ANTIMICROBIAL FACTOR FROM GRAPES

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Abstract

Food-borne pathogens and in particular the emergence of new food-borne bacterial pathogens, which includes *Listeria monocytogenes*, are a significant threat to public health. This thesis investigated a commercial red grape juice, derived from *Vitis vinifera* variety Ribier grapes, with a strong inhibitory action against *Listeria monocytogenes*. The commercial grape juice caused a 6-log reduction in *Listeria monocytogenes* numbers within 10 minutes exposure to the grape juice. The inhibitory effect was specific to all *Listeria* species. The grape juice was bactericidal and inhibitory at pH $\leq$ 5.0. The addition of protein (BSA), PVPP, and metal ions (Fe^{3+} and Mg^{2+}) suppressed the antilisterial activity of the grape juice. The antilisterial factor in the commercial grape juice was identified as pigmented condensed tannin (proanthocyanidin oligomers). The antilisterial pigmented condensed tannin in the commercial grape juice was likely derived from the skin of Ribier grapes. Fractionation of the commercial grape juice pigmented condensed tannin by molecular weight revealed that the antilisterial activity was distributed over a range (mean degree of polymerisation 2.8 through 8.5) of tannin fractions. Condensed tannin fraction (G) had a mean degree of polymerisation of 5.9 and was found to have the strongest antilisterial activity. The molecular weight of oligomers identified in fraction G ranged from MW 867 to MW 2905. Fraction G contained the highest percentage of polymeric colour with 90% the pigmented moieties (anthocyanins) in the fraction incorporated into the polymer structure. Fraction G was comprised of the flavonoid subunits catechin, epicatechin, epigallocatechin, and epicatechin-3-*O*-gallate.

Ribier grape seed condensed tannin had strong antilisterial activity. The antilisterial condensed tannin derived from Ribier grape seed was comprised of the flavonoid subunits catechin, epicatechin, and epicatechin-3-*O*-gallate and increasing antilisterial activity was associated with increasing mean degree of polymerisation of the tannin polymers. The efficacy of the pigmented condensed fraction of the commercial grape juice and Ribier grape seed condensed tannin as natural food preservatives were assessed. In the presence of *Listeria monocytogenes* contaminated cabbage the inhibitory activity of the pigmented condensed tannin from commercial grape juice was suppressed. The antilisterial activity of the Ribier grape seed condensed tannin was reduced but not eliminated when *Listeria monocytogenes* was associated with cabbage. The grape seed tannin may be effective at eliminating *Listeria monocytogenes* from food.

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List of Abbreviations

API	Analytical Profile Index
AWRI	Australian Wine Research Institute
BHI	Brain Heart Infusion broth
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
ESI-MS	Electrospray Ionising Mass Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
IES-MS	Infusion Electrospray Spectroscopy Mass Spectroscopy
LC-MS	Liquid Chromatography Mass Spectroscopy
mDP	Mean Degree of Polymerisation
MGJ	Model Grape Juice solution
MIC	Minimum Inhibitory Concentration
MPN	Most Probable Number
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
MWS	Model Wine Solution
PVPP	Polyvinylpolypyrrolidone
OD	Optical Density
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

1 INTRODUCTION

1.1 Food-borne Disease

The World Health Organisation (WHO) (1997) reported "hundreds of millions of people world-wide suffer from diseases caused by contaminated food." In industrialised countries that have safe water supplies, sound hygiene standards and food technologies, such as pasteurisation, the incidence of food-borne disease is increasing. WHO statistics show that 5-10% of the populations of industrialised countries are affected by food-borne disease. Although many food-borne infections have been controlled the emergence of new food-borne pathogens, in particular *Listeria monocytogenes*, *Escherichia coli* O157, and multi-antibiotic resistant *Salmonella* Typhimurium, are now a significant threat to public health (World Health Organisation, 1997).

Food-borne disease can be caused by a number of different agents. The most common cause is bacterial infection. Common food-borne bacterial pathogens include *Aeromonas hydrophila, Brucella* species*, Bacillus cereus* and other *Bacillus* species*, Campylobacter* species*, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Listeria monocytogenes, Plesiomonas shigelloides, Salmonella* species*, Shigella* species*, Staphylococcus aureus, Vibrio* species*,* and *Yersinia enterocolitica* (Adams & Moss, 1995). Non-bacterial agents may also cause disease by transmission from food. These include helminths and nematodes, protozoa, toxigenic algae, toxigenic fungi, food-borne viruses such as Norovirus and Hepatitis A, and chemical toxins like agricultural chemical residues.

