and sinapic acids could be separated, but were not found in detectable levels (Burns et al., 2000). Salagoityauguste and Bertrand (1984) reported ferulic acid at 0.1 mg·L⁻¹ in Bordeaux young red wine.

1.3.2 Flavonoids

Grape flavonoids are found in the skin, seeds and tissue portion of the fruit. Most of the flavonoids are believed to occur naturally as glycosides, with their base structure (the aglycone) consisting of two aromatic rings, A and B, joined via a pyran ring (Fig. 1.4). The flavonoids are substituted with e.g. hydroxyl, methoxyl, acyl, or glycosyl groups at different positions and may have double bonds or a carbonyl group in the C ring. The most abundant flavonoid classes in grapes are anthocyanins, flavanols, tannins, and flavonols. These
Chapter 1

compounds all have OH-substitutions in position 5 and 7 of the A-ring in the flavonoid ring structure, and mainly vary in their hydroxylation pattern and other substitution patterns in the B and C rings.

Figure 1.4 Flavonoid base structure and standard numbering system.

1.3.2.1 Anthocyanidins/Anthocyanins

The wine colour is attributed largely to the presence of anthocyanins. The content, distribution, and accumulation of anthocyanins in grape skin is largely influenced by variety (Mazza et al., 1999). The anthocyanidins (also referred to as aglycones) generally occur as glycosides (primarily glucosides), in which specific hydroxyl functions are linked with sugar residues. Anthocyanins in grape species are 3-glucosides (Zoecklein et al., 1990; Haslam, 1998). Five anthocyanins are generally found in red grapes: malvidin-, delphinidin-, peonidin-, cyanidin-, and petunidin-3-D-glucoside (Fig. 1.5). Of these, malvidin (as the 3-glucoside) is the most common pigment in grape. (Bakker et al., 1998; Gil-Munoz et al., 1999; Gomez-Plaza et al., 1999; Castellari et al., 2001).

Grape pigments are often acylated with acetic, caffeic, or p-coumaric acids (Gao et al., 1997) (Fig. 1.6).
Figure 1.6 Hydrolysis of a typical grape anthocyanin, malvidin-3-β-(6-p-coumaronyl)-D-glucose, yielding the aglycone malvidin (anthocyanidin), glucose, and p-coumaric acid. Modified from (Zoecklein et al., 1990).

1.3.2.2 Flavanols (flavan-3-ol)

Within the flavan-3-ol group (+)-catechin and (-)-epicatechin (monomer), as well as their oligomers procyanidin B1, B2, and B3, are the most widely occurring components in red wine grape. Proanthocyanidins are mainly composed of (+)-catechin and (-)-epicatechin association, (see Fig. 1.7a). These compounds are responsible for the astringency and bitterness of the grape as well as in wine (Peleg et al., 1999). They are mainly located in the grape seeds (Fuleki and Ricardo-da-Silva, 1997) and the remainder in the skin (Haslam, 1998). As a general rule, the quantities of (+)-catechin and (-)-epicatechin are roughly equivalent in the grape seeds, but in the skins (+)-epicatechin is predominant (Haslam, 1998). According to Meyer et al. (1997), when seed crushing and longer extraction times of grapes are employed, high amounts of flavan-3-ols are extracted. Burns et al. (2000), reported the presence of epigallocatechin, epigallocatechin gallate and epicatechin gallate in red wine grape (Fig. 1.7 b). However, only (+)-catechin and (-)-epicatechin were present in detectable quantities in wines.
1.3.2.3 Flavonols

In grapes flavonols are localized in the solid parts of the grape, particularly in the skin and herbaceous parts. The flavonols, occurring in glycosidic forms (glucoside, galactoside and glucuronide) are, as a rule, an o-glycosidic bond through a hydroxyl group at the position three. However, flavonols, which constitute a small portion of the phenolic compounds in wine, can be found in the form of aglycone (Fig. 1.8).

![Flavonol Structures](image)

**Figure 1.8** Flavonols (aglycone-form) found in red wine grape.

Quercetin and rutin (quercetin 3-o-rutinoside) are the most abundant flavonols in red wine grape, followed by myricetin, kaempferol and isorhamnetin (Gil-Munoz et al., 1999; Bakker et al., 1998).

1.3.2.4 Tannins

In general, tannins refers to a fraction of phenolic compounds whose fundamental characteristic is the capacity to precipitate proteins, in particular the salivary proteins in the oral cavity, which is believed to give them an astringent character that can be easily recognized (Santos-Buelga and Scalbert, 2000). Structurally, tannins possess 12-16 phenolic groups and 5-7 aromatic rings per 1000 units of relative molecular mass (Haslam, 1998). This feature, together with their high molecular weight, clearly makes the tannins different...
both in structure and properties from the low weight molecular phenolic acids and monomeric flavanols. Tannins are classically divided into two groups: Hydrolysable tannins and Condensed tannins.

1.3.2.4.1 Hydrolysable tannins

Hydrolysable tannins have a simpler structure compared with condensed tannins. They exist as esters and, as such, can be degraded or hydrolysed. They contain a central core of polyhydric alcohol, which is usually glucose, and hydroxyl groups, which are esterified either partially or completely by phenolic acids (Fig. 1.9a). The phenolic acids are either gallic acid in gallotannins or other phenolic acids derived from the oxidation of galloyl residues in ellagitannins. After hydrolysis by acids, bases or certain enzymes, gallotannins yield glucose and gallic acid.

![Figure 1.9](image_url) Representative structure of a) Hydrolysable tannins; b) Condensed tannins (proanthocyanidin), (Escarpa and Gonzalez, 2001).

1.3.2.4.2 Condensed tannins

More important compounds in winemaking are proanthocyanidins (PAs), also known as condensed tannins, which form the second group of tannins (Fig. 1.9b). Structurally, they are more complex than hydrolysable tannins. They are polymers made of elementary flavan-3-ol units (Fig. 1.9b). The most recurrent of these structures are proanthocyanins, which are based on (+)-catechin and (-)-epicatechin, and which in turn form structural units. A key feature of PAs is that they yield anthocyanidins upon heating in acidic media, hence their name. According to Santos-Buelga and Scalbert (2000), proanthocyanidin values in various red wines of different varieties and vintages could range from 0 to 500 mg L⁻¹.
1.4 Red wine grape phenolics as dietary antioxidants

1.4.1 Antioxidant activity \textit{in vitro} of different phenolics in red wine grape

Antioxidant activity is the property most studied as far as the health benefits of wine consumption are concerned. Various \textit{in vitro} methods have been used to examine the antioxidant activity of different classes of wine phenolics.

1.4.1.1 Total phenolic compounds

The antioxidant activity of red wine grape is distributed widely among different phenolic substances belonging to different classes (Frankel et al., 1995). Moreover, the high antioxidant activity of red wine grape has been attributed to the synergistic effects of different phenolic compounds (Kanner et al., 1994). The resulting oxidised phenolics (OxPh) may be reduced and possibly regenerated by less active phenolics:

\[
\text{Ph (1) + ROO}^- \rightarrow \text{OxPh (1) + ROOH}
\]

\[
\text{OxPh (1) + Ph (2) \rightarrow Ph (1) + OxPh (2)}
\]

This could be the explanation why the antioxidant activity of plant extracts, including extraction of grape for (red) wine, are highly correlated with total phenolics determined as gallic acid equivalent (GAE) rather than for any individual phenolic compound (Frankel et al., 1995; Meyer et al., 1997).

1.4.1.2 Cinnamates and hydroxybenzoates

The antioxidant activities of the simple phenolic acids of benzoic acid and cinnamic acids have during the past few years been studied in many different model systems. In a recent study, the correlation between antioxidant activity and the hydroxycinnamic acid content in 13 different types of berries was found to be high (R² = 0.73) (Kahkonen et al., 2001).

Despite the different test systems used in determining the antioxidant activity of phenolic acids, the studies, including those involving human LDL as the oxidizing substrate (Natella et al., 1999), rather consistently show that the hydroxycinnamic acids have a higher antioxidant activity compared to the corresponding hydroxybenzoic acids. In studies with \textit{in vitro} human LDL oxidation, the antioxidant activity improves as the number of hydroxyl and methoxyl groups increases, and particularly the presence of the \textit{o}-dihydroxy group in the
phenolic ring, as in caffeic acid, consistently enhances antioxidant activity (Meyer et al., 1998; Natella et al., 1999).

1.4.1.3 Flavonoids

Numerous authors have investigated the antioxidant activity of flavonoids, and several attempts have been made to elucidate structure-activity relationships. There is a general agreement that flavonoids possess both excellent iron and/or copper chelating and scavenging properties (Burda and Oleszek, 2001; Fauconneau et al., 1997; Brown et al., 1998). Numerous studies have been carried out on structure-antioxidant activity relationships. The antioxidant activity by radical scavenging has mainly been confined to 3’ and 4’ hydroxylation of the B ring, i.e. catechol structure. Most of the problems encountered when describing flavonoid activity are inherent to the variance in the generation of radicals in the scavenging assays. Radicals can be generated either enzymatically, for example, by xanthine/xanthine oxidase-reductase (Valentao et al., 2001) or nonenzymatically by a transition metal alone or in combination with a reducing agent, such as ascorbate (van Acker et al., 1996). In both types of assays, flavonoids can interfere not only with the propagation reactions of the free radical, but also with the formation of the radicals, either by chelating the transition metal or by inhibiting the enzymes involved in the initiation reaction.

1.4.1.4 Anthocyanidins

The anthocyanins in red wine grape have been shown to contribute to the strong protection low-density lipoprotein oxidation (Frankel et al., 1995). Later, Meyer et al. (1997) compared the ability of phenolic extracts from fourteen different types of fresh grapes to inhibit LDL oxidation in vitro. Relative antioxidant activity was correlated with the concentration of anthocyanins ($R^2 = 0.56$). An even lower correlation ($R^2 = 0.3$) for the French wines was found using the 2,2’-azinobis-(3-ethylbenzothiazoline-6 sulfonic acid (ABTS) assay.

1.4.1.5 Flavanols (flavan-3-ol)

Along with flavonols, flavanols are reported to be among the most effective flavonoid subclass in red wine (Frankel et al., 1995). The relative LDL antioxidant activity correlated highly with the levels of flavan-3-ols ($R^2 = 0.86$) that were extracted from crushed grapes (Meyer et al., 1997). This clearly demonstrates their effectiveness as LDL oxidation inhibitors. Also, against lipid peroxyl radical species, catechins (catechin and epicatechin),
measured at 2.4 and 2.5 mM Trolox® Equivalent Antioxidant Activity (TEAC), were more than twice as effective as vitamins E and C (TEAC, 1.0 mM) (Kondo et al., 1999; Salah et al., 1995). The total antioxidant activity of investigated Italian wines was well correlated with the flavanol content ($R^2 = 0.93$) (Simonetti et al., 1997). A lower correlation value was reported between antioxidant activity and total catechins ($R^2 = 0.7$) for the French wines (Landrault et al., 2001).

1.4.1.6 Flavonols

Among various subclasses of flavonoids, flavonols have received great attention for their ability to act as natural antioxidants (Pulido et al., 2000). Quercetin and myricetin are the two most active compounds in retarding hydroperoxide formation. Recent studies have proved that quercetin is a powerful antioxidant in every system used, since it is able to protect lipid, protein, and DNA against oxidation (Burda and Oleszek, 2001; Makris and Rossiter, 2001). The aglycones of these flavonols have been reported to be more active than their glycosides (Chu et al., 2000). However, in biological systems, glycosides may undergo enzymatic hydrolysis, resulting in the formation of active aglycones (Waterhouse, 1995).
Chapter 2

Enzyme technology to upgrade grape skins by improving release of phenolics

2.1 Composition of polysaccharides in the primary cell wall of dicots

In dicotyledonous (dicots) plants, the plant cell walls are mainly made up of pectin polysaccharides, xyloglucan, cellulose, mannan, lignin and glycoproteins (Nunan et al., 1998). In dicots, including grapes and apples, the structural elements of pectin are known to be mainly made up of three structural units: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II), with HG and RG-I generally being the quantitatively most dominant (O’Neill et al., 1996). Xylogalacturonan is a fourth structural unit of pectin, which has been identified in cell wall extracts from e.g. soybean and watermelon (Mort et al., 2008; Nakamura et al., 2002), and which was recently presumed to be present in modified hairy regions of apple pectin, as a xylogalacturonan-RG-I linkage (oligomer GalA6Rha3Xyl1) was proposed (Coenen et al., 2007). However, no indications of the quantitative levels of xylogalacturonan have been given in any of these reports. Since xylogalacturonan has only been identified in few cases, we have presumed that xylogalacturonan might only make up a negligible amount of the plant cell wall material in the fruit skin samples studied here. Homogalacturonans are helical homopolymers of α-1,4-linked galacturonic acid monomers, which may be methyl-esterified and/or acetylated. Single α1,2 linked rhamnose residues may interrupt the long homogalacturonan chains, resulting in a bend, a “pectic elbow”, in the polygalacturonic acid structure (Grassin and Fauquembergue, 1996), but the galacturonic acid:rhamnose levels vary in different plant materials. In apple cell wall materials a typical ratio is 100 galacturonic acids to 1 rhamnose (Thibault et al., 1993). The backbone structure of RG-I is a heteropolymer consisting of an array of repeating disaccharides of alternating α-1,2-linked rhamnose and α-1,4-linked galacturonic acid residues (1:1) (O’Neill et al., 1996). The rhamnose residues in RG-I may be substituted with neutral side chains of galactan, arabinan and/or different arabino-galactan side-chains. RG-II has a homogalacturonan (HG) backbone rather than one of alternating galacturonic acid and rhamnose (as in RG-I) and RG-II uniquely has complex side chains attached to the galacturonic acid residues (O’Neill et al., 1996). RG-II acts as bridge to covalently cross-link two chains of HG in the cell walls of dicots, and predominantly exists
as a dimer (O'Neill et al., 2004) that makes up ~5% of the weight of buffer-soluble grape mesocarp polysaccharides (Nunan et al., 1997). The RG-II-HG network supports and stabilizes the cellulose microfibrils network that appears to be noncovalently bound to xyloglucan (O'Neill et al., 1996). HG typically accounts for about 80% by weight of the pectin (Vidal et al., 2001), whereas RG-I and RG-II together account for about 10% by weight of total grape skin material (Vidal et al., 2001).

2.2 Commercial enzymes used in wine and juice making

As early as the 1930s, pectinases have been used in the processing of fruits for juice products (Bhat, 2000). These enzymes facilitate an easier release of juice and other desirable compounds from the cells, with shorter and more effective press times. Pectinases also clarify the juice rapidly by breaking down the large polysaccharide units that would otherwise remain suspended in the solution, and by the same action pectinases reduce the juice viscosity and improve the filterability of the product. The addition of exogenous pectinase enzymes is beneficial because, although they occur naturally in the fruit, they exist in such small quantities that they are of little practical value over the short time and under the conditions of the must and juice handling processes (Felix and Villettaz, 1983). Where grapes are thermovinified, enzymes must be added after cooling as the temperatures used for thermovinification destroy the endogenous enzymes. Most pectinases for grape and wine processing contain pectinesterase and endopolygalacturonase activities, and may also contain pectin-lyase and/or proteases or protopectinases. Also adding small amounts of cellulase and hemicellulase enzymes was suggested to achieve further breakdown of the cells and the fruit structure (Plank and Zent, 1993). These macerating enzyme systems have increased the total juice yield of processed grape when compared to using pectinases alone (Haight and Gump, 1994). Macerating enzymes can be used for partial or total liquefaction of fruit in a relatively short time period.

It has been suggested that the application of pectolytic and macerating enzyme preparations to red grape musts could enhance the extraction of colour and phenolics from red grapes. A number of researchers have investigated this with some contradictory findings. Some reported that pectolytic and macerating enzymes promoted colour extraction and improved the quality of red wines (Haight and Gump, 1994; Plank and Zent, 1993; Felix and Villettaz, 1983). Others have found that pectinase and macerating enzymes can cause a decrease in the total yield of anthocyanins and a loss of the wine colour due to the β-
glucosidase side activity found in fungal enzyme preparations or the pectinase have no apparent effects (Wrolstad et al., 1994; Wightman and Wrolstad, 1996).

2.3 Activity of enzymes towards polysaccharides

2.3.1 Pectinases

Pectinases used in the mashing are usually mixtures of the endo-polygalacturonase (PG), pectin methylesterase (PE) and pectin lyase (PL). The combined action of these enzymes is capable of almost completely degrading the smooth regions of the pectic substances. PG hydrolyses the \( \alpha-(1\rightarrow4)\)-glucosidic bond between non-methylated galacturonic acid residues in the pectic backbone. PG therefore acts on pectin with a low degree of esterification, and if more than 60 – 70 % is esterified, PG is no longer active (Burns J.K., 1991; Grassin and Fauquembergue, 1996). In the present work PG is the abbreviation for the endo-polygalacturonase, but not referring to the exo-form of the polygalacturonase. The exo-form cleaves galacturonic acid of the non-reducing end of the polymer, while the endo-form acts in a random manner along the polymer. This is, however, not useful in pulp mashing, because of its incapability of reducing viscosity and the very limited action of this enzyme in fruit pulp (Burns J.K., 1991; Grassin and Fauquembergue, 1996).

PE hydrolyses the esterbond of the methylester of the galacturonosyl residues of the pectic backbone. The products are methanol and a free carboxyl group in the galacturonosyl residue. It seems that PE needs at least one unesterified carboxyl group next to the residue with the cleavage site, although the overall action occurs randomly along the chain (Burns J.K., 1991). The activity of PE itself has no viscosity reducing effect, but the demethylation of pectin by PE enhances the effect of PG, and therefore a synergistic effect of the combined use of these is present. If the effect of the enzymes is measured as a reduction of the viscosity of the pulp, then the synergistic effect is that viscosity is reduced more with both enzymes, than if the enzymes were used one at a time. Actually the effects of PE alone may even increase the viscosity, because of the possible gelation of the pectin in the presence of calcium (Ca\(^{2+}\)) and COO\(^-\) of the carboxyl acid forms, a so called egg-box structure.

The substrate of the PL is the \( \alpha-(1,4)\)-glycosidic bond between methylated galacturonosyl residues in the pectic backbone. The mode of action is a \( \beta \)-elimination, and it produces a 4,5-unsaturated galacturonosyl residue at the non-reducing end of the substrate cleaved. PL is an endo-enzyme attacking randomly along the chain, although it only attacks bonds between two methylated residues. This substrate specificity causes the rate of
degradation to increase as the degree of esterification increases (Burns J.K., 1991; Grassin and Fauquembergue, 1996). Different common pectinases used in fruit juice processing are displayed in Table 2.1.

Table 2.1 Mode of action for the pectinase enzymes with their substrate specificity (Pilnik and Voragen, 1991; Burns J.K., 1991; Grassin and Fauquembergue, 1996).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC Number</th>
<th>Substrate</th>
<th>Pectinases</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-polygalacturonase (Endo-PG)</td>
<td>3.2.1.15</td>
<td>Pectate</td>
<td>Hydrolyses α(1,4)-linkages between non-methylated galacturonosyl residues randomly. Produces pectin-oligomers of various sizes.</td>
<td></td>
</tr>
<tr>
<td>Exo-polygalacturonase (Exo-PG)</td>
<td>3.2.1.67</td>
<td>Pectate</td>
<td>Hydrolyses α(1,4)-linkages between non-methylated galacturonosyl residues at the non-reducing end of the pectin, and produces galacturonic acid.</td>
<td></td>
</tr>
<tr>
<td>Pectin methylesterase (PME)</td>
<td>3.1.1.11</td>
<td>Pectin</td>
<td>Hydrolyses the methylester of methylated galacturonosyl residue, producing methanol and a free carboxyl group in the galacturonosyl residues.</td>
<td></td>
</tr>
<tr>
<td>Pectin acetyl esterase</td>
<td>3.1.1.6</td>
<td>Pectin</td>
<td>Hydrolyses the acetyler of acetylated galacturonosyl residue, producing ethanol and a free carboxyl group in the galacturonosyl residues.</td>
<td></td>
</tr>
<tr>
<td>Endo-pectin lyase (PL)</td>
<td>4.2.2.10</td>
<td>Pectin</td>
<td>Cleaves α(1,4)-linkages between methylated galacturonosyl residues by random β-elimination. Produces pectin-oligomers of various size with a 4,5-double bond at the non-reducing end next to the cleavage-site.</td>
<td></td>
</tr>
<tr>
<td>Endo-pectate lyase</td>
<td>4.2.2.2</td>
<td>Pectate</td>
<td>Like PL, but cleaves between non-methylated galacturonosyl residues.</td>
<td></td>
</tr>
<tr>
<td>Rhamno-galacturonase</td>
<td>not classified</td>
<td>Rhamno-galacturan</td>
<td>Cleaves between galacturonosyl and rhamnosyl residues, producing pectin-oligomers of various sizes with rhamnose as the non-reducing end.</td>
<td></td>
</tr>
<tr>
<td>Endo-arabinase</td>
<td>3.2.1.99</td>
<td>Arabinans</td>
<td>Hydrolyses α(1,5)-linkages between arabinosyl residues randomly, producing oligosaccharides of various size.</td>
<td></td>
</tr>
<tr>
<td>Exo-arabinase</td>
<td>3.2.1.55</td>
<td>Arabinans</td>
<td>Hydrolyses α(1,2), α(1,3) and α(1,5)-linkages between arabinosyl residues from the non-reducing end of the chain, producing arabinose.</td>
<td></td>
</tr>
<tr>
<td>Endo-β1,4-galactanase</td>
<td>3.2.1.89</td>
<td>Galactans</td>
<td>Hydrolyses β(1,4)-linkages between galactosyl residues</td>
<td></td>
</tr>
<tr>
<td>Endo-β1,3-galactanase</td>
<td>3.2.1.90</td>
<td>Galactans</td>
<td>Hydrolyses β(1,3)-linkages between galactosyl residues.</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2 Cellulases and hemicellulases

Cellulases and hemicellulases are important ingredients in the multicomponent enzyme systems, especially if a complete liquefaction is desirable or necessary. In juice and red wine production, such a complete liquefaction is not desirable, but still it seems beneficial to use limited amounts of these enzymes during mashing. Partly degradation of the cellulosic and hemicellulosic substances softens the cell wall structure and facilitates the release of juice. The use of aggressive enzymes in a complete liquefaction releases colloid
fragments. First of all, this affects the filterability during pressing by reducing the amount of
free run juice, and thereby reducing the capacity of the press. Secondly the colloid fragments
are undesirable themselves, because colloids contribute to the turbidity of otherwise clear
juices. Different common cellulases and hemicellulases used in fruit juice processing are
displayed in Table 2.2.

Table 2.2 The cell wall degrading enzymes cellulases and hemicellulases. Mode of action and
their substrate specificity are given (Grassin and Fauquembergue, 1996; Ward and Mooyoung,
1989).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>E.C. Number</th>
<th>Substrate</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellulases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endo-glucanase</td>
<td>3.2.1.4</td>
<td>Amorphous cellulose</td>
<td>Hydrolyses the β(1,4)-linkages between glucosyl residues.</td>
</tr>
<tr>
<td>Endo-glucanase</td>
<td>3.2.1.74</td>
<td>Cellulose</td>
<td>Hydrolyses the β(1,4)-linkages from the non-reducing end of the chain, and produces glucose.</td>
</tr>
<tr>
<td>Cellobiohydrolase</td>
<td>3.2.1.91</td>
<td>Crystalline cellulose</td>
<td>Hydrolyses the β(1,4)-linkages from the non-reducing end of the chain, and produces the disaccharide cellobiose.</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>3.2.1.21</td>
<td>Cellobiose</td>
<td>Hydrolyses the β(1,4)-link of cellobiose, and produces glucose</td>
</tr>
<tr>
<td><strong>Hemicellulases</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xyloglucan-specific</td>
<td>3.2.1.151</td>
<td>Cellulose</td>
<td>Like endo-glucanase, but hydrolyses the backbone of xylolguccans as well as cellulose.</td>
</tr>
<tr>
<td>Endo-β-1,4-glucanase</td>
<td></td>
<td>Xyloglucan</td>
<td>Hydrolyses the β(1,4)-linkages between xylosyl residues.</td>
</tr>
<tr>
<td>Endo-xylanase</td>
<td>3.2.1.8</td>
<td>Xylans</td>
<td>Hydrolysis occurs randomly throughout the chains, and the products are xylan-fragments of various length.</td>
</tr>
<tr>
<td>Exo-xylanase</td>
<td>3.2.1.37</td>
<td>Xylans</td>
<td>Hydrolyses the β(1,4)-linkages from the non-reducing end of the chain, and produces xylose.</td>
</tr>
<tr>
<td>Mannanase</td>
<td>3.2.1.78</td>
<td>Galactomannan, Glucomanan</td>
<td>Hydrolyses the β(1,4)-linkages between mannosyl residues. Hydrolysis occur randomly throughout the chains, and the products are mannan-fragments of various length.</td>
</tr>
</tbody>
</table>
2.4 Side activities in commercial enzyme preparations

Commercial pectolytic enzyme preparations are most commonly produced from the extracellular material from *Aspergillus* or *Trichoderma* species. These enzyme preparations are rather crude, containing various pectinase activites as well as containing various side activities that may attack both the phenolic glycoside bonds and other bonds in the plant material. These activities are not clarified, but it known that the levels and types of the side activities in mixed pectinases vary considerably.

The variable and contradictory results, of pectolytic preparation used for colour extraction and stability in red wines, have been attributed to side activities in the enzyme preparations, most notably is the side activity of β-glucosidase. This activity was first identified in *Aspergillus* sp. enzyme extracts by Huang (1955), who found it to cause decolourising activity to anthocyanin in different fruits. Wrolstad et al. (1994) have screened some commercial red grape processing enzymes for this destructive β-glucosidase activity. They identified some enzymes that appear to have this side activity, and some enzymes that do not have side activity.

β-glucosidase cleaves the glucose from position three of the anthocyanin, producing anthocyanidin and glucose, followed by the spontaneous transformation of the unstable aglycone to the colourless chalcone form, then degradation products. The mechanism for degradation of an anthocyanin by the β-glucosidase enzyme is shown in Fig. 1.6.

A second common side activity that can be present in enzymes from *Aspergillus* sp. is cinnamate esterase activity causing deacylation of acylated anthocyanins (Fig. 1.6) (Barbe and Dubourdieu, 1998). Most often either a decrease in chlorogenic acid is observed accompanied by an increase in caffeic acid (Madani et al., 1997), or a decrease in the tartaric acid esters of the grape hydroxycinnamic acids, accompanied by an increase in the free acids content.

2.5 Review

This chapter is extended in a form of a published review article:
Chapter 2

Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release.
Pinelo, M., Arnous, A., & Meyer, A. S.


2.6 Key points

Optimal retrieval of phenolics from the grape skin into the juice and wine is crucial for acceptable colour and for sensory, and health properties of both juices and wines. In addition, the grape byproduct from wine making is a rich source of physiologically beneficial phenolic compounds having potential applications in functional foods and beverages. Detailed knowledge of composition, localisation, and interaction of phenolics with cell wall matrix polysaccharides is a fundamental prerequisite for the eventual revalorization of grape byproducts mainly coming from the wine industry.

The purpose of this review is to fill a knowledge gap about how phenolics are distributed and bound in the grape skin matrix. This knowledge is significant for efficient ultimate extraction. A comprehensive and updated coverage of the available information on phenolics in grape plant cell walls was written with particular focus on:

- The localisation and bonding of phenolics in relation to the plant cell wall matrix.
- Factors affecting the release and exploitation of the phenolics from complex fruit skin matrices.

2.7 Conclusion

Degradation of the cell wall polysaccharide structure is a fundamental step in accomplishing release of phenolics from fruit skins, notably those phenolics that are bound to the cell wall, but also those contained or interlocked within cell vacuoles.

Grapes were used as a model for other fruits and berries, and the knowledge attained for grape skin phenolics may be extrapolated to other fruit matrices with positive economic implications in several food industries.

This review was formed the step stone for following studies aiming at using a science-based approach for achieving optimal recovery of grape skin phenolics.
Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release

Manuel Pinelo, Anis Arnous and Anne S. Meyer*

Bioprocess Science & Technology Group, BioCentrum-DTU, Building 221, Technical University of Denmark, 2800 Lyngby, Denmark (Tel.: +45 45498822; fax: +45 45884922; e-mail: am@biocentrum.dtu.dk)

In grape skins, phenols may be classified as (1) cell-wall phenols, which are bound to polysaccharides by hydrophobic interactions and hydrogen bonds, and (2) non-cell-wall phenols, encompassing phenols confined in the vacuoles of plant cells and phenols associated with the cell nucleus. The phenolic composition of wines determines the colour quality, the sensory, and the potential health promoting properties of wines, and the extraction of phenols from the grapes into the must and wine is to a large extent governed by how the phenols are bound and entangled in the grape skins. Degradation of cell-wall polysaccharides is a fundamental step to improve the release of phenols from grape skin whether this is in wine-making or in upgrading of wine pomace. Cellulases, hemicellulases, pectinases, and other enzymes able to catalyze the hydrolysis of bonds in plant cell-wall polysaccharides can be employed to decompose the cell-wall structure. In addition, novel extraction principles and optimization of extraction conditions such as temperature, solvent-to-solid ratio, use of supercritical fluids and new extraction cell designs have shown promise for optimizing the release of phenols from grape skins for valorization of wine pomace. An in-depth knowledge of how phenols are bound in grape skins will allow us to employ the most suitable techniques to release phenols in order to optimize the phenol-related properties of wine and maximize the phenol recovery from grape byproducts.

Introduction: importance of grape phenols in the wine industry

Grape is the world’s largest fruit crop, with a reported annual production higher than 42 million tons (FAOSTAT-FAO Statistical Database, 2005). The economical importance of grapes and products obtained therefrom, such as wine, grape juice, jams and raisins, is therefore obvious. The most important grape producers are France, Spain, Italy and the United States. About 80% of the produced grape quantity is used in winemaking (Kammerer, Claus, Carle, & Schieber, 2004). The red colour and the sensory quality attributes of wines, notably of red wines, are to a large extent ascribable to the phenolic substances, aromas and precursors located in the cell walls of the grape skin cells. Phenols contained in grapes and wine can in general be classified into three main groups: (1) phenolic acids (mainly benzoic and hydroxycinnamic acids), (2) simple flavonoids (catechins, flavonols and anthocyanins) and (3) tannins and proanthocyanidins. During the red wine-making process, the transfer of phenolic compounds from the red grapes to the must mainly takes place from the grape skins during the maceration step, which for red wines takes place directly on the crushed berries (Salas, Fulcrand, Meudec, & Cheynier, 2003). However, the actual rate and extent of transfer of the phenols i.e. the extraction from the grape skins (and partially from the grape seeds and stems) to the must and into the gradually fermenting wine are very much determined by the winemaking procedure. The phenolic content of grape skin ranges from 285 to 550 mg phenols/kg grape skin, depending on the grape variety and type of pre-treatment (Pinelo, Rubilar, Jerez, Sineiro, & Núñez, 2005). The positive physiological effects associated with the consumption of wine, grape and grape derivatives are currently believed to be mainly due to the antiradical and antioxidant properties of the occurring phenols.
phenolic species (Lurton, 2003; Tomera, 1999). The potent antioxidant activity of wine and grape extracts on oxidizing human low-density lipoproteins in vitro thus correlates significantly to the phenolics (Meyer, Yi, Pearson, Waterhouse, & Frankel, 1997; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). In addition, various anti-inflammatory and anti-platelet aggregating effects and other potentially disease preventing cellular actions of wine phenolics have been amply documented (Damianaki et al. 2000; Kammerer, Schieber, & Carle, 2005; Maggi-Capeyron et al., 2001). Recently, grape and wine phenols, notably those obtained from winery byproducts, have also received attention for their potential application as food antioxidants (González-Paramás, Esteban-Ruano, Santos-Buelga, de Pascual-Teresa, & Rivas-Gonzalo, 2004).

**Valorization of winery byproducts**

Due to the beneficial health and lipid antioxidant properties, the recovery of phenolic compounds from grape byproducts coming from the winemaking industry has attracted great interest in the past couple of years (González-Paramás et al., 2004; Pinelo, Del Fabbro, Manzocco, Núñez, & Nicoli, 2005). When grapes are processed for red winemaking, the skins and seeds are usually in contact with the fermenting wine for several days. In this way, the grapes, including the skins, seeds, and some stems are subjected to a mild, but prolonged ethanolic extraction providing red wine with a high content of phenols. Nevertheless, the residues remaining after fermentation, i.e. the wine pomace, which mainly consists of skins and seeds, still contain high levels of phenols with most of the phenolic compounds retained in the skin matrix. These phenols are putative antioxidants, which justifies the use of grape skin as a good source for phenol recovery (Kammerer, Schieber, et al., 2005). 14.5 million tons of grape byproducts from wineries result annually in Europe and alternatives to the utilization of this wine pomace as soil conditioner or fertilizer are required, since high levels of phenols are known to cause germination problems (Negro, Tommasi, & Miceli, 2003).

Recent studies have attempted to unravel, on the one hand, the extraction conditions favouring the release of phenols from grape byproducts into different solvents (Meyer, Jepsen, & Sørensen, 1998; Pinelo, Rubilar et al., 2005), and, on the other hand, the complex composition and linkages between phenols and the different components of grape skin (Doco, Williams, Pauly, O’Neill, & Pellerin, 2003; Vidal, Williams, O’Neill, & Pellerin, 2001). However, an overview bringing the results of both subjects together is lacking, despite their obvious interrelation, and despite the potential advantages attainable by combining the available knowledge in order to improve extraction efficiency. The purpose of this review is therefore to fill a knowledge gap about how phenols are distributed and bound in the grape skin matrix with the main aim of understanding the mechanisms involved in the release of phenols from grape skin during juice and wine manufacture as well as in upgrading of wine pomace.

**Occurrence of different types of phenols in grape berries: an overview**

During ripening of grapes, environmental factors and endogenous enzymes promote changes that affect the composition and structure of the sugars and phenols contained in the grape berries. Previous reports have shown that wines made from more mature grapes in general have a higher content of anthocyanins, a lower anthocyanin/flavan-3-ol ratio, and a higher quantity of some simple phenols like gallic and syringic acids (Pérez-Magaríño & González-San José, 2006). The general distribution and contents of phenolic compounds found in the different fractions of grapes and wine pomace are depicted in Fig. 1 and in Table 1, respectively.

The grape skin: In general, even though phenolic composition can strongly vary as a function of the variety and cultivation conditions, the skin contains the highest amounts of tannins in the grape berry and these tannins differ from the other grape fractions by having a higher polymerization degree (DP) and a lower amount of gallates (Souquet, Cheynier, Brossaud, & Moutounet, 1996). Catechin, epicatechin and epigallocatechin gallate are the main constituent units of skin tannins, although galloantocatechin and epigallocatechin are also present in minor quantities. The average polymerization degree (mDP) for skin tannins is ~28, with 80 being the maximum DP detected, and the percentage of gallates in the tannins is only 5.16% (Souquet et al., 1996; Yilmaz & Toledo, 2004). Anthocyanins in grape skins, which are responsible for the red grape and wine colours include delphinidin, cyanidin, petunidin, peonidin and malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-coumaryl)-glucosides,peonidin and malvidin 3-(6-cafeoyl)-glucosides and some puyruvates (Monagas, Garrido, Bartolomé, & Gómez-Cordovés, 2006; Revilla, Pérez-Magaríño, González-SanJosé, & Beltrán, 1999). Quercetin and kaempferol glucosides and gluconorides, gallic acid and its glucosides, resveratrol, caftaric and coumaric acid complete the phenol composition of grape skins (Lu & Foo, 1999; Pascual-Martí, Salvador, Chafer, and Berna, 2001). Apart from the presence of anthocyanins in red skins (and another minor phenol like vitexin in white skins), the phenolic composition of red and white grape skins does not seem to differ to a great extent (Borbála, Zóro, Guilèn, & Barroso, 2003). The seeds and stems: Seed tannins have the same constitutive units as the skin tannins, but the mDP is only ~11 in seed tannins. Tannins in grape seeds tend to be in monomeric form rather than polymerised. Their quantity has been found to decrease remarkably during ripening following a second-order kinetic course (Kennedy, Matthews, & Waterhouse, 2000). The levels of gallates in the seeds are ~30% higher than those in the skin and stems (Souquet, Labarbe, Le Guernevé,
Cheynier, & Moutounet, 2000). Procyanidin B1 and B2 have been detected in seed extracts as well, along with minor quantities of epigallocatechin and gallic acid (Guedez, Kalithraka, Makris, & Kefalas, 2005; Yilmaz & Toledo, 2004). Stems: The grape stems are also an attractive source of phenol species and it is known that wines made from a non-destemmed crop generally contain higher levels of phenolic compounds than those prepared from destemmed grapes. Souquet et al. (2000) have concluded that tannins are the most abundant phenolic constituents of grape stems (w80%); the seed tannins mainly consist of epicatechin units along with smaller amounts of catechin, epicatechin gallate, and epigallocatechin. The mDP for the stems' tannins is 9, i.e. the lowest compared to the mDP of tannins in the skins and seeds. Other compounds like quercetin 3-glucuronide have also been detected in considerable amounts in seeds, followed by catechin, caftaric acid and astilbin (Souquet et al., 2000).

**General composition of grape skin and grape skin cell walls**

Grape skins represent about 5–10% of the total dry weight of the grape berry and act as a hydrophobic barrier to protect the grapes from physical and climatic injuries, dehydration, fungal infection and UV light. The grape skin can be divided into three superimposed layers (Fig. 2): (1) the outermost layer, the cuticle, is composed of hydroxylated fatty acids called cutin, and is covered by hydrophobic waxes; (2) the intermediate epidermis, assumed to consist of one or two layers, which appears as a regular tilling of cells; and (3) the inner layer, the hypodermis, which is the layer closest to the pulp, and which is composed of several cell layers that contain most of the phenolics in grape skin (Lecas & Brillouet, 1994). The cell wall (CW) of grape berries forms a barrier to the diffusion of components including aromas and phenols and acts as a protection against external factors (Doco et al., 2003). Botanically, the grape plant is classified as an angiosperm and shares the same structural CW features as the rest of dicotyledones (type I CW according to Carpita & Gibeaut, 1993). The CW of grape skins is made up of 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan and mannann), 20% of acidic pectin substances (of which 62% are methyl esterified), ~15% insoluble proanthocyanidins, and <5% structural proteins (Lecas & Brillout, 1994). The CW is built of three general layers (Raven,
the third part consists of structural proteins (10%) (Bidlack, 1992). Recent studies concerning linking of phenols in other dicotyledones like apple and flower petal CWs, and thus presumably valid for grape and flower petal CWs, have reported phenols to be linked or entangled in the CW, have reported phenols to be linked or entangled in the CW, have reported phenols to be linked or entangled in the CW.

Table 1. General content of the main phenolics occurring in different fractions of grape

<table>
<thead>
<tr>
<th>Compound</th>
<th>Grape pomace</th>
<th>Skin</th>
<th>Seed</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>0.03–0.11d</td>
<td>0.03i</td>
<td>0.10–0.11j</td>
<td>–</td>
</tr>
<tr>
<td>Catechin</td>
<td>0–0.18j</td>
<td>0.01–0.18j</td>
<td>2.14–2.15j</td>
<td>0.06j</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0–0.05k</td>
<td>Traces</td>
<td>0.05k</td>
<td>0.07k</td>
</tr>
<tr>
<td>Epigallocatechin 3-gallate</td>
<td>0–0.05l</td>
<td>–</td>
<td>0.06–0.09m</td>
<td>–</td>
</tr>
<tr>
<td>Total flavan-3-ols</td>
<td>0.34–4.25</td>
<td>0.12–3.38</td>
<td>3.56–6.15</td>
<td>0.22–0.89f</td>
</tr>
<tr>
<td>Delphinidin 3-glcc</td>
<td>0.44–1.11d</td>
<td>0.44–1.11d</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyanidin 3-glcc</td>
<td>1.51–3.81d</td>
<td>1.51–3.81d</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Petunidin 3-glcc</td>
<td>0.51–1.34d</td>
<td>0.53–1.34d</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peonidin 3-glcc</td>
<td>0.99–2.49f</td>
<td>0.99–2.49f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Delphinidin 3-acglcc</td>
<td>0.08–0.19f</td>
<td>0.08–0.19f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Petunidin 3-acglcc</td>
<td>0.11–0.28f</td>
<td>0.11–0.28f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peonidin 3-acglcc</td>
<td>0.27–0.32f</td>
<td>0.27–0.32f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malvidin 3-acglcc</td>
<td>0.62–1.74f</td>
<td>0.62–1.74f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyanidin 3-glcc</td>
<td>0.07–0.22f</td>
<td>0.07–0.22f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Petunidin 3-glcc</td>
<td>0.19–0.49f</td>
<td>0.19–0.49f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peonidin 3-acglcc</td>
<td>0.43–1.72f</td>
<td>0.43–1.72f</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malvidin 3-acglcc</td>
<td>2.11–6.29g</td>
<td>2.11–6.29g</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total anthocyanin contentb</td>
<td>11.47–29.82d</td>
<td>11.47–29.82d</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Quercetin 3-glucoside</td>
<td>0.01–0.22d</td>
<td>0.15–0.24d</td>
<td>0.01–0.02d</td>
<td>0.02d</td>
</tr>
<tr>
<td>Myricetin 3-glucoside</td>
<td>Tracesg</td>
<td>–</td>
<td>–</td>
<td>Tracesg</td>
</tr>
<tr>
<td>Quercetin 3-glucosineno</td>
<td>0.01–0.29f</td>
<td>0.22–0.29f</td>
<td>0.01–0.02f</td>
<td>0.2f</td>
</tr>
<tr>
<td>Kaempferol 3-glucoside</td>
<td>0.01–0.14f</td>
<td>0.11–0.14f</td>
<td>0.04f</td>
<td>Tracesf</td>
</tr>
<tr>
<td>Myricetin 3-glucosineno</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Tracesf</td>
</tr>
<tr>
<td>Total flavonols</td>
<td>0.03–0.63</td>
<td>0.48–0.63</td>
<td>0.02–0.05</td>
<td>0–0.22</td>
</tr>
</tbody>
</table>

All values are given in mg/g.

- : non-detected; glc: glucose; ac: acetyl.

Evert, & Eichhorn, 1999). (1) The middle lamella, which binds the cells together, is mainly composed of pectin. (2) The primary CW, which is thicker than the middle lamella and is formed just after the cell division. It comprises three structurally independent, but interacting parts: the two first parts include the fundamental cellulose (8–25%)-xylo-

glucan (25–50%) framework that is embedded in a matrix of pectin polysaccharides (10–35%). The third independent part consists of structural proteins (10%) (Bidlack, Malone, & Benson, 1992; Carpita & Gibeaut, 1993). (3) The secondary CW, which is much thicker than the primary one, is largely made up of cellulose microfibrils, organized in parallel bundles (40–80%). The secondary CW also contains hemicelluloses (10–40%), pectins and some lignin (5–25%) (Bidlack et al., 1992). Recent studies concerning linking of phenols in other dicotyledones like apple and flower petal CWs, and thus presumably valid for grape CW, have reported phenols to be linked or entangled in the polysaccharides of the cell walls, to be confined in the cell vacuoles, or to be associated with cell nuclei through...
different chemical bindings or unions of physical nature depending on the composition and disposition of both phenols and polysaccharides, as will be discussed below (Hutzler et al., 1998; Le Bourvellec, Bouchet, & Renard, 2005a; Markham, Gould, & Ryan, 2001).

Localization of phenolic compounds in plant cells
Cell-wall linked phenols

The retention of phenols in the CW depends on compositional and structural parameters like stereochemistry, conformational flexibility, molecular weight or percentage of galloylation of the phenolic molecule. Besides, physical traits of the CW like surface topography, porosity, and chemical composition can also influence the eventual aggregation between conformational CW polysaccharides and phenolic substances (Le Bourvellec, Guyot, & Renard, 2004; Riou, Vernhet, Doco, & Moutounet, 2002). The most abundant data about complexation of phenols with plant cell-wall polysaccharides have been obtained using model compounds (e.g. cyclodextrins or polysaccharides in solid forms) or apple CW prepared by certain chemical treatments (Renard, Baron, Guyot, & Drilleau, 2001). Two mechanisms of association have been proposed to explain the formation of the complex polysaccharide—phenol entities: (1) hydrogen bonds between the hydroxyl groups of phenols and the oxygen atoms of the cross-linking ether bonds of sugars present in the CW polysaccharides. In this way dextran gels would be able to encapsulate phenols inside their pores (Freitas, Carvalho, & Mateus, 2003; Le Bourvellec, Bouchet, & Renard, 2005b). (2) Hydrophobic interactions occurring as a result of the ability of some polysaccharides to develop secondary structures, i.e. nanotubes or gels, which result in hydrophobic regions. The formed hydrophobic pockets or cavities may be able to encapsulate and complex phenols, as have been shown to occur between β-cyclodextrin and different phenolic compounds like caffeylquinic acid and flavonoids (Ficarra et al., 2002; Le Bourvellec et al., 2005b). Aggregation of anthocyanins to yeast cell walls during the fermentation of different red wines has also been explained to occur as a result of hydrophobic interactions between sugars and phenols, although this adsorption of phenols to yeast cells has been found to strongly depend on the degree of methoxylation and acylation of the (anthocyanin) compounds (Morata et al., 2003). Being the same system phenol—sugar, it is therefore probable that the latter factors also affect the interaction between phenolic compounds and sugars in grape CW. No evidence for hydrophobic interactions between hydroxycinnamic acids and CW polysaccharides has been found (Renard et al., 2001).

Affinity between procyanidins and CW polysaccharides

Renard et al. have carried out a complete study about the interaction between procyanidins and apple cell-wall material, and have attained the following main conclusions: (1) the apparent affinity constants between procyanidins and apple CW polysaccharides were found to decrease as follows: pectin > xyloglucan > cellulose. (2) The amount of procyanidins bound increased with the polymerization degree, and the proportion of (+)-catechin. (3) A decrease of the CW porosity by drying also decreased the apparent affinity and saturation levels between CW polysaccharides and procyanidins (Le Bourvellec et al., 2004, 2005a,b).

Non-cell-wall phenols

Phenols occurring in plants are not always associated with the plant cell walls, and recent works have demonstrated that phenols can also be found within the cell cytoplasm, inside the cellular vacuoles or even in, or very near, the cell nucleus.

Cytoplasmic and vacuolar phenols: Most reports regarding vacuolar phenols are focused on the study of the colour
Polysaccharide structures in grape skin

The cellulose–xyloglucan framework

Cellulose is composed of chains of \((1\rightarrow4)\beta\)-linked \(\alpha\)-glucan. Hydrogen bonds hold about 40 of these glycan chains together to form a cellulose microfibril (Albersheim, 1975; Goodwin & Mercer, 1983). In grape skin CW, hemicellulosic polysaccharides consist mainly of xyloglucans whose structures are based on a \((1\rightarrow4)\beta\)-\(\alpha\)-glucan backbone qualitatively identical to cellulose, but unlike cellulose, about 75% of the glucose residues carry \((1\rightarrow6)\) \(\alpha\)-linked \(\alpha\)-xylose residues, approximately 35% of which are substituted with galactose residues, many of which are themselves fucosylated (Thompson & Fry, 2000). Other neutral sugars like mannans (mannose–glucose backbone with galactose units attached), xylans (xylose backbone with arabinose units attached), and arabinogalactans (arabinose and galactose chains) have also been detected in minor quantities in grape skin CW (Lecas & Brillouet, 1994). In flowering plants in general, xyloglucans are believed to interact non-covalently with cellulose and occupy two distinct regions forming a cross-linked network: one binds tightly by hydrogen bonds to the exposed parts of the glucan chains in the cellulose microfibrils, and a second spans the distance to the next microfibril or simply interlocks with other xyloglucans, as in a chain-link fence, to space and lock the microfibrils into place (Albersheim, 1975; Carpita & Gibeaut, 1993; Doco et al., 2003). Thompson, Smith, and Fry (1997) have pointed out the possibility of covalent unions between segments of xyloglucans into the CW architecture. In any case, cellulose and hemicellulose are less structurally organized in the primary CW than in the secondary CW; in the latter walls ester and ether bonds may connect hemicellulose to non-core lignin (Jung, 1989).

Pectin polysaccharides

The main pectin polysaccharides occurring in grapes, and hence presumably also in grape skin are homogalacturonan, consisting of a galacturonic acid backbone attached to some glucose and xylose, rhamnogalacturonan I, carrying arabinans, galactans and highly branched arabinogalactans linked to rhamnose residues to form a ramified pectin network, and rhamnogalacturan II which consists of a galactopyranosyluronic acid backbone with rhamnose, glucose, arabinose and galactose substituents, and capable of covalently cross-link between each other through borate diester dimers (Nunan, Sims, Bacic, Robinson, & Fincher, 1998; O’Neill et al., 1996; Vidal et al., 2001). Different theories based on investigations on diverse plant materials have been proposed in the attempt of explaining the linkage of pectin to the cellulose–xyloglucan framework. The existing data pertain to various plants, while no detailed studies intended to describe the plant cell-wall polysaccharide linkages solely on grapes are available. The current knowledge has been deduced by using different chemical and physical techniques and some of them could even seem contradictory. The earliest model has been proposed by Keegstra, Talmadge, Bauer, and Albersheim (1973), who concluded that xyloglucans and pectin polysaccharides could be covalently linked to each other by glycosidic bonds between the reducing end of xyloglucan and a side chain of pectin. The latter, in turn, are held together partly by Ca\(^{2+}\) bridges (Carpita & Gibeaut, 1993). From the 70’s and onwards, however, evidence for the formation of hemicellulose–pectin cross-linked complexes has been reported in several different plant cell systems by several authors (Brown & Fry, 1993; Kim & Carpita, 1992; Thompson & Fry, 2000). No studies about possible bindings between hemicelluloses and pectins focused on grape skin CW could be found.
However, it is probable than linkage among sugars can be explained as a combination of all these different theories.

Role of ferulic acid in cross-links among sugars

Ferulic acid also plays an important role in stiffening the structure of plant CWs by taking part in the binding among polysaccharides and, in turn, between polysaccharides and the lignin constituents. Most recent knowledge about linking ferulates has been made employing graminaceous plants like maize as a raw material. Ferulic acid is esterified to the C5-hydroxyl of α-arabinose side chains on xylans. Xylans become ester-linked by peroxidase/H₂O₂-mediated radical coupling of ferulate into 8−8′, 8−5′, 8−O−4, and 5′−5′-coupled dehydrodimers (Grabber, Ralph, & Hatfield, 2002).

Lignin

Lignin is not a polysaccharide, as it is originally made of p-coumaric acid, ferulic acid, diferulic acid, sinapic acid, cinnamic acid and p-hydroxybenzoic acid resulting from certain enzymatic reactions. Subsequently, other enzymes catalyze the formation of the p-coumaryl-, coniferyl-, and sinapyl-alcohols that polymerize to form lignin in the secondary cell wall (Bidlack et al., 1992). Lignification, which adds further rigidity to the plant matrix, arises from generation of free radicals resulting from the breaking of the covalent bond between the phenolic oxygen and the hydrogen in the alcohol. These free radicals then react spontaneously to form lignin and may even form linkages to cell-wall polysaccharides (Brett & Waldron, 1990). Free radical linkages between lignin monomers and polysaccharides may produce what is referred to as non-core lignin (by ether and ester bonds) while polymerization of monomeric free radicals results in highly condensed core lignin (Jung, 1989). The diverse and complex nature of lignin monomers and hemicellulosic moieties in ligno-hemicellulosic bonds make stereotypic conceptualizations of secondary cell structures for all plants extremely difficult. Due to this difficulty, literature regarding this subject is mainly confined to grasses (family Gramineae). These studies have shown that lignin is tightly linked to polysaccharides in the cell walls of plants by various linkage types, and most commonly, the covalent linkage is the ether bond of the hydroxyl group at the α-position of the lignin side chain with the alcoholic hydroxyl of the sugar residue (Sun, Sun, Sun, & Su, 2004). During lignification, the CWs are further stiffened by oxidative coupling of ferulate monomers and dimers with monolignols, forming additional cross-links between structural lignin and hemicellulose (Grabber, Ralph, & Hatfield, 2000). Ferulic and p-coumaric acids are the major non-core lignin monomers that link hemicellulose and core lignin (Mueller-Harvey & Hartley, 1986).