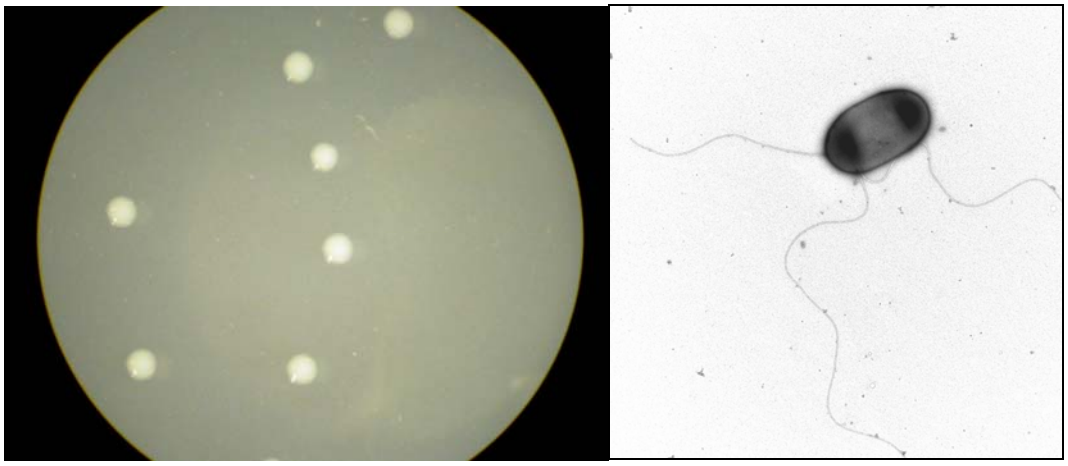
The World Health Organisation regards *L. monocytogenes* as one of three emerging food-borne pathogens that pose a significant threat to public health (World Health Organisation, 1997).

1.1.1 Listeria monocytogenes

Listeria monocytogenes is classified in the Bergey's manual under Regular, nonsporing gram-positive rods, which include the genera *Lactobacillus, Erysipelothrix, Brochothrix, Listeria, Kurthia, Caryophanon,* and *Renibacterium*. The *Listeria* genus contains the species *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi, L. murrayi* and *L.*

denitrificans (Kandler & Weiss, 1986). However *L. denitrificans* has recently been transferred to the new genus *Jonesia*, as *J. denitrificans* (Farber & Peterkin, 1991). Of the seven species of *Listeria*, *L. monocytogenes* is by far the most important human pathogen of the genus, although *L. seeligeri*, *L. welshimeri*, and *L. ivanovii* have occasionally been associated with human disease (Adams & Moss, 1995).

Listeria monocytogenes is ubiquitous in the environment, has been isolated from plant, soil, and water samples (Weis & Seeliger, 1975), and has been found in cattle, sheep, goats, and poultry (Gray & Killinger, 1966) and in the faeces of humans and cattle (McCarthy, 1990). *L. monocytogenes* is a facultative anaerobic bacterium able to grow between -1.5^{o}C (Hudson *et al*., 1994) and 45^{o}C with an optimum growth temperature of 30-37^{o}C (Kandler & Weiss, 1986). Cells are motile by peritrichous flagella when cultured at 20-25^{o}C (**Figure 1.1**).



1.1 **Figure 1.1** ***Listeria monocytogenes*** **colonies grown on tryptic soy agar (left) and transmission electron microscope image of** ***Listeria monocytogenes*** **(right).**