During lignification, ferulate and 5−5 coupled diferulate copolymerize more rapidly and form fewer ether-linked structures with coniferyl alcohol than 8−8′, 8−5′, 8−O−4′ coupled diferulates (Grabber et al., 2000; Xu et al., 2005). The reduction in ferulate−xylan, ferulate−pectin and ferulate−lignin cross-linking would significantly improve the enzymatic hydrolysis of CW (Grabber, 2005), presumably favouring the release of phenolic compounds retained in the CW plant matrix. Other phenolics like p-coumaric acids have also been reported to be responsible for ester and ether cross-linkages between sugars and lignin (Grabber, Ralph, Lapierre, & Barrière, 2004). It may be speculated that various phenolic substances, including flavonoids and tannins in grape skins, may be deposited and “caught” in the lignin—polysaccharide matrix during the free radical reactions taking place during lignification of the secondary plant cell wall or during the formation of the ferulic acid dimers or during formation of the ether links that interlock the lignin—polysaccharide structures in the CW. However, to our knowledge, there are no reports available that provide any evidence to this hypothesis. It thus still remains relatively unclear how exactly the phenols present in grape skins are bound and/or entangled in the lignin—polysaccharide matrix of the skin cell-wall material. Nevertheless, the recent accumulation of knowledge on the plant cell-wall polysaccharide and lignin chemistry and architecture may pave the way for new theories and experimental leads regarding how the polyphenols are bound in the structural cell-wall matrices of fruit skins, including grape skins.

**Influence of critical variables on efficiency of conventional extraction procedures for releasing phenols from fruit matrices**

Phenols more weakly linked to the CW structure and those contained in vacuoles are presumably the ones whose realise efficiency is more liable to be affected by variables like temperature, solvent-to-solid ratio, type of solvent used, etc., which are able to modify equilibrium and mass transfer conditions in the solid–liquid extraction. In fact, temperature is one of the most critical variables affecting the release of phenols from grape skin. Increases in the temperature favour extraction by enhancing both the solubility of solute and the diffusion coefficient. As a consequence, an increase of extracted phenols is observed at higher temperature values (Pinelo, Sineiro, & Núñez, in press). Despite the positive effects of higher temperatures on the extraction yields, temperature cannot be increased indefinitely; because instability of phenolic compounds and denaturation of membranes may take place at temperatures above 50 °C (Cacace & Mazza, 2003). Increasing solvent-to-solid ratio has also been found to work positively for enhancing phenol yields; however, an equilibrium between the use of high and low solvent-to-solid ratios, involving a balance between high costs and solvent wastes and avoidance of saturation effects, respectively, has to be found (Pinelo, Del Fabbro et al., 2005). The type of solvent is also one of the most influencing variables in the
extraction process; methanol, ethanol, and water the most widely employed solvents for extraction of phenols from grape skins and wine pomace. Among these solvents, methanol exhibits the highest capacity to extract phenolics, followed by ethanol and then water (Pinelo, Del Fabbro et al., 2005). When alcohols are employed as the extraction solvent, a progressive release of phenols from grape skin as a function of the extraction time is observed, whilst contact time is not as significant when water is used as the solvent. Other variables like lower particle sizes and higher sample quantities also favour the release of phenols, especially in continuous extraction. Packaging phenomena of grape skin particles may, however, occur during extraction, and this packaging can in turn beget preferential flow channels and offside zones, promoting a decrease in surface contact between grape skin solids and liquid and thus as a result decrease the extraction efficiency (Pinelo, Del Fabbro et al., 2005). Further studies have, however, demonstrated that these packaging phenomena can be minimized by varying the solvent flow into the skin bulk, for instance by use of pulsed flow (Pinelo, Rubilar, Sineiro, & Núñez, 2006). The extraction conditions may also promote the formation of phenolic compounds not naturally occurring in the grape skin. Pinelo, Del Fabbro et al. (2005) have observed the formation of flavan-3-ol polymers in the extractor bulk when grape skins were subjected to a continuous ethanolic extraction. Although some of these structural changes have been shown to be minimized by limiting the presence of oxygen in the extractor bulk, any structural changes of the phenols can in turn lead to variations in their properties (Pinelo, Manzocco, Núñez, & Nicoli, 2004; Pinelo et al., 2006). Fig. 3 shows the usual steps to follow for the valorization of winery byproducts, indicating some of the critical factors affecting the solid—liquid extraction of the phenols.

Fermentation conditions affecting phenolic extraction during winemaking

Apart from the general variables affecting solid—liquid extraction yields of phenolics, several variables and techniques have been specifically reported to have an influence on the phenolic concentrations in wines. Some of them, as indicated below, look for the bursting of the grape cells, thus promoting the breakdown of the linkages stiffening...
the structure of grape CW and allowing an increase in the release of the phenolic compounds.

Temperature: In general, higher fermentation temperatures increase phenolic extraction. Works with Pinot noir grapes have thus shown that the total phenol yields increase, but that there is only little difference in the anthocyanin content in wines over fermentation temperatures ranging from 15 to 30 °C (Girard, Yuskel, Cliff, Delaquis, & Reynolds, 2001). SO2 levels: At the levels of SO2 and temperatures normally used for red wine fermentations, SO2 does not dramatically affect the extraction of phenolics. However, Heatherbell, Dicey, Goldsworthy, and Vanhanen (1996) observed and increased extraction of phenols into experimentally produced wines with higher SO2 levels and lower temperatures. Cold soak and must freezing: during cold-soak treatment the must is held at low temperatures, usually at 10–15 °C, for several days before fermentation. The results obtained with Pinot noir have consistently showed that a cold soak alone has either no effect or a negative effect on the phenolic composition of the resulting wines (Heatherbell et al., 1996). Freezing the must before fermentation potentially has a much greater effect. Must freezing causes the berry cells to burst, breaking the cell membranes and thus releasing anthocyanins into the must (Sacchi, Bisson, & Adams, 2005). Thermovinification: In general, thermovinification consists of heating the skins to 60 or 70 °C for a short time, extracting them with the juice, pressing and then cooling before fermentation. The heat treatment damages the hypodermal cell membranes, releasing anthocyanins and also denaturing polyphenol oxidase, which prevents browning. Pectolytic enzymes: They are used to break down the middle lamella between the pulp cells and the pulp and skin cell walls, releasing pigments. An improvement in both juice yields and colour extraction by employing these enzymes has been reported (Ducruet, Dong, Canal-Llauberes, & Glories, 1997). Maceration time: Gomez, Gil, Lopez, Martinez, and Fernandez (2001) have concluded that prolonged maceration time (from 4–5 to 10 days) increases concentration of anthocyanins and tannins after one year in the bottle. Yeast selection: No pronounced differences in the phenolic profiles of wines were found when great differences were observed with five saccharomyces yeast in fermentation of four different grape varieties, Enantio, Lagrein, Merlot, and Teroldego (Nicolini, Mattivi, Larcher, & Volpini, 2003).

Enzymatically assisted extraction

Reduction of particle size of grape pomace has been shown to have a positive effect on the recovery of phenols from wine pomace by increasing the enzyme catalyzed polysaccharide hydrolysis accomplished by various mixed pectinolytic and cell-wall polysaccharide degrading enzyme preparations (Meyer et al., 1998). Other factors such as the time—temperature regime of the enzymatic treatment, enzyme/substrate ratio or the type of the extraction solvent employed also influence the phenol release from grape skins (Kammerer, Claus, Schieber, & Carle, 2005; Meyer et al., 1998). Meyer et al. (1998), when used 70% aceton as a solvent and 125–250 μm as particle size, obtained extracts from grape pomace of 6055 mg GAE/L phenol concentration after a Grindamyl pectinase 8-h extraction. When no enzyme was used, however, phenol concentration of extract decreased until 4615 mg GAE/L, confirming the enzymatically assisted extraction as one of the more effective techniques to increase phenol yields. Although significantly improved recoveries of phenols from grape pomace skins have been obtained with enzymatic treatment prior to a brief methanolic or aqueous extraction (Landbo & Meyer, 2001) the available data indicate that only 5–10% by weight of the skin dry matter has been degraded, suggesting that the degree of cell-wall polysaccharide breakdown achieved has indeed been low. Pectinas are the most usually employed enzymes during winemaking. Some studies have shown the positive effect of using pectinas during maceration on enhancing the phenolic and anthocyanin content of wine during processing and conservation. Pardo, Salinas, Alonso, Navarro, and Huerta (1999) reported an increase of ~40% in the amount of anthocyanins (from 220 mg/L to 305 mg/L) during vinification and conservation of Monastrell wines treated with pectinases. Likewise, Bautista, Martinez, Ros, Lopez, and Gomez (2005) reported significant differences in the amount of total phenols when the same wine was subjected to pectinase maceration. It is well known that the presence of lignin may retard the action of polysaccharide degrading enzyme activities via unproductive adsorption of enzyme protein to the lignin. Besides, enzymes may be also inhibited by the presence of tannins (Shofield, Mbugua, & Pell, 2001). Studies on enzymatic degradation of maize CW polysaccharides strongly suggest that diferulate cross-linking of cell-wall polysaccharides reduces the accessibility and/or action of hydrolytic enzymes to the structural polysaccharides in the cell walls (Grabber, Hatfield, & Ralph, 1998). A 70% reduction in diferulate cross-linking of arabinoxylans to lignin thus increased carbohydrate solubilization by 24–26% after 6 h of hydrolysis with a cellulase, hemicellulase and pectinase mixture (Viscozyme & Celluclast) (Grabber, Ralph, & Hatfield, 1998). Taken together, these results indicate that both the presence of lignin and the presence of diferulate, notably diferulate dimer cross-links, are the fundamental factors negatively affecting the enzymatic degradation of CW plant tissues including grape skins. Obviously, improved knowledge about the specific cell-wall polysaccharides, lignin coupled with better insight into how the phenols are embedded and/or bound in the cell-wall matrices of grapes would pave the ground for the use of more specific enzymes to accomplish the phenolic release and thus the upgrading of grape skin phenolics.
Future trends
Development of new insight into the molecular build-up of plant cells and cell walls has provided new theories about how phenols may be bound in grape skins. In addition, progress in general wine technology and plant physiology has provided new knowledge on phenol complexes as they occur in plant cells including grape cells. In the last several years, trends in order to improve the phenolic extraction efficiency have been mainly focused on the use of enzymes, either independently or in combination with cell disruption techniques used to increase the phenolic yield during the extraction process. Degradation of the cell-wall polysaccharide structures is a fundamental step in the release of phenolics from grape skins, notably those phenols that are linked to the CW, but also those contained or associated with cell vacuoles. Ferulic acid linkages are that are linked to the CW, but also those contained or associated with cell vacuoles. Purification of enzymatic mixtures as well as the synthesis of new ones would definitely promote an enhancement of the phenolic yields during extraction (Kammerer, Claus, et al., 2005).

Recommendations and conclusions
In order to accomplish a major specificity and efficiency in the extraction process of phenols from grape skin, a combination of knowledge of distribution and linkage of these compounds and techniques used to extract them is necessary. Degradation of the cell-wall polysaccharide structures is a fundamental step in the phenol release from grape skins, notably those phenols that are linked to the CW, but also those contained or associated with cell vacuoles. Ferulic acid linkages are known to play a fundamental role in maintaining the sugar structure in grape skin CW and minimization of the effect of the ferulate cross-links, or enzyme catalyzed breakage of these cross-links, could be a first step in order to increase the phenolic yield during the extraction process. A detailed study about the effects of the phenolic compounds of interest both on the sensory properties of wine and on the human health would also help to discern what kind of compounds are the most suitable to be extracted.

References


Chapter 3

Compositional characterization and carbohydrate profile of grape and apple skin

This chapter is extended in a form of two published articles:

Comparison of methods for compositional characterization of grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins.

Arnous, A. & Meyer, A. S.

*Food and Bioproducts Processing*, 86 (2008), pp. 79-86.

Quantitative prediction of cell wall polysaccharide composition in grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins from acid hydrolysis monosaccharide profiles.

Arnous, A. & Meyer, A. S.

*Journal of Agricultural and Food Chemistry*, 57 (2009), pp. 3611-3619.

3.1 Key points

The first paper: No common or standardized method(s) exists to determine polysaccharide compositions of fruit skin and other plant materials and no direct comparisons between the structural carbohydrates make up and the phenolic compositions of apple and grape skins are available in the literature. In the literature there is an inconsistency of reported carbohydrate composition of (fresh) grape skin, which is closely influenced by the methodologies employed for preparation of the fruit skin/hydrolyses and later analysis methodology. For that a detailed compositional assessment of the carbohydrates and phenolics present in the grape and apple skin is needed. This assessment is a prerequisite for assessing which phenolics, or even which specific carbohydrate structures that can be enzymatically obtained from grape or apple skin.

The purpose of this study was to determine and compare the compositional carbohydrate and phenolics make up of grape and apple skins. As a first step we intended to prepare fruit skin in a mild way to retain the skin as close as possible to its native form (Fig 3.1). Assessment of the grape skin wall monosaccharide was done by means of acid hydrolysis where the efficiency of different acid hydrolysis methodologies was evaluated. Usually polysaccharide is determined by measuring the monosaccharides released after
acid hydrolysis by means of trifluoroacetic (TFA) or hydrochloric (HCl) acid De Ruiter (De Ruiter et al., 1992; Meseguer et al., 1999). Eventually the use of monosaccharide recovery values after acid hydrolysis of a standard mixture of monocarbohydrates was an essential step to develop a one-step acid hydrolysis method. This method compromise between on one hand obtaining sufficient hydrolysis, and on the other hand avoiding artefacts as a result of the hydrolysis treatment itself.

**Figure 3.1** Schematic diagram of the grape skin preparation steps.

The implemented method for chemical hydrolysis of polysaccharides to monocarbohydrates was easily incorporated with the separation and the quantification of monocarbohydrates using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Successful separation and quantification of neutral and acid monocarbohydrates was achieved (Fig. 3.2). This was accomplished in one single run without the need for pre-derivative treatment.
The second paper: No universal methods exist that can deduce the detailed composite polysaccharide structure of intact plant cell walls. Nor do methods exist that assess all the structures of the polysaccharides and their connection points which define the molecular architecture of complex plant cell walls. This is particularly the case for fibrous lignified materials present in polysaccharides of fruit skin cell walls.

The purpose of this study was to evaluate the options for calculating the most likely composition of the structural elements of the polysaccharides in the fruit skin cell walls by examining their monosaccharide profile. We report an iterative calculation methodology that combines current knowledge of dicot plant cell wall polysaccharide structures with the monosaccharide profile data we obtained in the present study by acid hydrolysis of grape and apple skin. The monosaccharide profile data gave information about the quantitative allocation of monomers into different structural carbohydrate polymer elements.

3.2 Conclusion

The iterative approach will not draw a full picture of the structure of the polysaccharides, but it is sufficient to provide knowledge about the profile of the polysaccharide building block in the plant cell wall. This knowledge is very crucial when we
wish to target the cell wall enzymatically. The data obtained demonstrated that it is possible to quantitatively predict the abundance of structural polysaccharide units in fruit skins from monosaccharide profiles obtained by a simple one-step acid hydrolysis. For both the grape and apple skin samples, including three different wine grape cultivars and two different apple cultivars, the skin cell wall polysaccharide matrices appeared to be mainly made up of pectins. The pectins were presented mainly by homogalacturonans, rhamnogalacturonan I, and rhamnogalacturonan II, and to a lesser degree xyloglucan and cellulose. The skins were high in Klason-lignin (35–45%) and notably the grape skins were very high in total phenolics, notably the 3-glucosides of anthocyanins. Apple skins turned out to be a potential source for the particular recovery of catechins (which were absent in grape skins), with Red Delicious skins containing the highest levels. Red Delicious skins also contained cyanidin-3-galactosides.
Comparison of methods for compositional characterization of grape (Vitis vinifera L.) and apple (Malus domestica) skins

Anis Arnous, Anne S. Meyer *

BioProcess Engineering Center, Department of Chemical Engineering,
Technical University of Denmark, Building 229, DK-2800 Kgs. Lyngby, Denmark

Article info
Article history:
Received 15 October 2007
Accepted 14 March 2008

Keywords:
Monosaccharides
Phenolics
Skin cell walls
HPAEC-PAD
HPLC-DAD
Trifluoroacetic (TFA) acid

Abstract
A fundamental prerequisite for upgrading of fruit skins in press residues from juice, cider, and wine processes to food ingredients or supplements is the provision of methods for evaluation of the phenolics and cell wall carbohydrates in the materials. This study compared the monosaccharide yields obtained for different wine grape skin (Cabernet Sauvignon, Merlot, and Shiraz) and apple skin (Red Delicious (RD) and Golden Delicious (GD)) samples by trifluoroacetic (TFA) acid vs. hydrochloric (HCl) acid hydrolysis. Recovery values of monosaccharide standards after the acid treatments were also compared. TFA hydrolysis (2 M, 121 °C, and 2 h) resulted in higher monosaccharide yields from the fruit skin samples than HCl hydrolysis under the same conditions—after recovery value adjustments. Analyses of the phenols after extensive, sequential aqueous methanol (60%, w/w) extraction of the fruit skins confirmed that grape skins are a good source of anthocyanin pigments, notably the 3-glucosides of malvidin and cyanidin, and demonstrated that apple skins are a potential source of catechins.

© 2008 The Institution of Chemical Engineers. Published by Elsevier B.V. All rights reserved.

1. Introduction

Each year the processing of grapes (Vitis vinifera L.) and apples (Malus domestica) for wine, juice, and cider production globally leave behind an estimated amount of at least 50 million tonnes of press residues, or "pomace" (Schieber et al., 2001). This pomace mainly consists of the fruit skins and seeds as it results from the pressing of the macerated fruit or the freshly fermented wine or cider. The skins of both grapes and apples, and notably of grapes, are known to be rich sources of phenolic compounds (Escarpa and Gonzalez, 1998; Pinelo et al., 2006). Several reports have suggested different solvent extraction methods for recovery of the phenolics from grape pomace to obtain extracts that may be used as natural health remedies, food supplements, or as novel nutraceutical food ingredients (Escarpa and Gonzalez, 1998; Negro et al., 2003; Pinelo et al., 2005). Nevertheless, the majority of the grape pomace is currently not upgraded, but used for composting or discarded in open areas potentially causing environmental problems. Apple pomace, left over from apple juice production, is widely used for pectin production, leaving the apple skin fraction as a secondary byproduct residue, which is currently not upgraded (Carle and Schieber, 2006).

Innovative valorisation of the processing byproducts is an important part of the development of sustainable production methods in large-scale food processes. One way to accomplish a sustainable valorisation, safe processing without use of organic solvents, and a high quality of the recovered products is to introduce non-solvent, biocatalysis-based techniques for upgrading of the low-value food processing byproducts. At the present time only sparse knowledge is available on the location and bonding of the phenolics within the fruit skin polysaccharide matrix, and on the relationship between carbohydrate composition and phenols release and yields. However, the conceptual use of enzymatic catalysis to digest plant cell wall polysaccharides in fruit skins of press residues to release phenolics have already been described in the scientific literature for upgrading of blackcurrant, and grape pomace phenolics (Meyer et al., 1998; Landbo...
and Meyer, 2001; Kammerer et al., 2005), and for improved use of enzymes in juice and wine production (Pinelo et al., 2004).

A first prerequisite for assessing the potentially obtainable yields in such enzyme-assisted recovery of phenols or specific carbohydrate structures from fruit skins is obtaining of a detailed compositional assessment of the carbohydrates and phenolics present. Several studies have already addressed the carbohydrate composition of (fresh) grape skins (Lecas and Brillouet, 1994; Vidal et al., 2001; Doco et al., 2003), but there are inconsistencies in the literature about the compositional make up of the skin of grapes and apples, and the data seem to differ depending on the methodologies employed. Polysaccharide compositions of fruits skins and other plant materials are usually determined by measuring the monosaccharides released after acid hydrolysis by means of trifluoroacetic (TFA) or hydrochloric (HCl) acid (De Ruiter et al., 1992; Meseguer et al., 1999), but no universal or standardized method(s) exist, and no direct comparisons between the structural carbohydrate make up and the phenolic compositions of apple and grape skins are available in the literature.

The purpose of this study was to evaluate the efficiency of different acid hydrolysis methodologies and to determine and compare the compositional carbohydrate and phenolics make up of grape and apple skins. We report on the differences in recovery factors after different acid hydrolysis methods. TFA acid was used by Nevis et al. (1967) for the hydrolysis of plant cell walls and has since been employed successfully for plant cell walls polysaccharides studies. The conditions of hydrolyses employed in our work were inspired by previous studies (Lecas and Brillouet, 1994; Vidal et al., 2001; Doco et al., 2003), and more specifically by De Ruiter et al. (1992) and Meseguer et al. (1999). We also report on the use of alkaline pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) for monosaccharide profiling of hydrolysates. This analytical method is simple and requires no sample preparation other than concentration/dilution and filtration. The method has nanomolar detection range and good precision (Weitzhandler et al., 2004).

2. Materials and methods

2.1. Chemicals and reagents

Folin–Ciocalteu (FC) reagent, sodium metabisulfite, methanol (HPLC grade), trifluoroacetic acid 99%, and sodium azide were from Merck (Darmstadt, Germany). Phenolic standards including gallic acid, caffeic acid, chlorogenic acid, catechin, epicatechin, rutin (quercetin 3-O-rutinoside), quercetin, p-coumaric acid, chloridrin, monosaccharides standards including d-(-)-fucose 99%, l-(+)-arabinose 99%, l-(+)-rhamnose monohydrate 99%, d-(+)-glucose 99.5%, d-(+)-galactose 99%, l-(+)-mannose 99%, d-(+)-fructose, d-(+)-glucuronic acid, d-(+)-galacturonic acid monohydrate 98%, acetonitrile (HPLC grade), and phosphoric acid (HPLC grade) were from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Anthocyanins mixture of delphinidin 3-O-β-glucoside, cyanidin 3-O-β-glucoside, peonidin 3-O-β-glucoside, petunidin 3-O-β-glucoside, and malvidin 3-O-β-glucoside was from Polyphenols (Biolink Group, Sandnes, Norway). NaOH standard solution HPLC grade was from Fluka/Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Plant materials and sample preparation

Three selected red wine grapes (V. vinifera L.) Cabernet Sauvignon, Merlot, and Shiraz were obtained from the Distell Group Ltd. (Stellenbosch, South Africa). Golden Delicious (GD) and Red Delicious (RD) apple varieties (M. domestica) were purchased from the local market in Lyngby, Denmark. Fruits were peeled manually under a continuous stream of nitrogen (N2) and the skins were carefully separated from the pulp using a scalpel. Skins were washed with cold, distilled water at 4 °C, and lyophilized immediately by use of a Lyovac GT 2 freeze-drier (Leybold-Heraeus, Germany) and then milled for 30 s under N2 at 20,000 rpm in an M 20 Universal mill (Jähne & Kunkel GmbH, Staufen, Germany). The chamber of the M 20 mill was cooled for the freezing water. Fruits skin particles of 125–250 μm were collected by means of a sieve tower, nominal aperture sizes of 500, 250 and 125 μm, respectively (Endecotts Ltd., London, UK). The fruits skin particles were kept in tightly closed glass jars under N2 at −20 °C until use.

2.3. 60% methanol extraction of phenolic compounds

Sample sizes of 40 mg of lyophilized skin particles were extracted with 1 mL 60% (v/v) aqueous methanol at ambient temperature. During the solvent contact the particles were swirled for 30 s then centrifuged at 14,000 rpm for 3 min at 4 °C. The supernatant was decanted and extraction was repeated sequentially seven and four times in total for the grape and apple skins, respectively and done in duplicate replica for each variety. Extracts were kept at 4 °C in screw-capped vials under N2 for max. 24 h until analysis.

2.4. Acid hydrolysis methods

The hydrolysis conditions with respect to moles concentrations of the acid and the temperatures were based on previous studies (De Ruiter et al., 1992; Meseguer et al., 1999). Moreover the exact applied conditions were selected after preliminary trials in our lab (data not shown). Three different hydrolysis methods each including five replicates of each grape and apple skin sample were compared. In the first method, 400 μL of 2 M TFA was added to 2 mg of lyophilized sample in a screw-cap vial. Each vial was tightly sealed and heated at 121 °C for 2 h in a drying oven. In the second method, 2 M HCl was used instead of TFA. The conditions of time, temperature and sample/acid ratio were the same as for the TFA hydrolysis. In the third method, 400 μL of 1.0 M HCl was added to 2 mg of sample in a screw-cap vial, which was then held at 100 °C for 1.5 h in a water bath. After hydrolysis the vials were cooled under tap water. Hydrolysates were lyophilized and kept at −20 °C under N2 until analysis. Prior to analysis by HPAEC-PAD, the fruit skin hydrolysates were re-dissolved in 5 mL of doubly deionised water containing 0.1% of sodium azide to prevent microbial growth. Just before injection for HPAEC analysis each hydrolysate was filtered through a 0.22 μm GH Polyprop Acrodisc® filter (Pall Life Sciences, Ann Arbor, USA).

Recovery values of the monosaccharides were estimated by exposing a mixture of monosaccharide standards (l-(+)-fucose, l-(+)-rhamnose, d-(+)-arabinose, d-(+)-
galactose, -(+)-glucose, -(+)-xylose, -(+)-mannose, -(−)-fructose, and -(+)-galacturonic acid) to the acid hydrolysis conditions mentioned above. The monosaccharide recovery values were expressed in percentage of the initial addition level and were used as correction factors for the quantitative monosaccharide assessment by acid hydrolysis.

2.5. Monosaccharides analysis by HPAEC-PAD

Separation and quantification of monosaccharides in the hydrolysates were performed using a BioLC system consisting of G50 gradients pumps/ED50 electrochemical detector/ASSO chromatography compartment coupled to an ASSO autosampler ( Dionex Corp., Sunnyvale, CA). Separations were performed using a CarboPac™ PA20 (3 mm × 150 mm) analytical column ( Dionex Corp., Sunnyvale, CA) according to the method of Ohro et al. (2004), with modifications: a two-eluent system comprising deionised water (18.2 mΩ cm at 25 °C) and 500 mM NaOH aqueous solution was used. Natural monosaccharides were eluted isocratically with 2.5 mM NaOH for 20 min followed by a second isocratic elution at high NaOH (500 mM) for 10 min to elute any present acidic monosaccharides (galacturonic and glucuronic) or the cellobiose disaccharide. At the same time, this high concentration of NaOH washed the column. Before each injection (10 μL) a column reequilibration program was run for 5 min with 100 mM NaOH followed by 5 min with 2.5 mM NaOH. The eluent flow rate was always kept at 0.5 mL/min. During chromatography, the eluents were kept under a blanket of N2 and the mobile phase was purged with N2 to minimize carbonate contamination, which would affect the retention times of the monosaccharides. The quantification was carried out by use of external standards. The different carbohydrate standards were mixed in proportion to resemble the matrix of the studied samples. The following pulse potentials and durations were used for detections: E1 = −0.1 V, t1 = 400 ms; E2 = −2 V, t2 = 20 ms; E3 = 0 V, t3 = 10 ms; E4 = −0.1 V, t4 = 70 ms; data collection rate 0.2 Hz. Data were collected and analyzed on computers equipped with Chromleon 6.80 Sp2 Build 1472 software ( Dionex Corp., Sunnyvale, USA).

2.6. Determination of total polyphenols (TP), total monomeric anthocyanins (TA), and Klasson-lignin content (%)

Total phenol contents of the fruit skins were determined by the Folin–Ciocalteu method (Singleton and Rossi, 1965). The total phenol concentration was calculated from a calibration curve using gallic acid as standard. Results were expressed as mg L−1 gallic acid equivalents (GAEs). Monomeric anthocyanins were assessed by the pH differential method (Lee et al., 2005), and calculated as malvidin-3-glucoside equivalents for grape samples and as cyanidin-3-gallocatechol equivalents for apple samples. Klasson-lignin was determined according to Theander and Aman (1979).

2.7. Analyses of phenolic compounds by HPLC-DAD

Phenolic extracts (10 μL) were analyzed using an HPLC system equipped with a diode array detector (DAD) (Hewlett-Packard 1100, Waldbronn, Germany) and a Nova-Pak C18 column (3.9 mm × 150 mm, Waters) at 40 °C. The mobile phase was made of three solvents delivered in a gradient at a flow rate of 0.5 mL/min essentially as described by Lamuela-Raventos and Waterhouse (1994). Identification and quantification of the phenolic compounds were carried out using an in-house UV–vis spectrum library of pure phenolic compounds. The library match factor, which is the result of the automated library search, was set to minimum 99.0% as a requirement to confirm the identity of the peaks. Quantification of the phenolic compounds was performed using external standards. The operation of the HPLC and the spectral match analyses were done by use of HP ChemStation B.01.01 software (Agilent Technologies Inc., USA).

2.8. Statistical analysis

All measurements were done in triplicate except for monosaccharides analysis with HPAEC-PAD and analyses of phenolic compounds by HPLC-DAD that were done in duplicates. Quadratic curve fit through the origin was used for monosaccharides standards analysis by HPAEC-PAD, and the calibration points were weighted by the factor 1/response7. For
<table>
<thead>
<tr>
<th>Variety</th>
<th>Hydrolysing method</th>
<th>Monocarbohydrates (μg mg⁻¹ lyophilized skins)</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fucose</td>
<td>L(-)-Rhamnose</td>
</tr>
<tr>
<td>Grape skins</td>
<td>TFA: 2 M, 121 °C, 2 h</td>
<td>13.55</td>
<td>13.96</td>
</tr>
<tr>
<td></td>
<td>HCl: 2 M, 121 °C, 2 h</td>
<td>0.85</td>
<td>4.71</td>
</tr>
<tr>
<td></td>
<td>HCl: 1 M, 100 °C, 1.5 h</td>
<td>0.02</td>
<td>4.09</td>
</tr>
<tr>
<td>Merlot</td>
<td>TFA: 2 M, 121 °C, 2 h</td>
<td>2.51</td>
<td>14.01</td>
</tr>
<tr>
<td></td>
<td>HCl: 2 M, 121 °C, 2 h</td>
<td>1.61</td>
<td>6.58</td>
</tr>
<tr>
<td></td>
<td>HCl: 1 M, 100 °C, 1.5 h</td>
<td>0.22</td>
<td>4.09</td>
</tr>
<tr>
<td>Shiraz</td>
<td>TFA: 2 M, 121 °C, 2 h</td>
<td>2.37</td>
<td>13.82</td>
</tr>
<tr>
<td></td>
<td>HCl: 2 M, 121 °C, 2 h</td>
<td>1.03</td>
<td>5.11</td>
</tr>
<tr>
<td>Apple skins</td>
<td>TFA: 2 M, 121 °C, 2 h</td>
<td>2.90</td>
<td>18.54</td>
</tr>
<tr>
<td>Red Delicious</td>
<td>HCl: 2 M, 121 °C, 2 h</td>
<td>0.58</td>
<td>5.25</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>HCl: 2 M, 121 °C, 2 h</td>
<td>0.87</td>
<td>3.59</td>
</tr>
</tbody>
</table>

* Corrected average of recoveries values. Average CVs for each one of the monocarbohydrates were as follows: D(+)-Fucose 9.9%, L(-)-Rhamnose 8.1%, D(-)-Arabinose 8.5%, D(+)-Galactose 7.0%, D(-)-Glucose 6.6%, D(-)-Xylose 7.8%, D(-)-Mannose 11.2%, D(+)-Fructose 0.0%, D(+)-Galacturonic acid 5.9%.
phenolic compounds by HPLC-DAD the curve fit consisted of a linear least squares fit through the data points including the origin.

3. Results and discussion

3.1. Monosaccharides recovery and analysis by HPAEC-PAD

The recovery values among the different monosaccharides varied, but were mostly in the range from 50 to 64% including all three acid treatments (Table 1). As expected, the recovery values were generally the highest for the weakest treatment with 1 M HCl at 100 °C for 1.5 h—the only exception was the recovery value of mannose after TFA hydrolysis, which was higher than the 1 M HCl treatment (Table 1). The recovery values of the same monosaccharide standards were relatively similar after acid hydrolysis with 2 M TFA and 2 M HCl, respectively, when compared at the same conditions of time and temperature with the TFA recovery values tending to be consistently highest—except for galacturonic acid (Table 1). TFA hydrolysis thus induced a pronounced loss of galacturonic acid, resulting in a recovery value of only ~15% for galacturonic acid. D-(−)-Fructose was completely destroyed with the 2 M acid treatments, but had a recovery value of 16% after treatment with 1 M HCl at 100 °C for 1.5 h (Table 1). Reduction of neutral sugars by HCl hydrolysis has been reported earlier where mannose and galactose were reduced by 30–50% (Hardy et al., 1988), but similar data for TFA are not available. Our TFA acid hydrolysis recoveries were generally lower than those reported earlier by Talaga et al. (2002), which could be due to differences in both hydrolysis and analysis methods. The finding that all the recovery values were considerably lower than 100% signifies the importance of employing recovery values when recalculating monosaccharide (and polysaccharide) levels after acid hydrolysis treatment.

3.2. Fruit skin polysaccharide acid hydrolysis and monosaccharide analysis by HPAEC-PAD

In order to evaluate the hydrolysing efficiency of different acid treatments on fruit skins, the Cabernet Sauvignon grape skins were hydrolysed by use of all the three different acid hydrolysis methods. Higher analytical values were obtained with TFA treatment than with HCl (Table 2). In particular, TFA appeared to have stronger hydrolysing efficiency towards the pectin fraction since TFA was able to release much higher amounts of D-(+)galacturonic and other structural monosaccharides of the pectin fraction including D-(−)-arabinose, D-(+)galactose, L-(−)-rhamnose, in addition to elevated levels of D-(−)-xylose and L-(−)-fucose than HCl (Table 2). The values of D-(+)fucose, L-(−)-rhamnose, D-(−)-arabinose, D-(+)galactose, and D-(+)xylose obtained after the TFA hydrolysis were in agreement with the compositional data for grape skins reported by Lecas and Brillouet (1994), and indicated that the skins contained significant amounts of pectin, apparently including both homogalacturonan (due to the high levels of galacturonic acid) and branched rhamnogalacturonans. The efficiency of TFA in hydrolysing the pectin fraction was consistent for both grape and apple fruits skins, which is in accordance with previous data for other plant materials (Smith and Harris, 1995; Benhura and Chidewe, 2002). TFA hydrolysis was more capable than HCl of disclosing the difference of monosaccharides profiles between different fruits skins. Cellulose has previously been reported to be poorly hydrolysed by TFA treatment (Mankarios et al., 1979; Carnachan and Harris, 2000). With glucose the marginal increase obtained with TFA as compared to HCl treatment was of a lower magnitude than that seen for the other compounds, but the glucose level obtained after TFA hydrolysis was still significantly higher than that obtained with HCl (Table 2). Based on the available knowledge (Smith and Harris, 1995; Benhura and Chidewe, 2002) the data might suggest that monosaccharides were mainly released from noncellulosic glucan polymers, e.g. xylloglucan, rather than from the cellulose fraction. TFA hydrolysis resulted in lower D-(−)-mannose values than those obtained with HCl. Our present experiment does not allow a firm conclusion to be drawn with respect to the mechanism behind this difference. However, since the recovery of mannose after TFA hydrolysis treatment was higher than the analogous recovery value after HCl treatment (Table 1), a tempting assumption may be that the bonds releasing mannose from the skin polysaccharide matrix during the acid hydrolysis were simply not accessible or attacked very well by the TFA.

The difference in monosaccharide profiles between grape and apple skins reflected the difference in polysaccharide cell structures between the two plants species. By TFA hydrolysis D-(−)-arabinose and D-(+)galacturonic acid values were higher in apple skins while D-(−)-mannose was higher in grape skins regardless of hydrolysis method (Table 2). As could be predicted from the acid treatment of the standard monosaccharides (Table 1), D-(−)-fructose was completely lost with both the TFA and the HCl treatment at 2 M, 121 °C, and 2 h (Table 2). It is known that ketoses are completely reduced at harsh hydrolysis conditions (Schiller et al., 2002).

<p>| Table 3 – Determination of total phenolics, total anthocyanins, Klasson-lignin, and ash in grape, and apple fruit skins by spectrophotometric methods expressed in µg mg−1 lyophilized skins* |</p>
<table>
<thead>
<tr>
<th>Varieties</th>
<th>Total phenolics (µg mg−1 lyophilized skins)</th>
<th>Total anthocyanins (µg mg−1 lyophilized skins)</th>
<th>Klasson-lignin (µg mg−1 lyophilized skins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grape</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cabernet Sauvignon</td>
<td>102.3</td>
<td>29.6</td>
<td>40.6</td>
</tr>
<tr>
<td>Merlot</td>
<td>111.8</td>
<td>46.0</td>
<td>43.7</td>
</tr>
<tr>
<td>Shiraz</td>
<td>111.8</td>
<td>43.2</td>
<td>45.1</td>
</tr>
<tr>
<td>Apple</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Delicious</td>
<td>18.0</td>
<td>2.0</td>
<td>38.6</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>13.1</td>
<td>0.04</td>
<td>34.4</td>
</tr>
</tbody>
</table>

* The average of three replicates. Average CV was as follows: total phenolics 1.2%, total anthocyanins 3.5%, Klasson-lignin 3.4%.

* Total phenols (µg mg−1 lyophilized skins) were expressed as gallic acid equivalent (GAE).
A milder hydrolysis method is thus required in case assessment of fructose and other ketose sugars more liable to acid degradation are needed.

A high percentage of lignin expressed as Klason-lignin (%, w/w) was found in both the grape skins (40–45%, w/w) and in the apple skin samples (34–38%, w/w) (Table 3). The skin matrix plays a major role in protecting the fruit itself and the seeds and the lignin apparently contributes a mechanical support and barrier against microbial invasion. The lignin values agreed with previously published values for grapes (Femenia et al., 1998; Botella et al., 2005), but were a little higher than previously published lignin values for apple pomace (Nawirska and Kwasniewska, 2005).

3.3. Total phenolics and total anthocyanins

Total phenolic (TP) contents of the skins of the three different grape varieties were relatively similar and ranged from ~102 to 112 µg·g⁻¹ GAE and were almost fivefold higher than the TP levels of the apple skins, which ranged from 13 to 18 µg·g⁻¹ GAE (Table 3). The data for the grape skins were in good agreement with those reported previously for ‘Negro amaro’ red grape marc peel (Negro et al., 2003) and were also consistent with previously reported data for the phenolic content in Merlot grape skins (Yilmaz and Toledo, 2006). However, our TP values were markedly higher than those reported by Ortega-Regules et al. (2006) for skins of red grapes (Cabernet Sauvignon, Merlot, Syrah, and Monastrell). The difference is presumably due to Ortega-Regules et al.’s application of an alkaline extraction method (1 M NaOH 100 °C, 10 min) which might have caused partial degradation of the phenolics. Likewise, our TP values for the apple skins (Table 3) were higher than those reported previously for cider apples (Blanco-Gomis et al., 1998) and RD apples (Tsao and Yang, 2003), and we ascribe the difference mainly to the type of extraction method employed. However, quantitative differences between the apple varieties might also be a factor (Vrhovsek et al., 2004). Total monomeric anthocyanins as assessed by spectrophotometry (TA) accounted for one-third of the TP in the grape skins. As expected, anthocyanins were almost absent in GD apple skins and low in RD apples skins. The anthocyanin values agreed well with data published previously by Tsao and Yang (2003), despite differences in extraction method and extraction solvent. This might highlight that anthocyanins are easier to extract from grapes skins compared to other phenolics groups.

Phenolics may be extracted from plant biomass, incl. pomace, by use of different solvents (Kallithraka et al., 1995). Our choice was aqueous methanol 60% as it has consistently been shown to give the highest total extraction yield of phenolics from grape pomace as compared to other methanol–water mixtures (Yilmaz and Toledo, 2006). In addition, methanol has been demonstrated to provide efficient extraction of phenolic compounds from different zone tissues of cider apple fruits when compared with acetone or ethanol extracts (Guyot et al., 1998).

3.4. Phenolic compositions

HPLC analyses were carried out to provide a qualitative mapping of the phenolics present and a quantitative measurement of the phenolic profiles (Table 4). The difference in plant species, i.e. grapes vs. apples, reflected on the detailed individual phenolic profiles: As expected, grape skins had sig-

![Table 4: Detailed phenolics profile of grape and apple fruit skins methanolic extract by HPLC expressed in lyophilised skin](image-url)
nificantly higher content of phenolics than apple skins by HPLC (TPHPLC) (Table 4). Phenolics by HPLC were consistently lower than TP obtained with the Folin–Ciocalteu method; the ratio for grape skins varied from 1.6 to 3.7 while a lower ratio near 1 was noted for apple skins: 1.5–1.8. The FC method is based on the reaction of reducing groups, and in fact measures the reducing capacity of any phenolic compound relative to the reducing capacity of gallic acid as standard (Frankel et al., 1995). The reducing capacity is increased if two hydroxyl groups are in ortho or para positions and the molar response is nonproportional to phenolic hydroxyl groups (Frankel et al., 1995). In other words, the FC method is not an absolute measurement of the total phenolics content and that could explain the constant higher values of total phenolics obtained by the FC method relative to the quantitative determination of phenolics by HPLC. In complete agreement with previous findings (Ju and Howard, 2005) the HPLC analysis of the methanolic extracts of the grape skins revealed that anthocyanins (TAHPLC) made up most of TPHPLC in grape skins. Among the three grape varieties, the Merlot grape skin had the highest content of the major anthocyanins including Mv-3-gl, Dp-3-gl, Pt-3-gl, and Pn-3-gl. The absence of grape seeds from the grape skin samples could explain why the extract contained no measurable catechins. Both Vilimaz and Toledo (2006) and Kallithraka et al. (1995) suggested aqueous methanol as the best extraction solvent for catechin, epicatechin and epigallocatechin. The low levels of hydroxybenzoic acids and the absence of hydroxycinnamates and the relatively modest levels of flavonols in the grape skins confirmed that these phenolic compounds mainly reside in the grape seeds and pulp, respectively (hydroxycinnamates are believed to be localized in the grape pulp cells).

In general, RD apple skins had slightly higher TPHPLC relative to GD apple skins and the flavonols, including rutin and quercetin derivatives, were also higher in RD skins than in GD skins (Table 4). Cy-3-galact was the only anthocyanin detected in apple skins, and only in the RD. Hydroxycinnamic acids and the absence of hydroxycinnamates and the relatively modest levels of flavonols in the grape skins confirmed that these phenolic compounds mainly reside in the grape seeds and pulp, respectively (hydroxycinnamates are believed to be localized in the grape pulp cells). To detect polymeric procyanidins using aqueous methanol as extraction solvent. Highly polymeric procyanidins may escape reverse phase C8 HPLC analysis, but since we have previously detected procyandin families by this same HPLC method the absence of polymeric procyanidins in our apple skin extracts may simply be a result of these particular apple varieties not containing any. Phlorizin, a dihydrochalcone, has been claimed to be one of the major glycosides present in apple seed (Durkee and Paogst, 1965) associated with seed dormancy (Grochows, 1986). Phlorizin was detected in low amounts and only in GD skins (Table 4). The values were similar to the values detected in apple seeds (Iham, 1996).

4. Conclusions

Acid hydrolysis by TFA (2M, 121°C, and 2h) turned out to be superior to HCl for assessing the monosaccharide composition and the potentially obtainable carbohydrate yields. The monosaccharide compositions were relatively similar for apple and grape skins. As judged from the high galacturonic acid levels, both grape and apple skins were dominated by complex pectinaceous structures. The glucose yields obtained indicated that the skins also contained significant amounts of glucans. The skins were high in Klason-lignin (55–45%) and notably the grape skins were very high in total phenols, notably the 3-glucosides of anthocyanins. Apple skins turned out to be a potential source for the particular recovery of catechins (which were absent in grape skins), with Red Delicious skins containing the highest levels. Red Delicious skins also contained cyanidin-3-galactosides, while phlorizin was only detected in Golden Delicious.

Acknowledgment

The Distell Group Ltd. (Stellenbosch, South Africa) is acknowledged for supplementation of the wine grape samples.

REFERENCES


On the basis of monosaccharide analysis after acid hydrolysis of fruit skin samples of three wine grape cultivars, Vitis vinifera L. Cabernet Sauvignon, Merlot, and Shiraz, and of two types of apple, Malus domestica Red Delicious and Golden Delicious, an iterative calculation method is reported for the quantitative allocation of plant cell wall monomers into relevant structural polysaccharide elements. By this method the relative molar distribution (mol %) of the different polysaccharides in the red wine grape skins was estimated as 57–62 mol % homogalacturonan, 6.0–14 mol % cellulose, 10–11 mol % xyloglucan, 7 mol % arabinan, 4.5–5.0 mol % rhamnogalacturonan I, 3.5–4.0 mol % rhamnogalacturonan II, 3 mol % arabinogalactan, and 0.5–1.0 mol % mannans; the ranges indicate minor variations in the skin composition of the three different cultivars. These cell wall polysaccharides made up ∼43–47% by weight of the skins (dry matter), the rest mainly being lignin. The predicted relative molar levels of the polysaccharide elements in the apple skins, which made up ∼49–64% by weight of the skins (dry matter), appeared to be similar to those of the grape skins. The apple skins were estimated to be relatively richer than grape skins in arabinan, total levels 10–13 mol %, and relatively lower in mannan content, total levels ≤0.3 mol %. The data also demonstrate the superiority of trifluoroacetic acid to hydrochloric acid for hydrolysis of plant cell wall material to monosaccharides, notably with respect to the galacturonic acid levels and, in turn, in relation to predicting the relative contents of structural pectin elements in the plant cell wall substrates.

KEYWORDS: Plant cell wall polymers; polysaccharides; composition; pectin

INTRODUCTION

Microbially derived pectinolytic enzyme preparations are widely used for prepress fruit maceration in the industrial production of apple and berry juices, ciders, and red wines to increase juice yields (1). Recently, there has been an increased interest in using plant cell wall degrading enzymes to enhance the release of colored pigments and antioxidant phenolic compounds during the prepress treatment in juice and wine processing (2, 3). Such enhanced release may take place via more aggressive enzyme-catalyzed degradation of the complex plant cell wall material, notably the fruit skins (4).

Each year the processing of grapes (Vitis vinifera L.) and apples (Malus domestica) for wine, juice, and cider production globally leaves behind an estimated amount of at least 50 million metric tons of press residues, or “pomace” (5). This pomace consists of fruit skins, remnants from the fruit pulp, seeds, and, in certain cases, some stems, with the skins and seeds making up the major part. Grape seeds and grape skins are rich sources of phenolic compounds and/or dietary fibers (6, 7), and grape pomace from wine processing is already used for the extraction of anthocyanins on an industrial scale (8). Increased efforts are now directed toward more extensive valorization of the press residues from fruit juice and wine processing to obtain high-value products such as natural health remedies, food supplements, and novel nutraceutical food ingredients or to use the material for enzyme production by solid state fungal cultivation (8). Apple pomace, left over from apple juice production, is used for pectin extraction, leaving the apple skin fraction behind as a secondary byproduct residue. This secondary apple byproduct residue is currently not upgraded to high-value products (9).

To rationally design and tailor enzymatic treatments to upgrade fruit skin residues and/or to increase the enzyme-catalyzed degradation of the fruit skins to release additional color or antioxidant phenolics during prepress treatments in juice and wine processes, a first essential step is to obtain quantitative knowledge about the structural makeup, including the glycosidic bonds, of the fruit skin polysaccharides. Several studies have already addressed certain characteristics...
of different carbohydrate polymers in grape skins (10–12), whereas surprisingly few reports have addressed the quantitative occurrence and structural features of apple skin cell wall polysaccharides (13, 14). The available data on grape and apple skins either focus on unique structural characteristics of particular polysaccharides (11, 15) or employ the monosaccharide composition to elucidate compositional differences among fruit tissues or of different cultivars in relation to processing or product quality (10, 11, 16). There are currently no stand-alone methods available for deducing the detailed composite structure of intact plant cell walls nor for assessing the full structures of the polysaccharides and the possible connection points that define the molecular architecture of composite plant cell walls. Hence, despite advances in, for example, electron microscopy, chromatographical methods, mass spectrometry, combinations of different sequential extraction methods and several analytical methods are required to obtain the pieces of the puzzle and to obtain an insight into the glycosyl linkage compositions (see e.g (11, 12, 16)).

The particular sequential extraction and precipitation approaches and the particular analytical methods employed may even affect the results obtained. The recovery of monosaccharides after acid hydrolysis is a widely used strategy to at least obtain an overview of the composition of the plant cell wall building blocks (17–19). The monosaccharide composition of fruit skins and other plant materials is usually determined via chromatographical analysis of the monosaccharides released after acid hydrolysis with either trifluoroacetic (TFA), hydrochloric acid (HCl), or in some cases sulfuric acid (H2SO4) (17, 18). Monosaccharide profiles are used to provide a rough overview of the types of polysaccharides present: for example, arabinose signifies the putative presence of “pectin”, and galacturonic (or uronic) acid levels indicate the presence of “pectin” even though, in the latter case, pectin is known to encompass several different structural elements. The monosaccharide profiles are rarely used to calculate the quantitative levels of the different types of structural polysaccharides in the plant material. An important question in relation to the rational design of enzyme treatments for efficient degradation of the cell wall polysaccharides is whether it is possible to combine the available knowledge of polysaccharide type structures, including their glycosidic bonds, in (dickut) plant materials with the now readily obtainable monosaccharide profiles and use the monosaccharide data to provide a picture of the quantitative levels of the different polymeric structural elements in plant materials without having to include mischievous, time-consuming procedures such as polysaccharide linkage analysis. This study was undertaken to evaluate the options for calculating the most probable structural elements from monosaccharide data. We report an iterative calculation strategy to at least obtain an overview of the composition of the full structures of the polysaccharides and the possible connection points that define the molecular architecture of composite plant cell walls.

MATERIALS AND METHODS

Chemicals and Reagents. Trifluoroacetic acid 99% (TFA) and sodium azide were from Merck (Darmstadt, Germany). Monosaccharide standards (L (+)-fructose 99%, D (+)-galactose 99%, L (+)-arabinose 99%, D (+)-glucose 99.5%, D (+)-xylose 99%, D (+)-mannose 99%, D (+)-xylose 99%, D (+)-fructose, and D (+)-galacturonic acid monohydrate 98% were from Sigma-Aldrich Chemical Co. (St. Louis, MO). HCl and the NaOH standard solution (HIPLC grade) were from Fluka; Sigma-Aldrich Chemical Co. (St. Louis, MO).

Plant Materials and Sample Preparation. Three selected red wine grapes (V. vinifera L.), Cabernet Sauvignon, Merlot, and Shiraz, were obtained from the Distell Group Ltd. (Stellenbosch, South Africa), Golden Delicious (GD) and Red Delicious (RD) apple varieties (M. domestica) were purchased locally in Lyngby, Denmark. Fruits were peeled manually under a continuous stream of nitrogen (N2), and the skins were carefully separated from the pulp using a scalpel. Skins were washed with cold, distilled water at 4 °C, and lyophilized immediately by use of a Lyovac GT 2 freeze-drier (Leybold-Heraeus, Germany) and then milled for 30 s under N2 at 20000 rpm in an M 20 Universal mill (Jahnke & Kunkel GmbH, Stauften, Germany). The chamber of the M20 mill was cooled with running tap water. Fruit skin particles of 125–250 μm were collected by means of a sieves tower, nominal aperture sizes of 500, 250, and 125 μm, respectively (Endecotts Ltd., London, U.K.). The fruit skin particles were kept in tightly closed glass jars under N2 at −20 °C until use.

Acid Hydrolysis Methods. Two different hydrolysis methods, each including five replicates of each grape and apple skin sample, were compared. In the first method, 400 μL of 2 M TFA was added to 2 mg of lyophilized sample per milligram of lyophilized sample. Each sample was tightly sealed and heated at 121 °C for 2 h in a drying oven. In the second method, 2 M HCl was used instead of TFA. The conditions of time, temperature, and sample/acid ratio were the same as for the TFA hydrolysis [a more detailed comparison of acid hydrolysis methods for fruit skin analyses has been published previously (19)]. Hydrolysates were lyophilized and kept at −20 °C under N2 until analysis. Prior to analysis by HPAEC-PAD (see below), the fruit skin hydrolysates were redissolved in 5 mL of doubly deionized water containing 0.1% sodium azide to prevent microbial growth. Just before injection for HPAEC-PAD analysis, each hydrolysate was filtered through a 0.22 μm GH Polypro Acrodisc filter ( Pall Life Sciences, Ann Arbor, MI). Recovery values of the monosaccharides were estimated as described previously (19) by exposing a mixture of monosaccharide standards L (+)-fructose, D (+)-rhamnose, D (+)-arabinose, D (+)-galactose, D (+)-glucose, D (+)-xylose, D (+)-mannose, D (+)-fructose, and D (+)-galacturonic acid to the corresponding acid hydrolysis conditions.