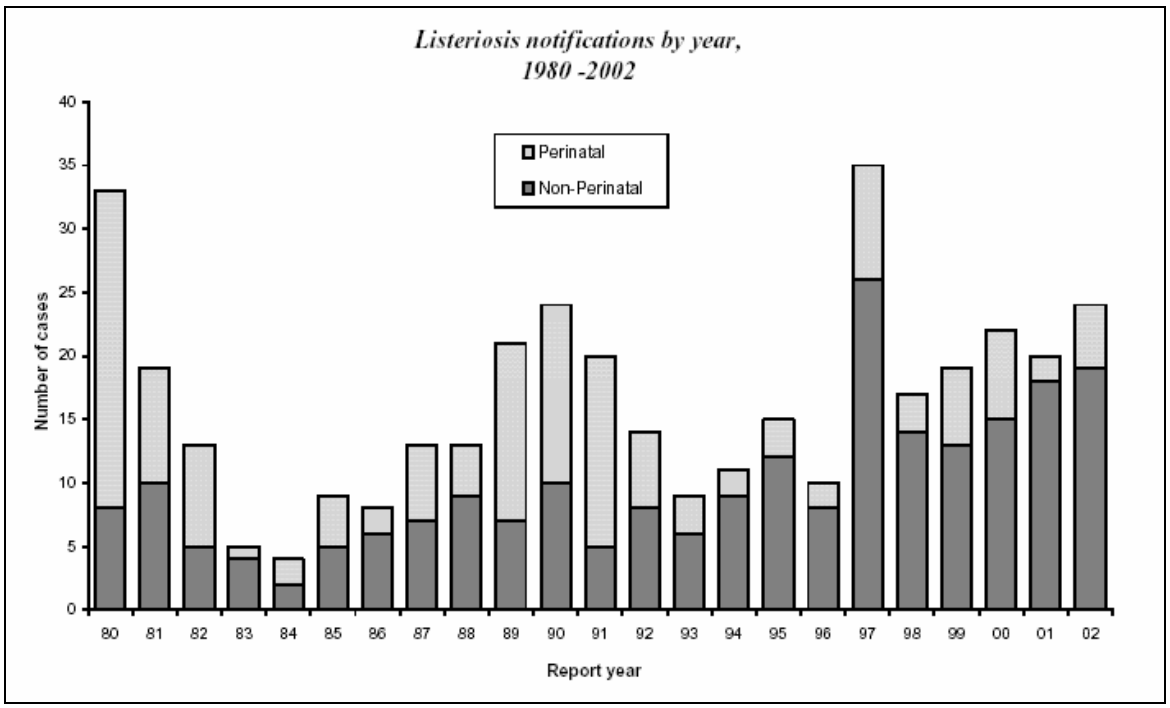
Listeria monocytogenes has an optimum pH for growth of 7.0 but is able to grow over the range of 4.4-9.4. Farber *et al*., (1989) reported that four strains of *L. monocytogenes* grown in brain heart infusion broth were more susceptible to organic acidulants such as acetic, lactic and citric acid than to mineral acidulant hydrochloric acid. *L. monocytogenes* is able to grow in nutrient broth supplemented with up to 10% NaCl. Characteristic biochemical reactions of *L. monocytogenes*

include production of β-hemolysis, catalase positive, oxidase negative, Voges-Proskauer positive, methyl red positive, aesculin hydrolysis, and fermentation of a number of sugars including glucose and rhamnose (Kandler & Weiss, 1986). There are 13 serovars of *Listeria monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7) and the majority of *L. monocytogenes* isolated from food belong to serogroup 1 (Gilot *et al*., 1996). Most outbreaks of food-borne listeriosis have been caused by *L. monocytogenes* serovar 4b (Farber & Peterkin, 1991), though sporadic cases of listeriosis have been linked to serovars 1/2a and 1/2b (Gilot *et al*., 1996)

1.1.2 Listeriosis

Listeria monocytogenes has been identified as an emerging food-borne pathogen (Tauxe, 2002) and although it has been known as a pathogen for a number of decades it has only recently been found to be transmissible by ingesting contaminated food (Schlech *et al*., 1983). *L. monocytogenes* can cause the disease listeriosis, which can take on two forms, a serious invasive form and a non-invasive febrile gastroenteritis form. The serious invasive form can affect individuals with weakened or underdeveloped immune systems such as the immunocompromised, the elderly and perinatals (3 months before birth and 1 month post birth) and can result in a number of clinical conditions including meningitis, encephalitis, septicaemia, endocarditis, abortion, abscesses and lesions. In New Zealand this form of listeriosis had a mortality rate of 11.1% (2001 and 2002 data) (Sneyd & Baker, 2003). The disease-causing dose of *L. monocytogenes*, causing the invasive form of listeriosis, is not known.

The gastroenteritis and flu-like form of listeriosis can effect otherwise healthy individuals that have consumed foods containing more than 10^7 cells *of L. monocytogenes* (Sim *et al*., 2002). A small percentage (2-6%) of the human population are asymptomatic carriers of *L. monocytogenes* (Sutherland *et al*., 2003) and there were 347 cases of invasive listeriosis in New Zealand between 1980 and 2002 (Sneyd & Baker, 2003) with perinatal infection comprising a significant proportion of cases (**Figure 1.2**).



2.1 **Figure 1.2 Invasive Listeriosis notifications in New Zealand from 1980 to 2002** (Sneyd & Baker, 2003).

Listeria monocytogenes is a facultative intracellular pathogen. The mode of action of *L. monocytogenes* infection causing listeriosis was recently clarified by the work of Lecuit *et al*., (2001) who discovered the role of a protein produced by *L. monocytogenes* that enables it to cross the human intestinal barrier. Using transgenic mouse and guinea pig models of listeriosis the authors reported that *Listeria* is able to enter the gut epithelial cells by binding its surface protein, internalin, to an epithelial trans-membrane protein called E-cadherin. The bacterium then expresses a virulence factor p60, a 60kDa protein, which induces the endocytosis of the bacterium by the gut cell. Once inside the phagocytic vacuole the bacterium produces the proteins, haemolysin and listeriolysin O, which allow it to escape the vacuole and multiply in the host-cell cytoplasm.