Monosaccharides Analysis by HPAEC-PAD. Separation and quantification of monosaccharides in hydrolysates were performed by use of a BioLC system, equipped with a CarboPac PA20 (3 mm × 150 mm) analytical column, and an ED50 electrochemical detector and controlled via Chromelon 6.60 SP2 Build 1472 software (Dionex Corp., Sunnyvale, CA) as reported previously (19). Monosaccharides were expressed as micrograms per milligram of lyophilized fruit skin. The values were then translated into micro-stoles of monosaccharide from milligram of lyophilized fruit skin for easier reconstruction according to the molar ratio between different monosaccharides in each type of polysaccharide. The data are reported as relative molar levels in percent.

Determination of Klason Lignin. Klason lignin was determined according to the method of Thonander and Aman (20).

Statistical Analysis. All measurements were done in triplicate except for monosaccharides analyses with HPAEC-PAD that were done in duplicate. Quadratic curve fit through the origin was used for monosaccharides standards analysis by HPAEC-PAD, and the calibration points were weighted by the factor 1/response².

RESULTS AND DISCUSSION

Monosaccharide Compositions. Grape Skins. Acid hydrolysis with TFA consistently gave higher analytical values than HCl hydrolysis with respect to the levels of monosaccharides after HPAEC-PAD analysis (after adjustment for recovery factors) (Table I). The high galacturonic acid levels in the TFA hydrolysates of the grape skins, > 300 μg mg⁻¹ of
lyophilized skins (Table 1), indicated that the grape skins contained significant amounts of pecticaceous polysaccharides. When consideration of the rhamnose, arabinobiose, galactose, and fucose levels (Table 1) was included, it appeared that these pecticaceous polysaccharides included both homogalacturonan (HG) and branched rhamnogalacturonans. The higher galacturonic acid levels of the TFA-treated samples as compared to the HCl-treated samples indicated that TFA had a stronger hydrolyzing efficiency than HCl toward pectin (all values had been adjusted for recovery). The galacturonic acid values obtained from the TFA hydrolysis were somewhat higher than those reported by others using other methods of hydrolysis and on other grape varieties (Table 2). Previously, De Ruiter et al. (17) demonstrated that the method of hydrolysis has a significant influence on the monosaccharide results and in turn on the determined composition of the plant materials. Our high galacturonic acid values with TFA hydrolysis were thus presumably a result of the superior efficiency of TFA to HCl for hydrolysis of pectin but perhaps also the extended treatment time (19) and the use of a highly sensitive chromatographic method of detection. Furthermore, we used recovery factors to compensate for monosaccharide losses during hydrolysis. However, with TFA hydrolysis, our values for mannose were lower than those found by others using the Saeman procedure (involving sulfuric acid hydrolysis) (Table 2) and lower than those obtained with HCl hydrolysis (Table 1), but similar to those reported previously for grape pulp cell wall material hydrolyzed by TFA combined with methylation and sonication (16). For grape berry skins it has previously been shown that mannose yields may be lower with TFA hydrolysis than with the Saeman hydrolysis procedure (10).

The analyzed levels of fucose, rhamnose, arabinose, galactose, and xylose in the TFA hydrolysates of the grape skins (Table 1) were similar to those reported by others, even for other grape varieties, but the glucose values of our TFA-treated grape skin samples were 2–3 fold lower than those reported previously by others (10, 11, 27) (Table 2). It is well-known that TFA has a relatively poor hydrolyzing capability against cellulose (22, 23), but this alone cannot fully explain the lower glucose levels, because TFA was also used by others for the hydrolysis (Table 2). We consider it unlikely that cellulose contents will vary this significantly among different grape varieties. We therefore ascribed the relatively low glucose values of our grape skin samples (Table 1) to be a result of the deliberate inclusion of washing of the skins prior to the acid hydrolysis treatment in our procedure. Washing will free the skin material from intercellular liquid, rich in glucose and fructose, and remove any free mono- saccharides (24). Hence, we presume that the high values of other samples may in fact be a result of the (artifact) presence of free glucose from disrupted pulp cells in the grape skin samples. When an anhydro correction factor of 0.88 was used, the polysaccharides together made up ∼43–47% by weight of the grape skins; the levels varying slightly among the grape cultivars.

**Apple Skins.** For the apple skins, hydrolysis with TFA, as opposed to with HCl, also consistently resulted in higher monosaccharide values, notably for galacturonic acid—but except for mannose, which was lower with TFA than with HCl hydrolysis, exactly as seen for the grape skins (Table 3). Hence, apparently the TFA hydrolysis was more capable than HCl hydrolysis of disclosing differences between monosaccharide profiles of different grape and apple skin samples (Tables 1 and 3). In general, the monosaccharide profiles of the TFA hydrolysates of the apple skins were quite similar to those of the grape skins, but the galacturonic acid level in the TFA hydrolysate of the Red Delicious apple skin was ∼30% higher than the levels found in the Golden Delicious and in the grape skin samples (Tables 1 and 3). Likewise, the arabinobiose and galactose levels and—a to a lesser extent—the xylose levels were higher in the TFA hydrolysates of the apple skins than in the corresponding grape skin hydrolysates, notably for the Red Delicious (Table 3). Partial monosaccharide analyses of apple marc have previously indicated that arabinobiose and galactose are particularly abundant in apple cell wall materials, with values reaching 85 and 40 μg g⁻¹, respectively (13). The relatively high arabinose levels found in the apple skin hydrolysates were consistent with the presence of highly branched α-L-arabinofuranans (“arabinans”) linked to rhamnogalacturonan I in apple cell walls (25, 26). When an anhydro correction factor of 0.88 was used, the polysaccharides made up ∼64% of the skin weight of the Red Delicious skins and ∼47% by weight of the Golden Delicious fruit skins.

**Lignin.** Lignin constituted 40–45% by weight of the grape skins (Table 1) and 34–39% by weight of the apple skins (Table 3). These relatively high levels of lignin agree well with previously published data for grapes (21, 27), whereas the lignin values for the apple skins were a little higher than those previously reported for apple pomace (28). The relatively high values may be because in our study the skins were carefully separated from visible pulp material prior to acid hydrolysis. The high levels of lignin found in both grape and apple skins are noteworthy and categorize the grape and apple

### Table 1. Analyzed Monosaccharide Composition of Grape Fruit Skins

<table>
<thead>
<tr>
<th>grape variety</th>
<th>method</th>
<th>L- (+)-fucose (μg g⁻¹)</th>
<th>L- (+)-mannose (μg g⁻¹)</th>
<th>D- (+)-arabinose (μg g⁻¹)</th>
<th>D- (+)-galactose (μg g⁻¹)</th>
<th>D- (+)-glucose (μg g⁻¹)</th>
<th>D- (+)-xylose (μg g⁻¹)</th>
<th>sum of total monosaccharides (μg g⁻¹)</th>
<th>Klonin lignin (μg g⁻¹)</th>
<th>sum of Klonin lignin and total monosaccharides (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet</td>
<td>TFA</td>
<td>2.1</td>
<td>14.0</td>
<td>32.8</td>
<td>17.4</td>
<td>94.9</td>
<td>15.5</td>
<td>2.4</td>
<td>324.0</td>
<td>494.1</td>
</tr>
<tr>
<td>Sauvignon</td>
<td>HCl</td>
<td>0.9</td>
<td>4.7</td>
<td>9.3</td>
<td>8.4</td>
<td>73.6</td>
<td>2.4</td>
<td>11.2</td>
<td>17.6</td>
<td>128.1</td>
</tr>
<tr>
<td></td>
<td>TFA</td>
<td>2.5</td>
<td>14.0</td>
<td>40.8</td>
<td>22.0</td>
<td>88.2</td>
<td>21.1</td>
<td>4.7</td>
<td>345.0</td>
<td>538.3</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>1.6</td>
<td>6.6</td>
<td>11.8</td>
<td>12.2</td>
<td>67.7</td>
<td>2.5</td>
<td>9.9</td>
<td>20.5</td>
<td>132.8</td>
</tr>
</tbody>
</table>

Table 1: Analyzed Monosaccharide Composition of Grape Fruit Skins
Delicious Golden residues (Delicious Red of alternating galacturonic acid residues; RG-I, made up of a backbone structural units: HG, made up of a backbone of acid residues were distributed among the three main pectin files: Methodology. 
matic attack and degradation. 
skins as lignin-rich biomass materials with respect to enzymatic attack and degradation.

Quantitative Calculation of Predicted Polysaccharide Profiles: Methodology. During the development of the iterative calculation method to quantitatively predict the cell wall polysaccharides and, implicitly, the bonds building the structural elements from monosaccharide profiles, we first allocated monosaccharides to a relatively simple and well-defined, homopolymeric molecular structure, namely, mannan (Figure 1). In grape pericarp, mannan is basically composed of chains of mannose, linear chains made up of $\beta$-1,4-linked mannose units. Mannose is not a constituent of any other plant cell wall polysaccharides when it is assumed that mannose is not a side-chain substituent of rhamnogalacturonan I (RG-I) (29). Then, the galacturonic acid residues were distributed among the three main pectin structural units: HG, made up of a backbone of $\alpha$-1,4-linked galacturonic acid residues; RG-I, made up of a backbone of alternating $\alpha$-1,2-linked rhamnose and $\alpha$-1,4-linked galacturonic acid residues (the rhamnose residues may be substituted with neutral side chains of galactan, arabinan and/or different arabinogalactan side chains); and rhamnogalacturonan II (RG-II), which consists of a backbone of HG with clusters of four different side chains containing apiose, aceric acid, 2-O-methylfucose, 3-deoxy-lyxo-2-heptulosonic acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO). The distribution between the three main structural pectin units was done by keeping in mind a relative molar ratio of HG:RG-I:RG-II in grape skins of approximately 16:3:1 (80:15:5) (11). To distribute the galacturonic acid as correctly as possible, we found it expedient to first reconstruct RG-II, the least abundant of the structural pectin units. This was done by using all of the available fucose monomers and then allocating the required galacturonic acid in a fucose:galacturonic acid ratio of 1:6 (Figure 1). This ratio was determined from the available data for RG-II structures in dicots (30, 31). Fucose is uniquely present in the side chain of RG-II (31), which is why the quantitative prediction of RG-II could be based on allocating the total fucose to RG-II and then allocating the required galacturonic acid proportionally to the fucose level (Figure 1). The total level of fucose thus established the corresponding consumption of galacturonic acid to RG-II as well as the predicted molar amount of RG-II. Other unique constituents of RG-II such as apiose, DHA, and KDO were not detected in the hydrolysates by our HPAEC-PAD method. By first allocating galacturonic acid to RG-II, that is, to the minor pectin unit, any marginal differences between the available galacturonic acid monomers and the correct distribution among the different pectin elements would have less impact on the subsequent galacturonic acid allocation to HG and RG-I, whereas if the distribution of the galacturonic acid to the major pectin components first is accomplished, the potential relative error on the estimated amounts of the minor component, in this case RG-II, would be maximized.

Table 2. Previously Reported Monosaccharide Compositions of Grape Fruit Skins

<table>
<thead>
<tr>
<th>grape variety</th>
<th>hydrolysis method</th>
<th>$\mu$g $\mu$g $\mu$g $\mu$g $\mu$g $\mu$g $\mu$g</th>
<th>sum of total monosaccharides (mg $^{-1}$)</th>
<th>Klaion lignin (mg $^{-1}$)</th>
<th>sum of Klaion lignin and total monosaccharides (mg $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grenache Noir</td>
<td>2 M TFA, 121 °C, 1 h/Saeman procedure $^a$</td>
<td>2$^b$ 10$^{c}$ 42$^{d}$ 20$^{e}$ 189$^{f}$ 22$^{g}$ 20$^{h}$ 195</td>
<td>491</td>
<td>ND$^i$</td>
<td>ND$^j$</td>
</tr>
<tr>
<td>Carignan</td>
<td>extraction of CIW by HEPES + treatment with glycollic hydroses</td>
<td>2.9 4.7 32.2 17.6 168.0$^{k}$ 19.4$^{k}$ 17.0$^{k}$ 224.0</td>
<td>485.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Red Globe</td>
<td>Saeman procedure and 1 M H$_2$SO$_4$, 100 °C, 2.5 h or 1 M H$_2$SO$_4$, 100 °C, 2.5 h on AIRs$^k$</td>
<td>7.3 13.4 59.4 34.2 285.7 31.4 32.4$^a$</td>
<td>227.4</td>
<td>692.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Cell wall material (CWM) (mg $^{-1}$) was obtained by Selvendran's method (10). $^b$ By TFA hydrolysis. $^c$ By Saeman hydrolysis. $^d$ ND, not determined. $^e$ AIRs, alcohol insoluble residues (21).

Table 3. Analyzed Monosaccharide Composition of Apple Fruit Skins

<table>
<thead>
<tr>
<th>apple variety</th>
<th>hydrolysis method</th>
<th>$\mu$g $\mu$g $\mu$g $\mu$g $\mu$g $\mu$g</th>
<th>sum of total monosaccharides (mg $^{-1}$)</th>
<th>Klaion lignin (mg $^{-1}$)</th>
<th>sum of Klaion lignin and total monosaccharides (mg $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Delicious</td>
<td>TFA</td>
<td>2.9 18.5 92.7 32.0 95.1 25.3 0.90 456.8</td>
<td>724.2</td>
<td>386</td>
<td>1110</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>0.6 5.2 14.6 11.7 49.0 1.90 5.50 21.10</td>
<td>109.6</td>
<td>386</td>
<td>496</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>TFA</td>
<td>3.3 11.8 56.9 23.2 90.6 22.1 1.5 348.4</td>
<td>557.8</td>
<td>344</td>
<td>902</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>0.9 4.0 11.2 8.8 47.1 1.6 6.0 15.9</td>
<td>95.5</td>
<td>344</td>
<td>440</td>
</tr>
</tbody>
</table>
In the next step, the rhamnose was used to reconstruct HG and RG-I. This was done by first setting the rhamnose distribution ratio between HG and RG-I to 1:6.7 (13:87%) and then distributing galacturonic acid to all of the available rhamnose by using ratios of rhamnose:galacturonic acid of 1:135 for the HG unit and 1:1 for RG-I (Figure 1). These latter ratios were defined from the available data for pectinaceous polymers in cell wall materials of dicots (16, 26, 32, 33). The influence on the predicted profile of structural pectin units of employing different ratios for rhamnose:galacturonic acid between HG and RG-I and within the slightly rhamnose-interrupted HG will be discussed below.

To our knowledge there are no literature reports on the quantitative or qualitative occurrence of xylogalacturonans in grape and apple skins, and the literature data on xylogalacturonan in dicots do not imply that xylogalacturonan is significantly abundant when present (34, 35). Hence, for the calculations we presumed that xylogalacturonan might constitute only a negligible fraction of the pectin in grape and apple skins—if present at all. Consequently, we decided to allocate all of the analyzed xylose to the xyloglucan in the fruit skins and in turn calculated that any surplus galacturonic acid—not assigned to RG-I, RG-II, or HG according to the calculations outlined above—was part of a homopolymeric HG structural unit composed of galacturonic acid only (Figure 1).

Reconstruction of xyloglucan was based on assuming a xyloglucan structure composed of a glucan backbone made up of β-1,4-bonded glucose units with intermittent substitutions at C6 with single α-1,6-xylopyranosyl residues or with disaccharide β-galactose(1→2)-α-1,6-xylopyranosyl substitutions and a set ratio between xylose, glucose, and galactose of 1:7:4:1.2 (12) (Figure 1). We opted for this ratio of glucose relative to xylose instead of the ratio suggested for an acidic xyloglucan fraction found previously in grape skins, which was proposed to be a linear β-1,4 xylose—glucose backbone structure in hemicellulose A-I in the grape skins with a molar xylose:glucose ratio of 1:0.1 (15). The rest of the glucose was then allocated to cellulose, and arabinogalactan was reconstructed from galactose and arabinose using a galactose:arabinose ratio of 1.5:1 (32) (Figure 1). Finally, the eventual “surplus” of arabinose was allocated as a pure arabinan.
Predicted Polysaccharide Profiles from Monosaccharide Analyses. Grapes. For the skin samples from different red wine grape varieties the iterative calculation method resulted in a predicted relative molar profile (mol %) of polysaccharides in the skins of red wine grapes of 55–61 mol % homogalacturonan, 1–10 mol % cellulose [the true levels may rather be in the range of ~5–10 mol %, as the predicted cellulose levels in the Merlot skin samples were very low (Table 4)], 16–20 mol % xyloglucan, ~7 mol % arabinan, ~5 mol % rhamnogalacturonan II, 3.5–4.0 mol % rhamnogalacturonan I, ~0.3 mol % arabinogalactan, and 0.5–1.0 mol % mannan; the ranges indicate minor variations among the three different grape cultivars (Table 4). The data thus indicated that homogalacturonan and xyloglucan were by far the most dominant polysaccharides of the grape skin cell wall polysaccharides. This comprehension is consistent with previous data that have elucidated the structures and tissue distribution of pectic polysaccharides in grapes (11) (Table 5).

The defined ratio for the fucose:galacturonic acid in RG-II, and in turn the distribution of rhamnogalacturonan in slightly rhamnose-interrupted HG, obviously determined the predicted relative molar levels of HG and RG-I. In this way the rhamnose monomer level in effect defined the relative levels of HG and RG-I. A scenario analysis of the consequences of changing the rhamnose distribution in RG-I HG from, for example, 80:20 to 87:13 for Merlot grape skins, while keeping the rhamnogalacturonan acid set at 1:60 in the slightly rhamnose-interrupted HG, clearly illustrated how such a change in the RG-I HG ratio resulted in a decrease in the relative percentage level of the slightly rhamnose-interrupted HG and concomitantly increased the relative molar percent levels of RG-I and RG-II, whereas the surplus galacturonic acid to be allocated in smooth homogalacturonan went up (Table 6). Oppositely, when the rhamnose:galacturonic acid ratio in the HG was changed from 1:60 to 1:135, the estimated relative level of this HG went up, whereas RG-I, RG-II, and surplus galacturonic acid for smooth, homopolymeric HG all went down (Table 6)—all as a result of the rhamnose being the limiting monomer. The final fit of the galacturonic acid/fucose ratio in RG-II, the rhamnose distribution between RG-I and HG, and the ratio of rhamnogalacturonan HG to HG was determined by iteration to result in the minimal amount of “surplus” galacturonic acid (Table 6). The choice of a low rhamnose:galacturonic acid ratio is of course, in effect, almost the same as leaving more surplus galacturonic acid to be distributed in smooth, homopolymeric galacturonic acid, but the relative distribution among HG, RG-I, and RG-II will vary (Table 6). In grape mesocarp cells, that is, grape pulp cell wall material, cellulose has previously been reported to make up ~30 mol % of the cell wall polysaccharides (29); in this light, the here estimated relative levels of cellulose seemed low (Tables 4 and 5). The lower hydrolysis efficiency of TFA toward cellulose relative to the efficiency of H2SO4 as used in the Saemann procedure (36) could be a factor explaining this difference. Alternatively, the lack of washing of the skins prior to acid hydrolysis in other studies might have contributed to artifacts causing higher analyzed glucose levels and in turn, perhaps, relatively high cellulose estimates.

Table 4. Theoretical Percentage of Major Polysaccharides in Lyophilized Grape Skin Cell Walls

<table>
<thead>
<tr>
<th>grape variety</th>
<th>hydrolysis method</th>
<th>mannan</th>
<th>HG</th>
<th>RG-I</th>
<th>RG-II</th>
<th>xyloglucan</th>
<th>arabinogalactan</th>
<th>arabinan</th>
<th>surplus GalA* balanced as HG</th>
<th>cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabernet Sauvignon</td>
<td>TFA</td>
<td>0.9</td>
<td>54.9</td>
<td>5.0</td>
<td>3.8</td>
<td>18.9</td>
<td>0.3</td>
<td>7.5</td>
<td>3.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>7.4</td>
<td>104.7</td>
<td>9.5</td>
<td>9.9</td>
<td>9.0</td>
<td>11.4</td>
<td>4.9</td>
<td>-102.4</td>
<td>45.7</td>
</tr>
<tr>
<td>Merlot</td>
<td>TFA</td>
<td>1.0</td>
<td>60.0</td>
<td>5.4</td>
<td>4.0</td>
<td>19.7</td>
<td>0.2</td>
<td>7.3</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>11.5</td>
<td>101.0</td>
<td>9.1</td>
<td>7.9</td>
<td>12.8</td>
<td>11.5</td>
<td>6.4</td>
<td>-96.9</td>
<td>36.6</td>
</tr>
<tr>
<td>Shiraz</td>
<td>TFA</td>
<td>0.5</td>
<td>60.8</td>
<td>5.5</td>
<td>3.5</td>
<td>16.4</td>
<td>0.3</td>
<td>6.7</td>
<td>-3.8</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>HCl</td>
<td>8.7</td>
<td>164.8</td>
<td>14.9</td>
<td>5.4</td>
<td>8.9</td>
<td>7.3</td>
<td>4.7</td>
<td>-162.9</td>
<td>52.3</td>
</tr>
</tbody>
</table>

*GalA, galacturonic acid.

Table 5. Previously Reported Percentages of Major Polysaccharides in (Dried) Grape Skin Cell Wall Material

<table>
<thead>
<tr>
<th>grape variety</th>
<th>hydrolysis method</th>
<th>mannan</th>
<th>HG</th>
<th>RG-I</th>
<th>RG-II</th>
<th>xyloglucan</th>
<th>arabinogalactan</th>
<th>arabinan</th>
<th>surplus GalA* balanced as HG</th>
<th>cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carignan Noir (10)</td>
<td>2 M TFA, 121 °C, 1 h; or Saemann procedure*</td>
<td>4.1</td>
<td>43.0</td>
<td>3.9</td>
<td>3.3</td>
<td>21.6</td>
<td>-0.6</td>
<td>8.8</td>
<td>-8.0</td>
<td>24.0</td>
</tr>
<tr>
<td>Grenache Blanc (11)</td>
<td>buffer extraction of CWM + glycosyl hydrolase treatment</td>
<td>3.5</td>
<td>20.4</td>
<td>1.8</td>
<td>4.9</td>
<td>19.2</td>
<td>-0.5</td>
<td>6.8</td>
<td>20.5</td>
<td>23.3</td>
</tr>
<tr>
<td>Red Globe (23)</td>
<td>5 μg mg⁻¹ of AII; Saemann procedure: 1 M H2SO4, 100 °C, 2.5 h; or 1 M H2SO4, 100 °C, 2.5 h</td>
<td>4.7</td>
<td>41.2</td>
<td>3.7</td>
<td>8.7</td>
<td>22.0</td>
<td>0.7</td>
<td>8.4</td>
<td>-17.5</td>
<td>28.0</td>
</tr>
</tbody>
</table>

*GalA, galacturonic acid.
Apples. The calculated relative molar distributions of the polysaccharides in the apple skins (Table 7) appeared to be similar to those obtained for the grape skins. However, in agreement with available knowledge on apple cell wall pectins, the apple skins were predicted to be relatively richer in arabinan (10–13 mol %) and to have a relatively lower mannann content than grape skins (0.1–0.3 mol %) (Table 7). The cellulose levels of 3.2–5 mol % in the apple skins were slightly lower than the cellulose levels of 5.3–10 mol % in the grape skins except for the level in Merlot (Tables 5 and 7).

Significance of the Acid Hydrolysis Method. TFA was consistently more efficient than HCl in hydrolyzing the pectin fraction in both grape and apple fruit skins (Tables 1 and 3). This finding was in accordance with previous data for other plant materials (37, 38) and resulted in the prediction of relatively abundant amounts of pectin, HG, RG-I, or RG-II, in the fruit skins and availability of galacturonic acid levels to match the presented ratio distributions. For the TFA hydrolysates, there was even a galacturonic acid “surplus”, which we calculated to be part of extended chains of HG made up exclusively of galacturonic acid (Figure 1 and Table 6). In contrast, after HCl hydrolysis, the deficit in “required” galacturonic acid for the structural pectin elements exceeded more than 100% of grape skin samples and ≈82–88% in the apple skins samples (Tables 5 and 7). These differences in the levels of galacturonic acid reflected the strong influence of the acid hydrolysis method on the quantitative prediction of pectinaceous structures. In other words, the acid hydrolysis method has a strong influence on the comprehension of the different structural elements in plant cell wall matrix and, in this particular case, especially with respect to predicting the levels of the different pectinaceous polysaccharides.

In dicotyledonous plants, the plant cell walls are mainly made up of pectin polysaccharides, xyloglucan, cellulose, mannann, lignin, and glycoproteins (29). In dicots, including grapes and apples, the structural elements of pectin are known to be mainly made up of three structural units: homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II)—with HG and RG-I generally being the quantitatively most dominant (39). Xylogalacturonan is a fourth structural unit of pectin, which has been identified in cell wall extracts from, for example, soybean and watermelon (34, 35), and which was recently presumed to be present in modified hairy regions of apple pectin, as a xylogalacturonan–RG-I linkage [the oligomer structure GalA(Rha)Xyl; was proposed (40)]. However, no indications of the quantitative levels of xylogalacturonan have been given in any of these papers. Because xylogalacturonan has only been rarely identified, we have presumed that xylogalacturonan might only make up a negligible amount of the plant cell wall material in the here studied fruit skin samples.

Homogalacturonans are helical homopolymers of α-1,4-linked galacturonic acid monomers, which may be methyl-esterified and/or acetylated. Single α-1,2-linked rhamnose residues may interrupt the long homogalacturonan chains, resulting in a bend, a “pectic elbow”, in the polygalacturonic acid structure (1), but the galacturonic acid:rhamnose levels vary in different plant materials; in apple cell wall materials a typical ratio is 100 galacturonic acids to 1 rhamnose (41). The backbone structure of RG-I is a heteropolymer consisting of an array of repeating disaccharides of alternating α-1,2-linked rhamnose and arabinose in the side chains of RG-I of grape pulp cell walls is 1.5:1 and that minor amounts of mannann and glucose might also be present (42). However, the presence of mannann and glucose in RG-I structures is not generally recognized, which is why in the present work we decided not to include mannann and glucose when reconstructing the levels of RG-I from the cell wall monomers. Despite its name, RG-II has a homogalacturonan (HG) backbone rather than one of alternating galacturonic acid and rhamnose (as in RG-I), and RG-II uniquely has complex side chains attached to the galacturonic acid residues (39). RG-II acts as bridge to covalently cross-link two chains of HG in the cell walls of dicots and predominantly exists as a dimer.
(30) that makes up ~5% of the weight of buffer-soluble grape mesocarp polysaccharides (16). The RG-II-HG network supports and stabilizes the cellulose microfibrils network that appears to be non-covalently bound to xyloglucan (39). Our quantitative data for the structural elements of pectin in the grape and apple skins are in full agreement with the recognition that HG typically accounts for about 80% by weight of the pectin (17), whereas RG-I and RG-II together account for about 10% by weight of total grape skin material (11).

Conclusions. The data obtained demonstrated that it was possible to quantitatively predict the abundance of structural polysaccharide units in fruit skins from monosaccharide profiles obtained by acid hydrolysis. For both the grape and apple skin samples, including three different wine grape cultivars and two different apple cultivars, the skin cell wall polysaccharides matrix appeared to be mainly made up of pectins presented mainly by homogalacturonans, rhamnogalacturonan I, and rhamnogalacturonan II, then xyloglucan and cellulose. The hydrolysis method, and thus in turn the resulting monosaccharide profiles, strongly affected the prediction and the reconstruction of the cell wall polysaccharides, notably with respect to the estimated levels of the different pectin structures versus cellulose. At this point in time no methods are available to examine the intact plant cell wall polysaccharides present in the true cell wall matrix, and the compositional analyses available thus only provide a picture of the building blocks—and the analytical, quantitative determination of the cell wall polysaccharides in fibrous, lignified plant materials such as fruit skins is complicated, if not impossible, to obtain with the currently available methods. The presented iterative calculation method may provide an important starting point for obtaining a better quantitative understanding of the polysaccharide structures in fibrous cell wall matrices. This may be useful for rationally designing enzymatic treatments, that is, selecting the relevant enzymes and estimating the expected yields, for obtaining maximal cell wall degradation in prepress treatments in fruit juice and wine processes. In addition, the quantitative prediction approach may provide a primary tool for designing efficient valorization of the skins in fruit juice and wine process. In addition, the quantitative prediction and the reconstruction of the cell wall polysaccharides matrix appeared to be mainly made up of pectins presented mainly by homogalacturonans, rhamnogalacturonan I, and rhamnogalacturonan II, then xyloglucan and cellulose. The hydrolysis method, and thus in turn the resulting monosaccharide profiles, strongly affected the prediction and the reconstruction of the cell wall polysaccharides, notably with respect to the estimated levels of the different pectin structures versus cellulose. At this point in time no methods are available to examine the intact plant cell wall polysaccharides present in the true cell wall matrix, and the compositional analyses available thus only provide a picture of the building blocks—and the analytical, quantitative determination of the cell wall polysaccharides in fibrous, lignified plant materials such as fruit skins is complicated, if not impossible, to obtain with the currently available methods. The presented iterative calculation method may provide an important starting point for obtaining a better quantitative understanding of the polysaccharide structures in fibrous cell wall matrices. This may be useful for rationally designing enzymatic treatments, that is, selecting the relevant enzymes and estimating the expected yields, for obtaining maximal cell wall degradation in prepress treatments in fruit juice and wine processes. In addition, the quantitative prediction approach may provide a primary tool for designing efficient valorization of the skins in fruit juice and wine process.

A B B R I E V I A T I O N S  U S E D
GD, Golden Delicious apple; HCl, hydrochloric acid; HG, homogalacturonan; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; RD, Red Delicious apple; RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT
The Distell Group Ltd. (Stellenbosch, South Africa) is acknowledged for supplementation of the wine grape samples.

L I T E R A T U R E  C I T E D


Chapter 4

Discriminated release of phenolic substances from red wine grape skin by multi component enzyme treatment

This chapter is extended in the form of a submitted article:

Discriminated release of phenolic substances from red wine grape skins (Vitis vinifera L.) by multi component enzyme treatment.

 Arnous, A. & Meyer, A. S.


4.1 Key points

This study was undertaken to examine in detail the evolution of phenolics release and cell wall carbohydrate degradation during extensive enzymatic degradation of grape skins under treatment with multicomponent pectolytic or cellulolytic enzyme preparations. We particularly wanted to assess if the release of certain phenolics is associated with the degradation of certain polysaccharide moieties in the grape skins and/or if the extraction of phenolics from grape skins is influenced by other factors.

Besides employing classical, extended enzymatic treatment the utilising of statically designed experiments turns to be of a great help to asses the significance and the interaction of different reaction parameters. The use of the kinetic approach is practically not possible to study the hydrolysis of complex substrates like plant cell walls by multi-enzyme preparation. Instead a statistical approach; full factorial setup was applied. The full factorial setup has the advantage of limiting the number of required experiments and examining rationally the influence of different reaction parameters (Fig 4.1). As phenolics in general are sensitive to elevated temperatures, which are usually used to stop enzymatic reactions, we had to use a different strategy. as an alternative the enzymatic treatments were terminated by adding high levels of an organic solvent as was suggested by Harborne (2003) (Fig 4.2).
Figure 4.1 Full factorial setup development by examining rationally the influence of reaction parameters including enzyme to substrate ratio (1, 5.5, or 10%), time (2, 4, or 6 h), and temperature (25, 40, or 55 °C) and relating the finding to surface response as the final step.
4.2 Conclusion

The results showed different release rates for different classes of phenolics but in general it was a function of polysaccharides cell wall degradation. Anthocyanins were instantly released from the skin during the early phase of the enzymatic treatments, and then degraded during further enzymatic treatment due to temperature as the main factor. Release of anthocyanins and flavanols did not correlate to monosaccharide production, and was thus not directly related to enzyme catalyzed degradation of the grape skin cell wall polysaccharides. Still it as interesting that flavonols underwent transformation from glycosylated (rutin) to deglycosylated (quercetin) during the enzymatic treatment (Fig. 4.3).
For phenolic acids, including hydroxybenzoic acids and hydroxycinnamic acids, their liberation was exponentially correlated to the liberation of galacturonic acid and arabinose at the same time. The liberation of especially hydroxycinnamates (notably $p$-coumaric acid) could be a result of release from acylated anthocyanins.

In general certain classes of phenolics in grape skin, anthocyanins and flavonols, are not in bound form with grape skin polysaccharide matrix. Never the less we would expect that degradation of grape skin cell wall will open up the cell and eliminate the physical barrier to leach anthocyanins and flavonols, and hence facilitate its extraction. On the other hand, the strong relation between the release/liberation of hydroxybenzoic acids and hydroxycinnamic acids, and the extensive degradation of pectin might indicate anthocyanins and flavonols have some relationship in bound form with the cell wall polysaccharide matrix.

**Figure 4.3** HPLC chromatogram recorded at 365 nm showing the transformation of glycosylated flavanol (rutin) to deglycosylated flavanol (quercetin) during the enzymatic treatment with Pectinex® BE Colour enzymatic preparation: a) at 0 hours b) after 2 hours, c) after 6 hours.
Chapter 4

The increase of quercetin and phenolic acids level under enzymatic treatment is an indication of the possibility to enhance antioxidant potential of grape juice or wine. Quercetin and its glycosides have been reported to exert numerous biochemical and pharmacological activities, such as free radical scavenging and anticarcinogenic effects (Andlauer et al., 2000; de Vries et al., 1998; Crespy et al., 1999). Moreover, both phenolic acids and flavanols were reported to as strong co-pigmentation agents in red wine (Boulton, 2001).

To conclude, these data signify the possibility for developing discriminated release of phenolics during differentiated enzyme catalysed plant cell wall degradation. Furthermore, the data shows that the ability to enzymatically modify the phenolics to optimize their health potentials is a realistic goal.
Discriminated release of phenolic substances from red wine grape skins (Vitis vinifera L.) by multicomponent enzymes treatment

Anis Arnous, Anne S. Meyer∗

Center for BioProcess Engineering, Department of Chemical and Biochemical Engineering, Building 229, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

ARTICLE INFO

Article history:
Received 2 June 2009
Received in revised form
12 September 2009
Accepted 22 November 2009

Keywords:
Phenols
Enzymatic extraction
Plants cell walls
Pectinase
Anthocyanins
Phenolic acids
Flavonoids

ABSTRACT

Detailed insight into the effects of enzymatic treatments on grape phenolics is of significant importance for grape processing for wine making. This study examined the release of phenols during enzymatic (pectinolytic and cellulolytic) degradation of the cell wall polysaccharides in skins of Merlot and Cabernet Sauvignon wine grapes (Vitis vinifera L.). Anthocyanins were released from skins during the early phases of the enzymatic treatments, but were then degraded during further enzymatic treatment; flavonols underwent transformation from glycosylated (rutin) to diglycosylated (quercetin) during the enzymatic treatment; phenolic acids, including hydroxybenzoic acids and hydroxycinnamic acids, were released as a function of monosaccharides liberation, i.e. as a function of the enzyme catalyzed cell wall degradation of the skins, and with some of the phenolic acids perhaps released from the lignin. The data moreover suggest that p-coumaric acid was also released during enzyme catalyzed degradation of acylated anthocyanins, probably as a result of cinnamate esterase activity. The data thus provided unexpected new clues as to how the enzymatic treatment with multicomponent pectinolytic enzymes may promote (a) discriminated release of phenols from grape skins, and (b) molecular changes in the phenols.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

In both red wine and fruit juice processing the use of enzymes is now expanding into various more targeted applications beyond the classic press yield and clarification purposes [1]. One line of these newer enzyme applications concerns the more aggressive use of multi-component pectinolytic enzyme preparations to enhance the extraction of coloured anthocyanin pigments and other desirable phenolics into the juice or red wine during pre-press maceration [1–6]. The concept of extensive enzyme catalyzed maceration to increase liberation of phenols is also being pursued in the upgrading of wine pomace and fruit juice press residues [7–9].

Previous results in these applications have shown increased phenols release with enzyme dosage [2,6,10,11]. Moreover, a strong, linear correlation has been found between the release of total phenols and the extent of plant cell wall carbohydrate degradation in the fruit skins – and as a function of increased enzyme dosage, elevated temperature (up to 60–65 °C), and extended enzymatic treatment time [2,8,10,11]. The available data thus suggest that the enzyme catalyzed release of phenolics from grape and berry skins is a result of a random liberation of phenols from the fruit skin cell wall matrix in response to the progressive enzyme catalyzed degradation of the cell wall polysaccharides [2,9,12].

The chemical nature of the phenolic substances in grapes has been amply studied, but the way the phenols may be integrated or bound in the cell wall matrix of grape skins has only been tentatively proposed in a few reports [9,13]. Certain of the phenolic acids, mainly the hydroxycinnamates ferulic acid and p-coumaric acid, are presumably esterified to the galactose and arabinose moieties in the pectin rhamnogalacturonan I side chains [14,15]. A portion of the tannins present in grape skins has also been proposed bound to the cell wall polysaccharides by glycosidic bonds [16]. The flavonols and hydroxybenzoic acids have been suggested to be integrated in the grape skin cell wall polysaccharides via hydrophobic interactions and/or hydrogen bonds [8,9]. The anthocyanins may be bound in insoluble phenol–protein–polysaccharide complexes as suggested for anthocyanins in flower petals [17], but the main portion of the anthocyanins, as well as some tannins, have long been believed to be located inside cell vacuoles that are surrounded by a proteinaceous membrane [3,9,13,16]. Microscopy of grape skins have corroborated that anthocyanins can be located within vacuolar inclusions named anthocyanoplast bodies in the epidermal and subepidermal cells of grape skins [18]. The easiness of anthocyanins extractability appears to be affected by the density of the cell wall polysaccharide matrix, so the cell wall apparently acts as a protective shield or barrier for extraction of these anthocyanins [3,19].
2. Materials and methods

2.1. Grape skin preparation

The grape varieties Cabernet Sauvignon (Cs.) and Merlot (Mt.) were selected as two representatives of red wine grapes (Vitis vinifera L.). The grapes were obtained from the Distell Group Ltd. (Stellenbosch, South Africa). The grapes had been picked manually using a scalpel. The skins were washed with cold, distilled water at 4 ◦C, and lyophilised immediately by use of a Lyovac GT 2 freeze-drier (Leybold-Haraeus, Germany) and then milled for 30 s under −30 ◦C until use. After gentle thawing, the grape skin particles of 125–250 µm were collected using sieves tower, nominal aperture sizes of 500, 250 and 125 µm, respectively (Endecotts Ltd., London, UK). The grape skin particles were kept in tightly closed glass jars under N2 at −20 ◦C until use.

2.2. Enzyme preparations

The three enzyme preparations, Pectinex® BE Colour, Vinozym® FCE G, and Celluclast® 1.5 L FG were obtained from Novozymes A/S (Bagsværd, Denmark). Pectinex® BE Colour and Vinozym® FCE G are pectinolytic enzymes that are already used in the juice and/or wine industry. Celluclast® 1.5 L FG is a cellulolytic enzyme employed for hydrolysis of cellulose and hemicellulose in food processing, and currently promoted for liquefaction of lignocellulosic feedstocks. A summary of the available information regarding the enzyme activities is given in Table 1.

2.3. Enzymatic hydrolysis procedure

Enzymatic hydrolysis of lyophilised grape skin samples was carried out in thermostatically controlled shaking water baths (JULABO SW-20C, Germany) with gentle agitation (150 rpm) in accordance with the reaction time and temperature given in the experimental plan (see below). A sample of 20 mg lyophilised grape skin was hydrolysed in 200 µL acetate buffer (0.2 M) pH: 3.6, and the relevant enzyme preparation was added at the enzyme/substrate ratio % volume/weight (E/S %) calculated on dry matter base required according to the experimental plan. At the end of each experiment 400 µL, equivalent to 50:50 vol/vol of ethanol (100%) and acetate buffer (0.2 M, pH: 3.6), were added to halt the enzymatic reaction. The sample was vortexed for 30 s. Following centrifugation at 19,280 × g for 3 min at 4 ◦C, the supernatant was decanted into a 1 mL vial and kept under N2 at 4 ◦C until the time of analyses.

2.3.1. Extended enzymatic treatment

Prolonged enzymatic hydrolysis experiments were run in duplicate using 5.5% E/S, at 40 ◦C, for 0, 2, 4, 6, 12, 18, or 24 h.

2.3.2. Full factorial experimental design

A full factorial two levels design with three center-points (orthogonal balanced design with all combinations of the factor levels) was run to evaluate the influence of three different reaction factors: temperature (25–55 ◦C), enzyme dose (1–10% E/S), and time (2–6 h). Experiments without enzyme addition were made separately. The individual and interactive effects of the different enzymatic treatments were evaluated by multiple linear regression analysis.

2.4. Analytical procedures

Each (enzymatic hydrolysis) sample supernatant was analysed for total phenols, total anthocyanins, quantitative phenolics profile, and monosaccharides profile. The yields are reported as amount of material per mg dry matter starting material, µg mg−1 lyophilised skins, whereas release is used as a term to describe the transfer.

2.4.1. Total phenols

The total phenols were determined by the Folin–Ciocalteu method [20]. The total phenol concentration was calculated from a calibration curve using gallic acid as standard. Results were expressed as µg gallic acid equivalents (GAE) per mg of lyophilised grape skin.

2.4.2. Phenolic profile analysis

The phenolics in the sample supernatants (10 µL) were analysed using an HPLC system equipped with a diode array detector (DAD) (Hewlett-Packard 1100, Waldbronn, Germany) and a Nova-Pak C18
Fig. 1. Monosaccharides yield μg g⁻¹ of lyophilised skins for Cabernet Sauvignon (Cs.) and Merlot (Mt.) grapes under the extended enzymatic treatment conditions by different enzymes: (a) Cs., Celluclast® 1.5 L FG; (b) Mt., Celluclast® 1.5 L FG; (c) Cs., Pectinex® BE Colour enzyme; (d) Mt., Pectinex® BE Colour enzyme; (e) Cs., Vinozym® FCE G; (f) Mt., Vinozym® FCE G. Monosaccharides: xylose (△), mannose (▽), rhamnose (⃝), galactose (□), glucose (/H1703), arabinose (▲), galacturonic acid (■).

column (3.9 mm × 150 mm, Waters) held at 40 °C. The mobile phase was made of three solvents delivered in a gradient at a flow rate of 0.5 mL/min essentially as described by Lamuela-Raventos et al. [21]. The operation of the HPLC-DAD and the spectral match analysis was done by use of HP ChemStation B.01.01 software (Agilent Technologies Inc, USA). Identification and quantification of the phenolic compounds were carried out using an in-house UV/vis spectrum library of pure phenolic compounds [22]. The library match factor, which is the result of the automated library search, was set to minimum 99% as a requirement to confirm the identity of the peaks. Quantification of the phenolic compounds was performed using external standards. Phenolics yields were expressed as μg per mg of lyophilised grape skin. The average coefficient of variation for each of the detected phenols was: gallic acid 0.7%, protocatechuic acid 1.6%, 2,4,6-trihydroxybenzoic acid 2.2%, vanillic acid 7.8%, caffeic acid 4.8%, syringic acid 2.4%, p-coumaric acid 6.8%, delphinidin-3-glucoside (dp-3-glc) 4.3%, cyanidin-3-glucoside (cy-3-glc) 4.3%, petunidin3-glucoside (pt-3-glc) 4.4%, peonidin-3-glucose (pn-3-glc) 4.6%, malvidin-3-glucoside (mv-3-glc) 4.1%, rutin 3.0%, myricetin 5.6%, quercetin 4.7%, kaempferol 5.4%, total monomeric anthocyanins 7.5%, flavonols 6.6%, hydroxycinnamic acids 3.5%, hydroxybenzoic acids 7.0%.

2.4.3. Monosaccharide analysis by HPAEC–PAD
Separation and quantification of monosaccharides in hydrolysates were performed by use of a BioLC system, equipped with a CarboPac™ PA20 (3 mm × 150 mm) analytical column, an ED50 electrochemical detector, and controlled via the Chromeleon 6.80 SP4 Build 2361 software ( Dionex Corp., Sunnyvale, USA) as reported previously [23]. Monosaccharides were expressed
as μg mg^{-1} of lyophilised grape skin. The average coefficient of variation for each of the monosaccharides was: D(+)-glucose 1.4%, L-ramnose 0.5%, D(-)-arabinose 3.5%, D(+)-galactose 2.9%, D(+)-galacturonic acid 4.4%, D(+)-xylose 1.3%, D(-)-mannose 1.0%, and D(-)-fructose 4.4%, D(+)-galacturonic acid 6.2%.

2.5 Statistical analysis

For the extended enzymatic treatments all measurements were done in duplicate. Quadratic curve fit through the origin was used for monosaccharide standards analysed by HPAEC-PAD, and the calibration points were weighted by the factor 1/Response². For phenolic compounds by HPLC-DAD the curve fit consisted of a linear least squares fit through the data points including the origin. Linear regression, calculation of coefficient of variation, design of the experimental templates, as well as the multiple linear regression calculations of the individual and interactive effects of the different enzymatic treatments were done with the aid of JMP 7.0.2 software (SAS Institute Inc., Cary, NC, USA). Significance of all the results was established at P < 0.05.

3. Results

3.1. Grape skin cell wall polysaccharides degradation with different enzymatic treatments

The monosaccharide releases, and hence the extent of cell wall degradation, were generally lower with Celluclast® 1.5 L treatment than with the pectinolytic enzyme treatments Pectinex® BE Colour and Vinozyme® FCE G (Fig. 1). This was evident for both the Cabernet Sauvignon (Cs.) and Merlot (Mt.) grape skins (Fig. 1). The more detailed comparison of the monosaccharides profiles of the grape skins samples showed that the Cs. and Mt. grape skin samples gave relatively similar monosaccharide release patterns when treated with the same enzyme preparation, but also showed some distinct differences: the levels of monosaccharides released from the Cs. grape skins were consistently higher than those released from the Mt. grape skins (Fig. 1). These findings are in good accordance with previous data [23,24] and indicate that Mt. grape skin cell walls may have a more firm skin cell wall structure than Cs. grape skins, making the Mt. cell wall polysaccharides less accessible to enzymatic attack. The only exception was for the Celluclast® 1.5 L FG treatment, where the glucose levels in the supernatants were higher for the Mt. grape skins than for the Cs. grape skins. This resulted in the total monosaccharides release values, mainly contributed by glucose, reached ∼70 μg mg^{-1} for the Mt. grape skins whereas it was ∼45 μg mg^{-1} for the Cs. grape skins (calculated from the data shown in Fig. 1a and b). For the Pectinex® BE Colour and Vinozyme® FCE G treated samples the galacturonic acid consistently increased the most during the enzymatic treatments. Although some galacturonic acid was released even during Celluclast® 1.5 L FG treatment, particularly from the Cs. grape skins samples, the maximum galacturonic acid levels obtained with Celluclast® 1.5 L FG treatment were in the order of 20 times lower than those obtained with the pectinolytic enzyme treatments (Fig. 1). This result was not surprising considering the enzymatic activities of the preparations (Table 1). The Pectinex® BE Colour preparation catalyzed the release of 100 and 85 μg mg^{-1} galacturonic acid from Cs. and Mt., respectively (Fig. 1). These values corresponded to approximately 1/3 and 1/4, respectively, of the D(+)-galacturonic acid liberated using TFA chemical hydrolysis for the same grape skin cultivars [23] and affirmed, that pectin, mainly homogalacturonic acid, was the most dominant structural carbohydrate unit in the grape skins [24]. The Pectinex® BE Colour preparation apparently had higher activity than the Vinozyme® FCE G preparation towards pectinaceous structures on both Cs. and Mt. grape skins. This could be explained by the presence of pectin-lyase activity in the Pectinex® BE Colour (Table 1). Moreover, the Pectinex® BE Colour enzyme was able to release most of the D(-)-arabinose in the Cs. skins (28 μg mg^{-1}) and ∼50% (21 μg mg^{-1}) of the theoretically maximal amount of arabinose in the Mt. grape skins relative to the level obtained by TFA chemical hydrolysis [23]. The same conclusion could be drawn for galactose. With the Pectinex® BE Colour treatment the released glucose level increased from 33 to 64 μg mg^{-1} over time for Cs. grape skins and from 44 to 68 μg mg^{-1} for the Mt. skins (Fig. 1); this evolution in glucose is likely a result of the presence of β-glucosidase [EC 3.2.1.21] as a secondary activity in the Pectinex® BE Colour preparation [25]. In contrast, the levels of glucose in the hydrolysates of the Celluclast® 1.5 L FG and the Vinozyme® FCE G treated grape skins remained relatively constant during the course of hydrolysis: for Cs. the glucose levels were ∼40 μg mg^{-1} throughout the hydrolysis while for the Mt. the glucose levels remained at ∼60 μg mg^{-1} (Fig. 1). This confirmed that these enzyme preparations were completely devoid of (at least) β-glucosidase to catalyze the release of glucose from celluloglycosaccharides, xylolucan-oligosaccharides, and glycosylated anthocyanins. D(+)-Mannose and L(+)-rhamnose liberation were only detected after the Pectinex® BE Colour enzyme treatment. We believe this release is due to the presence of undeclared (secondary) endo-β-mannanase [EC 3.2.1.78] and/or mannosidase [EC 3.2.1.25] and α-L- or β-L-rhamniosidase [EC 3.2.1.40, EC 3.2.1.43, respectively] activities in the Pectinex® BE Colour preparation. The maximum total monosaccharide release was reached with the Pectinex® BE Colour enzyme preparation, where, after 24 h of treatment, a total of ∼225 and ∼189 μg mg^{-1} monosaccharides were reached for the Cs. and Mt. grape skin samples, respectively (calculated from data in Fig. 1). Treatments with 50:50 mixtures of Celluclast® 1.5 L FG + Pectinex® BE Colour, Celluclast® 1.5 L FG + Vinozyme® FCE G, or Pectinex® BE Colour + Vinozyme® FCE G, respectively, did not produce any synergistic effects in the release of monosaccharides (data not shown).

During the enzymatic treatments, the release rates of monosaccharides from the grape skin cell wall generally increased up to 6 h, and subsequently flattened, providing typical progress curves for enzyme catalyzed reactions. However, the enzymatic degradation of plant cell wall polysaccharides do not follow Michaelis–Menten kinetics, so the flattening could be due to binding of the enzyme to the substrate or be a result of gradual enzyme inactivation after long treatment conditions at low pH, and high temperature. The drop in the release rate of monosaccharides after 6 h was taken into consideration when the full factorial setup was designed (see below). In general, the sum of released monosaccharides (excluding fructose) was 2.0–2.8 times lower than previously published data for grape skin cell wall samples of the same cultivars treated with trifluoroacetic acid (TFA) chemical hydrolysis [23], indicating that even during the prolonged enzymatic saccharification treatment, the cell wall polysaccharides were not fully degraded. Since the monosaccharide release from the Mt. grape skin samples were generally lower and fluctuated more than those of the Cs. skins we decided to focus mainly on the Cs. grape skins to examine the relation between polysaccharides hydrolysis and phenols.

3.2. Total phenols and anthocyanins releases from Cabernet Sauvignon grape skins during different enzymatic treatments

The course of the total phenols and total anthocyanins releases were quite similar in the different enzyme treatments, but very different from the monosaccharide releases. For the Pectinex® BE Colour and the Vinozyme® FCE G treated samples the initial total phenols and anthocyanins levels were at the same level, and these levels were about the double of those obtained with Celluclast®
1.5 L treatment (Fig. 2). The similarity in the evolution of the total phenols and anthocyanins was most likely a result of the quantitive dominance of anthocyanins in the phenolic profile (see later). All three enzyme treatments induced an initial decrease in the total phenols and total anthocyanins during the first few hours of hydrolysis (Fig. 2). For the Pectinex® BE Colour and the Vinozym® FCE G treated samples this dip in the phenols yields levelled off after 2–4 h, and the total phenols and anthocyanins levels then increased slightly during 4–6 h, notably for the Vinozym® FCE G treated skins (Fig. 2). However, beyond 6 h of enzyme treatment the total phenols levels again decreased, and for the Pectinex® BE Colour and the Vinozym® FCE G treated samples after 24 h of enzyme treatment the phenols and anthocyanins levels were only about 50% and 70% of the original yields, respectively (Fig. 2). A similar trend was seen with the Celluclast® 1.5 L FG treated samples, albeit to a lower extent – total levels dropped 20% while the values of the Pectinex® BE Colour treated samples dropped slightly more than 50% – and at lower total phenols and anthocyanins levels (Fig. 2). The course of the releases after 2–6 h might be a result of competitive reactions that simultaneously catalyzed the release from the grape skins and the degradation of the anthocyanins; the release would be a result of enzyme catalyzed liberation of mainly anthocyanins from the anthocyanoplast bodies during the enzymatic opening of the cell wall polysaccharide barrier (presumably mainly because of enzymatic degradation of pectin, Fig. 1), whereas the anthocyanin degradation might be a result of the formation of unstable anthocyanin aglycones by enzyme catalyzed deglycosylation caused by β-glucosidase activity (E.C. 3.2.1.21) and presumably cinnamate esterase activity (E.C. 3.1.1.1), notably in the Pectinex® BE Colour preparation as discussed in more detail later.

### 3.3. Evolution of flavonols and phenolic acids from Cabernet Sauvignon grape skins during different enzymatic treatments

At the onset of the enzymatic treatment, i.e. at time 0, the "released" rutin, i.e. quercetin-3-rutinoside or quercetin-3-O-α-L-rhamnosyl-β-D-glucose, was 3.5–4.5 μg·mg⁻¹ with all three types of enzyme treatment (Fig. 3); the similarity in the initial levels indicated that the measured rutin was most likely the result of instant, spontaneous diffusive leaching of rutin from the skins rather than the result of enzyme catalyzed release. Neither the Celluclast® 1.5 L FG or the Vinozym® FCE G treatment appeared to cause any further liberation of rutin, nor to cause any release of quercetin during the 24 h of enzyme treatment (Fig. 3). In contrast, the Pectinex® BE Colour treatment induced a gradual decrease in rutin levels and a concomitant increase in the quercetin levels during the enzymatic treatment (Fig. 3). This apparent conversion of rutin to quercetin has been observed earlier in pectinase treated berry extracts [26] and can be explained by the presence of both α-L-rhamnosidase and β-glucosidase activities in the Pectinex® BE Colour preparation. The action of these enzyme activities can cause a two-step deglycosylation of the rutin to the aglycone quercetin. The apparent Pectinex® BE Colour catalyzed transformation of rutin to quercetin via action of these activities are in full agreement with the monosaccharide release findings that Pectinex® BE Colour treatment, as the only one among the treatment, resulted in some liberation of rhamnose, and in significant liberation of glucose (Fig. 1).

A detailed analysis of the evolution of the hydroxybenzoic and hydroxycinnamic acid levels during extended treatment of the Cs. skins with the three different enzyme preparations, Pectinex® BE Colour, Celluclast® 1.5 L FG, and Vinozym® FCE G, showed that these phenolic acids were only released with Pectinex® BE Colour treatment, and that they were only released in relatively low amounts (0–1.6 μg·mg⁻¹) during 24 h of enzymatic treatment (Fig. 4). A closer look at the release of the anthocyanins, flavonols, and phenolic acids in response to the total monosaccharide release (the latter in fact signifying the extent of cell wall polysaccharide degradation) showed that while the anthocyanins and flavonol levels decreased, the hydroxybenzoic and hydroxycinnamic acid releases increased steadily with the overall extent of cell wall polysaccharide degradation with the Pectinex® BE Colour treatment (Fig. 5). When comparing the correlations between the released hydroxybenzoic acids and hydroxycinnamic acids with individual monosaccharides, the following was obvious (Fig. 6): (a) the release patterns of hydroxybenzoates and hydroxycinnamates vs. the release of individual monosaccharides were strikingly similar, (b) the releases (y) of hydroxybenzoic and hydroxycinnamic acids (x) were positively, approximately linearly (y = mx + b), correlated to the release of galactose, glucose, xylose, and mannose, with linear correlation coefficients ranging from 0.91 to 0.99 (discussed later), (c) the releases (y) of both hydroxybenzoic and hydroxycinnamic acids (x) were apparently exponentially correlated (y = a·ebx) to the release of galacturonic acid and arabinose with correlation coefficients of...
3.4. Reaction factors affecting the enzymatic hydrolysis of grape skin polysaccharides and phenolics release

In order to examine and understand the events taking place during 0–6 h in more detail (this time span was the most significant for the plant cell wall polysaccharide degradation, especially for the Pectinex® BE Colour treated samples, Fig. 1), we evaluated the influence of various enzyme reaction parameters on the Pectinex® BE Colour treatment of Cs. grape skin samples in an experimental design. We mainly focused on unravelling any possible correlations between the polysaccharide cell wall degradation and the release of specific phenolics. In order to compare the results of the factorial design experiments with those obtained in the extended enzymatic treatments, the reaction conditions employed in the extended treatments, i.e. pH 3.6, 5.5% E/S, and 40 °C, were the center points in the factorial design experiments. Neither the reaction temperature nor time had any significant effects on the release of different monosaccharides from the Cs. skins (Table 2). The enzyme to substrate ratio (E/S %), i.e. the enzyme dosage, however, had a significantly positive effect on the release of most of the monosaccharides (the only exceptions being the release of xylose and glucose) and hence on the enzyme catalyzed degradation of polysaccharides in the Cs. grape skins promoted by the Pectinex® BE Colour treatment (Table 2). The lack of fit of the xylose release was most likely a result of the negligible xylose release obtained with the enzymatic treatment (Fig. 1c), while the lack of fit of glucose to any of the reaction factors was surprising and contrasted the strong correlation with time using Pectinex® BE Colour preparation. An explanation may be that the Pectinex® BE Colour enzyme catalyzed glucose release actually represented a relatively small increment on top of a high level of glucose (Fig. 1).

Multiple linear regression analysis (Table 3), showed that the significance of the reaction factors on the phenolics releases varied, but generally confirmed the observations of the extended enzymatic reactions. The data signified that temperature, not the enzymatic reaction (E/S), was the main factor behind disappearance of anthocyanins during enzymatic treatment during the first 6 h (Table 3). This was true for the different individual major anthocyanins including malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside – for malvidin-3-glucoside, the major anthocyanin compound in grape skins, both the temperature and time and their interaction had adverse effects (data not shown). For the acylated anthocyanins, however, the enzymatic treatment alone (E/S) exerted a negative effect on the anthocyanins (Table 3). Since the Pectinex® BE Colour preparation apparently harboured β-glucosidase activity, and since we ran our experiments under N2, the enzyme catalyzed decay of the acylated anthocyanins mostly took place via a sequential enzyme catalyzed degradation as caused by both cinnamate esterase activity (E.C. 3.1.1.x) and β-glucosidase activity (E.C. 3.2.1.21): as a first step, cinnamate esterase catalyzes the stripping of the acylated anthocyanins from its shielding hydroxycinnamic acid (p-coumaric, caffeic, ferulic acids), which make it possible for β-glucosidase to deglycosylate anthocyanin releasing glucose and the more susceptible aglycone; anthocyanidin (Fig. 7). Although the Cellulclast® 1.5 L FG preparation is a cellulolytic enzyme preparation, the preparation is actually devoid of profound β-glucosidase activity [27].
One of the key factors among different enzymatic preparation was the absence of cinnamate esterase activity in Vinozyme® FCE G (Table 1), and this activity was never reported for Celluclast® 1.5 L FG. On the other hand, cinnamate esterase catalyzed deacylation of anthocyanins was reported previously with an industrial pectinase preparation from Aspergillus niger [28]. A detailed analysis of the decline of the acylated anthocyanins with time during treatment with the three different enzyme preparations clearly corroborated that the decline during the Pectinex® BE Colour treatment was most profound and longer, i.e. continued throughout the 24 h of enzymatic treatment, as compared to the other enzymatic treatments (data not shown). That the release of particularly p-coumaric acid (hydroxycinnamic acid) may be tied to the decay and deglycosylation of anthocyanins, is substantiated by the tight correlation between the loss in acylated anthocyanins and the increase in hydroxycinnamic acids and glucose during extended Pectinex® BE Colour treatment of Cs. skins (compare Figs. 2 and 4). These data gave a strong correlation between the release of both hydroxycinnamic acids and glucose reaching 0.96, and 0.83 (P<0.05), respectively.

The data thus suggest that 3-glycosylated anthocyanins may be the main source of the measured glucose and p-coumaric acid (Fig. 7).

For the rutin:quercetin reactions the enzyme dosage was a significant negative factor for the rutin levels and a significantly positive factor for the quercetin yields (Table 3). The reaction temp-

---

**Table 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>R² (Prob&gt;F)</th>
<th>Significant factor (coeff, P)</th>
<th>Constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>d- (+)-Fucose</td>
<td>0.62 (0.0025)</td>
<td>E/S (0.02, 0.0038)</td>
<td>0.03</td>
</tr>
<tr>
<td>d- (+)-Xylose</td>
<td>No fit</td>
<td>Not significant</td>
<td>No fit</td>
</tr>
<tr>
<td>d- (+)-Mannose</td>
<td>0.99 (&lt;0.0001)</td>
<td>E/S (0.57, &lt;0.0001)</td>
<td>0.64</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>0.55 (0.0305)</td>
<td>E/S (0.34, 0.0086)</td>
<td>0.67</td>
</tr>
<tr>
<td>d- (+)-Galactose</td>
<td>0.47 (0.0561)</td>
<td>E/S (0.46, 0.0263)</td>
<td>1.44</td>
</tr>
<tr>
<td>d- (+)-Glucose</td>
<td>No fit</td>
<td>Not significant</td>
<td>No fit</td>
</tr>
<tr>
<td>d- (-)-Arabinose</td>
<td>0.69 (0.0098)</td>
<td>E/S (1.93, 0.0021)</td>
<td>6.54</td>
</tr>
<tr>
<td>d- (+)-Galacturonic acid</td>
<td>0.51 (0.0467)</td>
<td>E/S (8.77, 0.008)</td>
<td>35.82</td>
</tr>
</tbody>
</table>

a) E/S: enzyme dose (1, 5.5, and 10% E/S), T<sub>temp.</sub> temperature (25, 40, and 55°C), T<sub>time.</sub> time (2, 4, and 6 h).
b) Coefficients of the linear model found by least squares.
c) P<0.05 indicates significance at 95% level.
Regression model summaries and regression coefficients of hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) with cell wall lyophilised grape skins with Pectinex® BE Colour enzyme.

Table 3

<table>
<thead>
<tr>
<th>Significant factors and their interactions (±)</th>
<th>Coefficient (±)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total monomeric anthocyanins</td>
<td>0.66 (0.0139)</td>
<td>T&lt;sub&gt;emp&lt;/sub&gt; (−3.31, 0.0039)</td>
</tr>
<tr>
<td>Anthocyanins-3-O-β-glucosides</td>
<td>0.92 (0.0055)</td>
<td>T&lt;sub&gt;emp&lt;/sub&gt; (−2.72, 0.0006), T&lt;sub&gt;ime&lt;/sub&gt; (−0.91, 0.0339), E/S × T&lt;sub&gt;ime&lt;/sub&gt; (−0.97, 0.0265), T&lt;sub&gt;emp&lt;/sub&gt; × T&lt;sub&gt;ime&lt;/sub&gt; (−0.71, 0.0645)</td>
</tr>
<tr>
<td>Acylated anthocyanins</td>
<td>0.43 (0.0764)</td>
<td>E/S (−0.18, 0.0754), T&lt;sub&gt;emp&lt;/sub&gt; (−0.20, 0.0572)</td>
</tr>
<tr>
<td>Mono-acylated anthocyanins</td>
<td>0.41 (0.0898)</td>
<td>E/S (−0.15, 0.0908), T&lt;sub&gt;emp&lt;/sub&gt; (−0.18, 0.0577)</td>
</tr>
<tr>
<td>Poly-acylated anthocyanins</td>
<td>0.51 (0.0479)</td>
<td>E/S (−0.05, 0.0237), T&lt;sub&gt;emp&lt;/sub&gt; (−0.02, 0.0757)</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.53 (0.0419)</td>
<td>E/S (−0.4, 0.0085)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.97 (0.0001)</td>
<td>E/S (0.21, &lt;0.0001), T&lt;sub&gt;emp&lt;/sub&gt; (−0.07, 0.0039), E/S × T&lt;sub&gt;emp&lt;/sub&gt; (−0.15, 0.001), E/S × T&lt;sub&gt;ime&lt;/sub&gt; (−0.05, 0.0226)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.91 (0.0005)</td>
<td>E/S (0.05, 0.0033), T&lt;sub&gt;emp&lt;/sub&gt; (−0.03, 0.049), E/S × T&lt;sub&gt;emp&lt;/sub&gt; (−0.04, 0.062)</td>
</tr>
<tr>
<td>Hydroxybenzoic acids</td>
<td>0.35 (0.1201)</td>
<td>E/S (0.04, 0.0266)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.40 (0.0937)</td>
<td>E/S (0.03, 0.0214)</td>
</tr>
<tr>
<td>Hydroxycinnamic acids</td>
<td>0.69 (0.0003)</td>
<td>E/S (0.13, 0.0004), T&lt;sub&gt;emp&lt;/sub&gt; (0.14, 0.0004)</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.79 (0.0024)</td>
<td>E/S (0.09, 0.0013), T&lt;sub&gt;emp&lt;/sub&gt; (0.06, 0.0092)</td>
</tr>
<tr>
<td>Kae fic acid</td>
<td>0.70 (0.0080)</td>
<td>E/S (0.04, 0.0049), T&lt;sub&gt;emp&lt;/sub&gt; (0.07, 0.003)</td>
</tr>
</tbody>
</table>

* E/S: enzyme dose (1, 5.5, and 10% E/S), T<sub>emp</sub>: temperature (25, 40, and 55 °C), T<sub>ime</sub>: time (2, 4, and 6 h).
* a Con.b c R<sub>2</sub> = 0.05 indicates significance at 95% level.
* b Coefficients of the linear model found by least squares.
* c P<0.05 indicates statistical significance at 95% level.

Fig. 7. Sequential hydrolysis of typical grape anthocyanin, malvidin-3-β-(6-p-coumaryl)D-glucose, by cinnamate esterase then β-glucosidase yielding the p-coumaric acid as a first step and then glucose and the more vulnerable aglycone form of anthocyanins.

Table 4

<table>
<thead>
<tr>
<th>Regression model summaries and regression coefficients of hydroxybenzoic acids (HBA) and hydroxycinnamic acids (HCA) with cell wall α-(+)-xylose, α-(+)-mannose, α-(+)-galactose, and α-(+)-glucose in the extended enzymatic treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E/S</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>HBA</td>
</tr>
<tr>
<td>HCA</td>
</tr>
</tbody>
</table>

* P<0.05 indicates significance at 95% level.
* a Constant of the linear regression model.
* b m is the slope of the regression line.
of dry matter) of these grape skins [24], secondly, the typical degradation of mannan, xyloglucan, rhamnogalacturonan-I side chains, their release was particularly exponentially correlated to the different monosaccharides analysed, while on the other hand their release correlated with the liberation of all the one hand their release correlated with the liberation of all the different monosaccharides analysed, while on the other hand their release was particularly exponentially correlated to the liberation of galacturonic acid and arabinose. The latter corre-
lation indicated a unique relation to the extensive degradation of pectin, mainly homogalacturonan and rhagomogalacturonan I arabinan side chains; at the same time, the liberation of espe-
cially hydroxycinnamates (notably p-coumaric acid) could be a result of release from acylated anthocyanins. These data thus signify the option for developing discriminated release of phenols during enzyme catalyzed degradation of grape skin polysaccharides, further experiments are required to assess whether the established correlations do, in fact, signify a differentiated relationship between the release of the phenolic acids and the enzyme catalyzed degradation of spe-
cific cell wall polysaccharides, anthocyanins, and/or perhaps even lignin, in grape skins. The gradual formation of quercetin provides a base for differentiated recovery of e.g. anthocyanins and formation of quercetin, respectively, during valorisation of e.g. grape pomace. The complex composition of the cell wall polysaccharides require the concerted action of different enzymatic activities, and the present data indicate that it might be possible to design

### Table 5: Regression model summaries and regression coefficients of hydroxybenzoic acids

<table>
<thead>
<tr>
<th>Acid</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Model fit</th>
<th>$y$-intercept</th>
<th>$R^2$</th>
<th>$P$</th>
<th>Model fit</th>
<th>$y$-intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBA</td>
<td>0.97</td>
<td>$&lt;0.0001$</td>
<td>y = 0.0531</td>
<td></td>
<td>0.98</td>
<td>$&lt;0.0001$</td>
<td>y = 0.0811</td>
<td></td>
</tr>
<tr>
<td>HCA</td>
<td>0.95</td>
<td>$&lt;0.0001$</td>
<td>y = 0.0493</td>
<td></td>
<td>0.98</td>
<td>$&lt;0.0001$</td>
<td>y = 0.0811</td>
<td></td>
</tr>
</tbody>
</table>

$* P<0.05$ indicates significance at 95% level. The present study showed that several different types of polysaccharides were degraded during multicomponentpecti

### 3.5. Correlations between the solubilised cell wall monosaccharide and phenols

The regression analysis confirmed the positive linear correlations between the enzyme catalyzed release of hydroxybenzoic and hydroxycinamic acids and the degradation of plant cell wall polysaccharides in the Cs. grape skins (Table 4). The released yields of both hydroxybenzoic acid and -cinamic acids thus correlated signific-
antly positively with the releases of xylose, mannose, rhamnose, galactose, and glucose (Table 4). In general, the correlation data for hydroxybenzoic and hydroxycinamic acids were similar. The highest slopes for the correlations were found for mannose, the lowest for glucose (Table 4). In contrast, the correlations between the release hydroxybenzoic and hydroxycinamic acids and arabinose and galacturonic acid releases were not linear, but best fitted with exponential curve fits (Table 5). The present data do not reveal the exact mechanism behind the difference in the corre-
lation models, but it is tempting to suggest that the hydroxybenzoic and hydroxycinamic acids were mainly released during extensive degradation, i.e. when a certain amount of pectin, as indicated by the galacturonic acid and arabinose, had been degraded. The kinetics thus indicate that the phenolic acids were only accessible when a certain amount of pectin had been catalytically removed from the cell wall matrix. Under the Pectinex® BE Colour treatment the release of hydroxybenzoic acids (Fig. 4), mainly syringic and vanillic acids, increased over time; this might reflect disintegration of lignocellulose structures. When coupling this to the correla-
tion data for acylated anthocyanins loss and hydroxycinamic acid release (Table 3), it seems that the release of hydroxybenzoic and hydroxycinamic acids were tied to the degradation of sev-
eral different structural polysaccharide elements. This suggests a relationship between the enzymatic activities catalyzing the degra-
dation of mannan, xylolucan, thamogalacturonan-I side chains, and homogalacturonan, and the release of the phenolic acids, but also indicates that the enzymatic access to the hydroxybenzoic and hydroxycinamic acids bound in the skin cell wall matrix, including the lignin, became more favourable when a certain amount of pectin had been catalytically degraded. The data indicate that the acylated anthocyanins were attacked randomly during the polysaccharide degradation.

### 4. Discussion and conclusions

The present study showed that several different types of polysaccharides were degraded during multicomponentpecti-

nolytic activities including pectin methyl esterase (E.C. 3.1.1.1), endo-polysaccharacease (E.C. 3.2.1.15), pectin-lyase (E.C. 4.2.2.10) (Table 1), but also contain several secondary activities including galactanases (E.C. 3.2.1.89 and 90), arabinases (E.C. 3.2.1.99), cel-
lulases (incl. E.C. 3.2.1.4, 21, 74, and 91), rhamnosidase (incl. E.C. 3.2.1.40 and E.C. 3.2.1.40), mannanase and mannosidase (E.C. 3.2.1.25 and 78), various esterases (E.C. 3.1.1.x), and perhaps other activities [9,12,29].

The present study provided several different, new results; some of the data confirmed our hypothesis that different phenolic classes may be bound to different specific cell wall polysaccharides, while other data completely changed our original comprehension that the phenolics were liberated during the progressive enzymatic catalyzed disintegration of the cell wall polysaccharides:

1. Anthocyanins were released first, but their release did not correlate to any monosaccharide production, and was thus not directly related to enzyme catalyzed degradation of the grape skin cell wall polysaccharides. The extended enzymatic treatments affected the anthocyanins negatively; the initial rapid loss of total anthocyanins was found to be mainly caused by the tem-
perature, while the loss of acylated anthocyanins might be tied to the formation of hydroxycinamic acids, notably p-coumaric acid (Fig. 7).

2. The flavonoids, mainly rutin, were not either enzymatically released during the enzymatic cell wall polysaccharide sacchar-
ification of the grape skins, but the forced enzymatic maceration induced conversion of rutin to quercetin, presumably as a result of a two-step deacylution of rutin. The correlation between the flavonoids and the extent of enzymatic polysaccharide degra-
dation were complex with simultaneous negative interactions between enzyme dosage ($E$/S of Pectinex® BE Colour) and tem-
perature and enzyme dosage ($E$/S of Pectinex® BE Colour) and time, that gave a negative net correlation between flavonoids and extent of enzyme catalyzed polysaccharide degradation. Hence, for discriminated recovery of flavonoids from grape skins, the flavonoids are recovered early, mainly as a result of leaching, and any later conversion, e.g. from rutin to quercetin, may then take place subsequently and perhaps separately.

3. The phenolic acids, i.e. hydroxybenzoic and hydroxycinamic acids, were released differently than the other phenols: on the one hand their release correlated with the liberation of all the different monosaccharides analysed, while on the other hand their release was particularly exponentially correlated to the liberation of galacturonic acid and arabinose. The latter corre-
lation indicated a unique relation to the extensive degradation of pectin, mainly homogalacturonan and rhagomogalacturonan I arabinan side chains; at the same time, the liberation of espe-
cially hydroxycinnamates (notably p-coumaric acid) could be a result of release from acylated anthocyanins.
release of certain phenolics during targeted degradation of the skin material. However, further insight into the location and bonding of phenolics in the cell walls of grape skins is still required before this potential may be fully exploited. Whether a further understanding can be achieved by promoting the separate enzymatic modification of specific polysaccharide fractions and carefully assessing the evolution of liberated phenols is part of our ongoing research.

Acknowledgments

The Distell Group Ltd. (Stellenbosch, South Africa) is acknowledged for supplementation of the wine grape samples. Donation of enzyme samples from Novozymes A/S (Bagvaerd, Denmark) is gratefully acknowledged.

References

Chapter 5

Grape skins catalyze the *in vitro* enzymatic hydroxylation of *p*-coumaric acid to caffeic acid

This chapter is extended in the form of a submitted article:

Grape skins (*Vitis vinifera* L.) catalyze the *in vitro* enzymatic hydroxylation of *p*-coumaric acid to caffeic acid
Arnous, A. & Meyer, A. S.

5.1 Key points

During previous studies to facilitate the release of phenolic compounds from grape skin using pectolytic enzyme preparation, we noticed an increase of total hydroxycinnamic acids with time. In particular there was an increase in caffeic acid that was parallel to decrease in *p*-coumaric acid.

The purpose of the present study was to investigate the possibility of converting *p*-coumaric acid to caffeic acid. Furthermore, we wished to study the conversion kinetics as a first initial evaluation of the possibilities of bioconversion strategies to produce juice and wine naturally enriched with caffeic acid.

5.2 Conclusion

We discovered that the grape skin itself harbour *o*-hydroxylation activity that catalyze the conversion of *p*-coumaric acid into caffeic acid (Fig. 5.1). The *in planta* conversion of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid (3,4 dihydroxycinnamic acid) via hydroxylation is still disputed, but has been described to be catalyzed by two possible hydroxylation pathways: by copper-containing polyphenol oxidases, including monophenol monooxygenase (EC 1.14.18.1) or by P450-dependent hydroxylases, respectively (Strack, 2001).
Figure 5.1 Reversed phase HPLC chromatogram of rapid separation of phenolics. Caffeic acid is produced from hydroxylation of added \( p \)-coumaric acid by catalytic activity in the grape skin.

The endogenous capacity of grapes to catalyze \( p \)-coumaric acid to caffeic acid has never been considered in relation to neither wine making nor other grape processing operations before. The deliberate promotion, by enzyme catalysis, enhancing the levels of caffeic acid from \( p \)-coumaric acid may be a new type of strategy to improve the antioxidant potency of grape juice and red wine, and may open the door for novel valorisation routes for grape pomace. This is very interesting as caffeic acid is believed to have superior antioxidant activity due to the presence of the \( o \)-dihydroxy group in the phenolic ring Meyer (Meyer et al., 1998; Andreasen et al., 2001). No similar pathways have been identified in animal or human tissues.
Grape skins (Vitis vinifera L.) catalyze the in vitro enzymatic hydroxylation of p-coumaric acid to caffeic acid

Anis Arnous · Anne S. Meyer

Abstract The ability of grape skins to catalyze in vitro conversion of p-coumaric acid to the more potent antioxidant caffeic acid was studied. Addition of different concentrations of p-coumaric to red grape skins (Cabernet Sauvignon) resulted in formation of caffeic acid. This caffeic acid formation (Y) correlated positively and linearly to p-coumaric acid consumption (X): Y = 0.5 X + 9.5; R² = 0.96, P < 0.0001. The kinetics of caffeic acid formation with time in response to initial p-coumaric acid levels and at different grape skin concentrations, indicated that the grape skins harboured an o-hydroxylation activity, proposedly a monophenol- or a flavonoid 3’-monooxygenase activity (EC 1.14.18.1 or EC 1.14.13.21). The K_m of this crude o-hydroxylation activity in the red grape skin was 0.5 mM with p-coumaric acid.

Keywords Antioxidant · Flavonoid 3’-monooxygenase · Ortho hydroxylation · Monophenol monooxygenase · Polyphenol oxidase

Introduction

Grape is the world’s largest fruit crop, with an annual global production higher than 66 million tons (FAO-UN 2009). The majority of grapes are processed into red wine. The red grape skins are particularly rich in extractable phenolics (Pinelo et al. 2006) and during red wine making, phenols localized in the grape skins, and partially those present in the seeds and stems, are extracted into the juice and the fermenting wine. The phenolic compounds influence the quality and sensory attributes of wines, and are suspected to exert physiological benefits via their antioxidant activity and presumably via a number of anti-inflammatory signaling cascades (Pan et al. 2009). Hydroxycinnamic acids (3-phenyl-2-propanoic acids) are ubiquitous phenolic compounds occurring in various bound, esterified, and conjugated forms in plant foods (Herrmann 1989). In grapes, the hydroxycinnamates predominantly occur as tartaric acid esters (Herrmann 1989). The antioxidant activity of monomeric hydroxycinnamic acids towards in vitro human low-density lipoprotein oxidation is: caffeic acid > sinapic acid > ferulic acid > p-coumaric acid (Meyer et al. 1998; Andreasen et al. 2001). The order is similar for the esterified tartaric acid derivatives (Meyer et al. 1998). The superior antioxidant activity of caffeic acid is believed to be due to the presence of the o-dihydroxy group in the phenolic ring (Meyer et al. 1998; Andreasen et al. 2001). The in planta conversion of p-coumaric acid (4-hydroxycinnamic acid) to caffeic
acid (3,4-dihydroxycinnamic acid) via hydroxylation is still disputed, but has been described to be catalyzed by two possible hydroxylation pathways: by copper-containing polyphenol oxidases, including monophenol monooxygenase (EC 1.14.18.1) or by P450-dependent hydroxylases, respectively (Strack 2001). No similar pathways have been identified in animal or human tissues. During our studies on upgrading of grape skin phenols, we discovered that the grape skin itself, i.e. an apparent \( o \)-hydroxylation activity in the skins, catalyzed the conversion of \( p \)-coumaric acid into caffeic acid. The purpose of the present study was to investigate this conversion and its kinetics as an initial evaluation of the possible workability of a bioconversion strategy to produce juice and wine naturally boosted with caffeic acid.

**Materials and methods**

**Chemicals and reagents**

Caffeic acid, \( p \)-coumaric acid, acetonitrile, and methanol, all of analytical or HPLC grade, were purchased from Sigma-Aldrich. The commercial enzyme preparation Pectinex BE Colour was supplied by Novozymes A/S (Bagsværd, Denmark). The enzyme preparation is a liquid formulation of pectin lyase/polygalacturonase derived from \( \text{Aspergillus niger} \). The pectinase strength is 3600 MOE units/ml (MOE: Most Einheit = Juice Must Unit; a measure of the reduction in the viscosity of apple juice); the pH and temperature optima of the main activities are 3.5–4 and 45–55°C, respectively. The preparation also harbours cinnamate esterase activity (Barbe and Dubourdieu 1998).

**Grape skin preparation**

Red wine grape (\( \text{Vitis vinifera} \) L. cv. Cabernet Sauvignon) were obtained from the Distell Group Ltd., Stellenbosch, South Africa. Grape skin particles of 125–250 \( \mu \)m were prepared according to the method previously reported (Arnous and Meyer 2008).

**Hydroxylase assay**

Hydroxylation of \( p \)-coumaric acid was achieved either by releasing \( p \)-coumaric acid after enzymatic treatment of lyophilized grape skins (3 g l\(^{-1}\)) with the Pectinex BE Colour preparation, or by addition of the substrate \( p \)-coumaric acid when no Pectinex BE Colour preparation was added. The level of 3 g grape skin l\(^{-1}\) was established based on preliminary experiments (data not shown). The hydroxylation experiments were carried out in 1 ml acetate buffer (0.2 M), pH = 3.6 in Eppendorf tubes at 40°C with stirring at 750 rpm.

**Effect of crude catalyst dosage**

The relation between grape skin concentration and \( o \)-hydroxylation activity measured as caffeic acid formation was measured with grape skin at 1, 3, 5, or 7 g l\(^{-1}\), with a constant (added) initial concentration of \( p \)-coumaric acid of 6 \( \mu \)M.

**\( K_m \) and \( V_{max} \) determination for hydroxylation by the grape skins**

Experiments were run in duplicate with grape skin (3 g l\(^{-1}\)) as catalyst. \( p \)-Coumaric acid ranged from 6 to 500 \( \mu \)M. Samples were taken from 3 to 360 min (see Fig. 4 below). The assay from the \( K_m \) value for \( p \)-coumaric acid was carried out over 3 min to give the initial velocity of reaction.

**Sampling**

In all cases, 50 \( \mu \)l was taken and the reaction was terminated by adding 50 \( \mu \)l 60% (v/v), methanol (containing 0.7% TFA). Samples were centrifuged immediately for 3 min at 16,000 g, and the supernatant was subsequently injected (10 \( \mu \)l) into the HPLC system.

**Identification of the reaction products**

The conversion of \( p \)-coumaric into caffeic acid was monitored using an IC-3000 HPLC system coupled to photodiode array detector equipped with Synergy Hydro-RP column (150 \( \times \) 4.6 mm i.d. 4 \( \mu \)m, Phenomenex, Torrance, CA, USA). Caffeic acid and \( p \)-coumaric acid were eluted isocratically according to the methods of Sachan et al. (2004), but with the modification that the eluent system consisted of three components (Table 1).
The UV/vis is spectrum of each of the pure phenolic compounds and their retention time was used to confirm the identities of the phenolics. Chromatograms were recorded at 334 and 308 nm for caffeic acid and p-coumaric acid, respectively. Quantification was based on linear regression of the area responses on different doses of authentic external standards of caffeic acid and p-coumaric acid. The calibration points were weighted by the factor 1/response². The spectral match analyses were accomplished by use of the Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp.).

Statistical analysis

All experiments were in duplicates. Linear regression and calculation of coefficients of variation were done with the aid of JMP 7.0.2 software (SAS Institute Inc., Cary, NC, USA). The enzyme kinetics modules fit were done with the aid of SigmaPlot Enzyme Kinetics Module 1.3, (Systat Inc., Chicago, IL, USA). Significance of all the results was established at $P < 0.05$.

Results and discussion

Detection of caffeic acid during enzymatic treatment of grape skin with pectolytic enzyme

When the red Cabernet Sauvignon grape skins were treated with the pectolytic Pectinex BE Colour enzyme preparation, there was a sharp increase in the released p-coumaric acid concentration for the first 60 min (Fig. 1).

During this time a concomitant increase in the caffeic acid concentration, up to $\sim 4 \mu M$, took place.

| Table 1 | HPLC elution program
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elution time (min)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Eluent A: deionised water (18.2 mΩ cm at 25°C) containing 0.7% TFA, eluent B: acetonitrile/water (1:1 v/v) containing 0.17% TFA, eluent C: methanol/water (1:1 v/v) containing 0.17% TFA</td>
<td>9</td>
</tr>
<tr>
<td>9.5</td>
<td>0.4</td>
</tr>
<tr>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>20.1</td>
<td>0.4</td>
</tr>
<tr>
<td>22</td>
<td>0.5</td>
</tr>
<tr>
<td>27</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fig. 1 Conversion of p-coumaric acid to caffeic acid as a result of grape skin "o-hydroxylation" activity (grape skin concentration 3 g l⁻¹), p-Coumaric acid release from the grape skin (treatment with Pectinex BE Colour enzyme preparation 4.4 E/S %) (●), caffeic acid resulting from conversion of p-coumaric acid by grape skin (treatment with Pectinex BE Colour enzyme preparation 4.4 E/S %) (○), evolution of the concentration of added p-coumaric acid (initial concentration 6 μM) in the presence of grape skin (no treatment with Pectinex BE Colour enzyme preparation 4.4 E/S %) (○), p-coumaric acid evaluation under the "assay" reaction conditions, but when grape skin was excluded (△).

After 60 min, a consistent linear decline in the level of p-coumaric acid was apparent, whereas the level of caffeic acid continued to rise (Fig. 1). When the same experiment was repeated in the absence of the Pectinex BE Colour preparation, no formation of either caffeic acid or p-coumaric acid from the grape skin was observed (data not shown).

These observations corroborated that p-coumaric acid may be catalytically released from acylated anthocyanins in grape skins, presumably as a result of...
Cinnamate esterase side activity present in the *Aspergillus niger* pectolytic enzyme preparation (Barbe and Dubourdieu 1998). The data also provided a first indication that *p*-coumaric acid was apparently converted to caffeic acid in the reaction system by *o*-hydroxylation catalytic activity. The experiments were conducted at pH 3.6 to simulate the conditions during wine maceration.

In a different set of experiments, conducted under the same experimental conditions, the Pectinex BE Colour enzyme was incubated in buffer with *p*-coumaric acid added at 6 µM but without grape skins. During this incubation no caffeic acid production was detected and the *p*-coumaric acid level remained unchanged for 6 h (Fig. 1). This finding was expected since *o*-hydroxylation has never been reported as a side-activity of fungal multicomponent pectolytic preparations derived from *Aspergillus niger*. As the *p*-coumaric acid level did not decrease during the treatment also confirmed the relatively high stability of *p*-coumaric acid reported earlier (Salameh et al. 2008).

Taken together, these results suggested that the presumed hydroxylation activity originated from the grape skin itself. To demonstrate this, *p*-coumaric acid (6 µM) was added to the assay mixture in the presence of grape skin (3 g l⁻¹) and the evolution of *p*-coumaric acid and caffeic acid was monitored for 6 h. Under these conditions, an immediate, steep increase in the caffeic acid concentration was found. At the same time, the *p*-coumaric acid declined exponentially (Fig. 1) and then levelled off reaching ~3 µM after 360 min, while the *p*-coumaric acid had completely disappeared after 180 min (Fig. 1).

These data corroborate the comprehension that *p*-coumaric acid was catalytically converted to caffeic acid by an *o*-hydroxylation activity present in the grape skin, but also indicated that the caffeic acid was subject to a slow, consistent decay during extended reaction beyond 60 min. Further support for the ability of grape skin to hydroxylate excess levels of *p*-coumaric acid (600 µM) into caffeic acid was tested in both the presence and absence of the Pectinex BE Colour enzyme preparation on grape skins. In both cases a strong and positive, linear relationship between the consumption *p*-coumaric acid and formation of caffeic acid was found, and the slopes of the linear regression lines were similar (0.5) irrespective of whether the Pectinex BE Colour preparation was present or not (Fig. 2).

The linear relations were statistically significant and were virtually similar when Pectinex BE Colour enzyme preparation was present (R² = 0.95, P < 0.0001) or when it was absent (R² = 0.96, P < 0.0001). In conclusion, the presence of the Pectinex BE Colour preparation had no promoting effect on the hydroxylation process and the hydroxylation was being catalyzed by one or more enzyme activities present in the grape skins. The hydroxylation of *p*-coumaric acid to caffeic acid is a first step in the biosynthesis of lignins, flavonoids and coumarins in higher plants.

Based on the available literature this hydroxylation reaction may be described as follows:

\[
p\text{-Coumaric acid} + O_2 + AH_2 \rightarrow \text{Caffeic acid} + H_2O + A
\]

in which AH₂ represents electron donors such as NADH, ascorbic acid or L-dopa. The activity is listed by International Union of Biochemistry and Molecular Biology as a monophenol monooxygenase (EC 1.14.18.1) (with L-3,4-dihydroxyphenylalanine as donor) and one of the first characterizations of this activity from plant material was done for the enzyme from spinach-beet leaves (*Beta vulgaris* L.) (Vaughan and Butt 1969). Several catechol oxidases also catalyse the reaction listed under EC 1.14.18.1

---

© Springer
monophenol monooxygenase, while specific catechol oxidase activity has EC 1.10.3.1. The monophenol monooxygenase activity that catalyzes the o-hydroxylation of cinnamates is also called cinnamate-4-hydroxylase. Cinnamate-4-hydroxylase and 4-coumarate:coenzyme A ligase have both been identified as key enzymes catalyzing the biosynthesis of hydroxylated cinnamate compounds during grape berry development (Chen et al. 2006). In view of these results, other enzymes may participate in the p-hydroxylation of p-coumaric acid. F3'H (EC 1.14.13.21) hydroxylates at the 3'-position of the B-ring of the anthocyanins and the carbon at 3'-position of the anthocyanins has its origin at the C-3 atom of the anthocyanin precursor, p-coumaric acid. F3'H enzyme requires NADPH as a cofactor in grapes (Bogs et al. 2006).

We propose that it may be an EC 1.14.18.1 or EC 1.14.13.21 hydroxylation activity that was responsible for the observed o-hydroxylation activity in the reaction.

Caffeic acid oxidation versus o-hydroxylation of p-coumaric acid in grape skins

Understanding the cause of the tendency of caffeic acid to decline with time was than investigated. Since the reaction conditions of the hydroxylation assay i.e. (temperature, pH, exposure to O₂) could be the reasons for oxidation of caffeic acid, the stability of caffeic acid was monitored during incubation in buffer, i.e. under the "hydroxylation conditions", but in both the absence and presence of grape skin, and with or without addition of ascorbic acid (Fig. 3). The data showed a high stability of caffeic acid in the buffer as the concentration of the added caffeic acid (6 mM) did not change over 6 h (Fig. 3).

This result indirectly indicated that the caffeic acid consumption might be evoked by the grape skins. When the exact same test was repeated in the presence of grape skins (3 g l⁻¹) a rapid decrease in caffeic acid (added at 6 mM) was evident (Fig. 3). The same was observed with 600 mM caffeic acid (Fig. 3). These data confirm that the intrinsic oxidation activity within grape skin toward caffeic acid is strong. This oxidation activity may be related to grape polyphenol oxidase (PPO) or catechol oxidase activity (EC 1.10.3.1) (Singleton et al. 1985; Yokotsuka et al. 1991). Both caffeic acid, and its tartrate esterified form, caftaric acid [caffeoyl-L-(+)-tartaric acid], which is present in the grape pulp, is susceptible to PPO activity which catalyzes the conversion of o-diphenols to o-quinones (catecholase activity). This reaction is usually followed by non-enzymatic polymerization of the oxidation products to dark products or, in grapes, by condensation of the o-quinone with glutathione to form S-glutathionylcaffeic acid-tartrate (Singleton et al. 1985; Cheynier and Moutounet 1992). The latter reaction results in the "disappearance" of caftaric acid in normal grape juice and wine processing operations (Singleton et al. 1985). NaF is a strong PPO inhibitor (Janovitzklapp et al. 1989) and its addition (0.12 M) to the reaction mixture of grape skins and caffeic acid stabilized the caffeic acid level to a degree comparable to the stability of caffeic acid in the absence of grape skin (data not shown). PPO activities, which are found in many plants as well as in grape, hydroxylates 4-hydroxy substituted aromatic compounds, including p-coumaric acid, in the presence of an electron donor such as L-ascorbic acid in vitro as the first step. The reaction may be followed by oxidation of the caffeic acid (Sato 1969; Vaughan and Butt 1969). However, there are contradictory results concerning the participation of PPO in p-coumaric acid o-hydroxylation since some authors have shown strong activity towards p-coumaric acid (Harel and Mayer 1971).
whereas others reported negative results for the same substrate (Nakamura et al. 1983). The contradictory results could support arguments raised by both Duke and Vaughn (1982), and later by Kojima and Takeuchi (1989), who found that tentoxin, a cyclic tetrapeptide toxin produced by Alternaria alternata (Fr.) Keissler, had no effect on the contents of ethanol-soluble caffeic acid derivatives in mung bean (Vigna radiate) seedlings. This was true despite of the fact that PPO activity and the activity to hydroxylate p-coumaric acid to caffeic acid was eliminated in the presence of L-ascorbic acid (Kojima and Takeuchi 1989).

When p-coumaric acid was added to the grape skins hydroxylation assay at 600 μM some protection towards the produced caffeic acid was seen (Fig. 2), whereas at 6 μM such protection was absent (Fig. 1). Moreover, the addition of the Pectinex BE Colour preparation to dark berry skins releases different phenolic compounds apart from p-coumaric acid (Landbo and Meyer 2001; Landbo et al. 2007). The release of a plethora of phenolics may shield caffeic acid against PPO oxidation because of substrate competition. L-Ascorbic acid at 600 μM gave some protection of caffeic acid (Fig. 3) and may both inhibit the PPO activity directly or reduce the quinone produced before it undergoes secondary reactions (Rapeanu et al. 2006). To conclude, p-coumaric acid, L-ascorbic acid, and any phenolics released from the grape skins by enzyme hydrolyses protected caffeic acid against PPO, while the o-hydroxylation, presumably catalyzed by monophenol monoxygenase or F3’H present in the grape skins, was not inhibited.

Evaluation of kinetic constants and reaction rates

From the initial rate (0–3 min) of the hydroxylation reaction (see Fig.4), the V$_{\text{max}}$ for caffeic acid formation was calculated as 22 μM min$^{-1}$ and the K$_m$ value for p-coumaric acid was 0.5 mM. The K$_m$ value compares favourably well with the K$_m$ value of 0.6 mM for p-coumaric acid reported for partially purified catechol oxidase from white table grape (Harel and Mayer 1971). The K$_m$ value also compares well with values for similar hydroxylation of p-coumaric acid by other types of plant-derived PPOs: in maize (Zea mays L.) a K$_m$ of 0.33 mM has been reported for p-coumaric acid o-hydroxylation (Larson and Robles 1981), while the value for PPO from sugar beet (Beta vulgaris L.) gave a K$_m$ of 0.27 mM (Vaughan and Butt 1969). Finally, a clear dose-response effect of the extent of conversion of p-coumaric acid to caffeic acid was seen in response to an increase in the concentration of grape skins (Fig. 5).

The progress of the grape skin-catalyzed o-hydroxylation fitted classical rect-angular hyperbola curves, characteristic for enzyme catalyzed reactions. Under the reaction conditions, the initial reaction velocity was maximal during the first minutes already at 3 g grape skins l$^{-1}$, although the maximal conversion was manifest with grape skin at 5 and 7 g l$^{-1}$.
An obvious future research priority is to isolate and characterise the enzyme(s) responsible for the \(\alpha\)-hydroxylation and to evaluate the rate and robustness of the \(\alpha\)-hydroxylation catalytic activity at different temperatures and pH values. Such enzyme isolation would probably moreover permit a more accurate kinetic analysis of the enzyme activity and the further catalytic conversion of the caffeic acid might be avoided.

**Conclusions**

Previous results obtained for enzymatic processing of grapes, dark berries and their skins (press residues) using multicomponent pectolytic enzyme preparations, including the *Aspergillus niger* pectolytic enzymatic preparation Pectinex BE Colour, have shown increased release of phenols with enzyme dosage (Landbo and Meyer 2001; Revilla and Gonzalez-SanJose 2003; Landbo et al. 2007). These studies have also indicated that the enzyme catalyzed release of phenolics from grape skins is a result of a random liberation of phenols from the fruit skin cell wall matrix in response to the progressive enzyme catalyzed degradation of the cell wall polysaccharides (Landbo and Meyer 2001; Revilla and Gonzalez-SanJose 2003; Pinelo et al. 2006). Significant changes of the phenols take place during wine making but possible changes during the initial stages of juice and phenols extraction have received surprisingly little attention. The results of the present study indicate that significant changes of (at least) the hydroxycinnamates may occur already during a few hours of contact between the grape skins and the released hydroxycinnamates present in the liquid (or juice) surrounding the grape skin material. The evolution of different phenolics, including \(p\)-coumaric and caffeic acid and other hydroxycinnamates, has been studied in Cabernet Sauvignon grapes (*Vitis vinifera* L. cv. Cabernet Sauvignon) in relation to grape berry development (Chen et al. 2006). Biotransformation of \(p\)-coumaric acid to caffeic acid by the white-rot fungus *Pycnoporus cinnabarinus*, grown with high feeding of \(p\)-coumaric acid (0.1–1 g \(l^{-1}\)), has been proposed as a possible alternative route to produce the antioxidant caffeic acid (Alvarado et al. 2003). However, to our knowledge, the endogenous capacity of grapes to catalyze the upgrading of \(p\)-coumaric acid to caffeic acid has never been considered in relation to neither wine making nor other grape processing operations. The deliberate promotion (by enzyme catalysis) enhanced levels of caffeic acid from \(p\)-coumaric acid may be a new type of strategy to boost the antioxidant potency of grape juice and red wine, and may open the door for novel routes to give added value to grape pomace. The caffeic acid obtained by biotechnological processing of products from plant origin, using endogenous enzymes, would be classified as natural by the US and EU legislation.

**Acknowledgments** The Distell Group Ltd (Stellenbosch, South Africa) is acknowledged for supplementation of the wine grape samples. Donation of the Pectinex BE Colour enzyme preparation from Novozymes A/S is gratefully acknowledged.

**References**


The quantitative compositional profiles of the carbohydrates and phenolics present in the grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins were assessed in detail. This assessment is a prerequisite for assessing which phenolics, or which specific carbohydrate structures that can be enzymatically obtained from grape or apple skin.

Assessment of the fruit skin cell wall monosaccharides was done by means of acid hydrolysis where the efficiency of different acid hydrolysis methodologies was evaluated. The use of trifluoroacetic acid (TFA) as hydrolysing agent was shown to be superior to hydrochloric acid (HCl) after the recovery value adjustment, and hydrolysis with TFA could be accomplished in one-step.

The acid hydrolysis of polysaccharides to monocarbohydrates was easily fully integrated with the chromatography method HPAEC-PAD. The separation and the quantification method using HPAEC-PAD were accomplished in one single run without the need for pre-derivative treatment.

Elucidation of the main building blocks of the cell-wall polysaccharide structures could be achieved based on iterative calculation methodology. The method involves the fitting of monosaccharide profile data (monosaccharides released by acid hydrolysis), to current knowledge of dicot plant cell wall structures to obtain a quantitative allocation of monomers into different structural carbohydrate polymer elements. The monosaccharides identified were mainly galacturonic acid, arabinose, glucose, galactose, and in minor amounts: xylose, rhamnose, mannose, and fucose.

For both the grape and apple skin samples the skin cell wall polysaccharide matrices appeared to be mainly made up of pectins. The pectins were presented mainly by homogalacturonans, rhamnogalacturonan I, and rhamnogalacturonan II, and to a lesser degree xyloglucan and cellulose. Surprisingly the skins were high in Klason-lignin (35–45%).

Analyses of the phenolics after extensive, sequential aqueous methanol (60 % w/w) extraction of the fruit skins confirmed that grape skins are a good source of anthocyanin
pigments, notably the 3-glucosides of malvidin and cyanidin, and demonstrated that apple skins are a potential source of catechins.

Enzymatic release of phenolics from the grape skin of two representatives of wine grapes, Merlot and Cabernet Sauvignon, was assayed. The treatments used were pectinolytic (Pectinex® BE Colour or Vinozyme® FCE G preparation) or cellulolytic (Celluclast® 1.5 L FG) degradation enzymes.

The present study showed several interesting new findings:

After treatment of the grape skin with pectolytic enzyme preparation, anthocyanins were released first, but did not correlate to any monosaccharide production, and were thus not directly related to enzyme catalyzed degradation of the grape skin cell wall polysaccharides. The extended enzymatic treatments affected the anthocyanins negatively due to temperature as the main factor. The loss of acylated anthocyanins might be related to the formation of hydroxycinnamates, notably p-coumaric acid.

The flavonols, mainly rutin, were not enzymatically released during the enzymatic cell wall polysaccharide saccharification of the skins, but the forced enzymatic maceration induced conversion of rutin to quercetin, presumably as a result of a two step deglycosylation. The correlation between the flavonols and the extent of enzymatic polysaccharide degradation were complex and mainly negative.

The phenolic acids, i.e. hydroxybenzoic and hydroxycinnamic acids were correlated with the liberation of all the different monosaccharides analysed, particularly exponentially correlation with galacturonic acid and arabinose. The latter correlation indicated a certain relation to the extensive degradation of pectin, mainly homogalacturonan and rhamnogalacturonan I arabinan side chains; at the same time, the liberation of especially p-coumaric acid could be a result of release from acylated anthocyanins.

The increases in the quercetin and phenolic acids levels under enzymatic treatment is a strong indication of the possibility to enhance the antioxidant potential of grape juice and wine by discriminate liberation of certain phenolic classes or by enzymatic modification. The data also shows that if mainly high colour extraction (in the form of anthocyanins) is desired, the treatment should be short, as the anthocyanins were only released in the initial phase of the extraction, and then subsequently may decay.
Ultimately we discovered an endogenous capacity of grape skin (Cabernet Sauvignon) to catalyze the in vitro enzymatic hydroxylation of p-coumaric acid to caffeic acid. Grape skins might harbour an o-hydroxylation activity, proposedly a monophenol- or a flavonoid 3′-monooxygenase activity (EC 1.14.18.1 or EC 1.14.13.21). The work signifies that the grape skins can be used as a "catalyst" to convert added p-coumaric acid to a more potent antioxidant, caffeic acid. Hence, the principle use of the grape skins as a catalyst to accomplish o-hydroxylation may open the door for a completely new line of enzymatic improvements in juice and wine processing.

6.1 Future perspectives

We established that the degradation of the cell-wall polysaccharide structures is a fundamental step in the release of phenolics from the grape skins, notably those phenolics that are linked to the cell wall, but also those contained within or associated with cell vacuoles. Knowledge about the structural makeup of fruit skin polysaccharides is a prerequisite for tailoring of enzymatic treatments. Enzymes that cut specific sites in the wall offer the potential for structural and mechanical analyses of the cell wall. The ultimate goal is to develop hydrolysing methods, which target certain structural polysaccharides by using mono-activity enzymes that cut specific sites. Thereby possibly freeing and releasing interlocked or bound phenolics without harming the phenolics itself.

Sequential release of different phenolic classes could be the future strategy for recovering phenolics from different plant sources. By discriminating extraction methods sensitive anthocyanins and flavonols could be recovered at an early stage of extraction as a result of gentle loosening of plant cell wall followed by leaching. The released sensitive phenolics could be stabilised using non-conventional and non-thermal preservation techniques like electrical pulsing to inactivate endogenous enzymes like polyphenol oxidase (PPO). The conversion of flavonol rutin to quercetin may then take place subsequently and perhaps separately in a following step. In a later phase hydroxycinnamic and hydroxybenzoic acids could be release using extended specific enzymatic treatment.

As both grape and apple skins contain high amounts of lignin the understanding of the interaction of the lignins with enzymatic hydrolysis is important. The possibility to induce lignin degradation and thereby possibly loosen the structure of the cell wall polysaccharides could be a key factor in controlled cell wall hydrolysis events.
Chapter 6

The deliberate promotion (by enzyme catalysis) enhancing the levels of caffeic acid from \(\mu\)-coumaric acid, may be a new type of strategy to improve the antioxidant potency of grape juice and red wine, and may open the door for novel valorisation routes for grape pomace. Detailed insight into the specificity and working conditions, of this endogenous enzyme responsible for \(\alpha\)-hydroxylation activity, is needed.
References


Caccetta RAA, Croft KD, Beilin LJ, Puddey IB (2000) Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect \textit{ex vivo} lipoprotein oxidizability. Am J Clin Nutr 71:67-74


Plank, P. F. H. and Zent, J. B. Use of enzymes in wine making and grape processing. Gump, Barry H. and Pruett, David J. Beer and Wine Production: Analysis, Characterization, and