1.1.3 Outbreaks of Listeriosis

Globally, *L. monocytogenes* has been isolated from various foods including dairy products, vegetables, seafood, and cooked meats (Hitchins & Whiting, 2001) and outbreaks of listeriosis have been linked to a diverse range of foods.

The consumption of raw vegetables has been linked to a number of outbreaks of listeriosis. Schlech *et al*., (1983) reported an outbreak of listeriosis in Canada in which infections occurred in 7 adults and 34 perinatals. Coleslaw contaminated with *L. monocytogenes* was identified as the vehicle of transmission of the disease. They found that the cabbage used for preparing the coleslaw was from a farm known to have had cases of ovine listeriosis and that raw ovine manure had been used to fertilise the cabbage crop. Aureli *et al*., (2000) reported a large outbreak of febrile gastroenteritis among students and staff at two Italian schools. The outbreak was caused by the consumption of a cold salad of corn and tuna contaminated with L. *monocytogenes*. A total of 292 people were hospitalised.

Outbreaks of listeriosis have been linked to dairy products that have not been pasteurised or have suffered post-pasteurisation contamination and temperature abuse. Dalton *et al*., (1997) reported an outbreak of febrile gastroenteritis in America which affected 45 people who had ingested chocolate milk contaminated with *L. monocytogenes*. This outbreak was caused by post-pasteurisation contamination of the chocolate milk and was exacerbated by inadequate milk holding temperatures (unrefrigerated), thus allowing the rapid growth of *L. monocytogenes*. Bille (1990) reported an increased rate of listeriosis in Switzerland from 1983 to 1987. Strains of *L. monocytogenes* associated with illness were found on the surface of a locally produced soft cheese. It was not stated whether the milk used to make the cheese was pasteurised. The cheese was withdrawn from the market and no new cases due to the endemic strain of *L. monocytogenes* have occurred.

The most publicised recent outbreak of invasive listeriosis in New Zealand occurred in 1992 when two perinatal cases of listeriosis were linked to the consumption of *L. monocytogenes* contaminated smoked mussels by pregnant women (Brett *et al*., 1998). Thirty-one recent cases of febrile gastroenteritis listeriosis incidents in New Zealand have been linked to the consumption of ready-to-eat meats such as corned beef and ham. The long shelf lives marked on these products may have allowed *L. monocytogenes* numbers to grow to high enough levels to cause disease (Sim *et al*., 2002).

1.1.3.1 Food Recalls in New Zealand

For the three-year August 2000 to August 2003 period, a total of 47 food products were recalled from the New Zealand domestic market because of contamination, with 10 of the recalls (ca. 20%) being attributed to pathogenic bacterial contamination. Of these 10 recalls, a significantly high percentage (70%) were associated with *Listeria* contamination, with three of the *Listeria* recalls

associated with dairy products, three with meat products and one with a seafood product (New Zealand Consumer, September 2003).

1.1.4 Control of Microorganisms in Food

There are a number of ways in which microorganisms are eliminated from food. Standard methods of elimination include heat processing, irradiation, high-pressure processing, low-temperature processing, modification of atmosphere, control of water activity, and chemical preservation.

Heat processing encompasses a number of different heating methods to inactivate the target bacteria to the desired extent. Pasteurisation involves heating the food product or material to a temperature range of typically between 60-80^{o}C for a given time period to eliminate both pathogens and spoilage organisms (Board, 2001). Generally, foods that are pasteurised to preserve them have other intrinsic antimicrobial attributes such as low pH or low water activity. The heating time of a food will depend on the type of organism to be eliminated. In the case of *L. monocytogenes* the thermal death time at 60^{o}C (D_{60}) is 5-8.3 minutes (Adams & Moss, 1995). However, many factors influence the heat resistance of *L. monocytogenes* including strain type, age of culture, and characteristics of the food such as the presence of other inhibitors, salt concentration, fat content, and water activity (Doyle *et al*., 2001). The heating of food at temperatures greater than 100^{o}C eliminates viable bacteria from food and drying and concentrating food at greater than 100^{o}C results in the removal of water from the food and the elimination of viable bacteria.

Irradiation of foods by microwave radiation, UV radiation, and ionising radiation can kill bacteria in food. Microwave radiation heats the water in the food and this generation of heat destroys any bacteria present in the food. UV radiation utilises wavelengths below 450 nm. The greatest lethality is shown by wavelengths around 260 nm, which damage the microorganism's DNA and prevents the microorganism from replicating. Ionising radiation has frequencies of greater than 10^{18} Hz and like UV radiation can damage the chromosomal DNA (Adams & Moss, 1995).

The use of high pressure (Pascalization) can be used to eliminate bacteria from food. High pressure compresses the lipid membranes of vegetative bacteria and fungi and alters membrane permeability. The commercial application of high pressure processing has been applied to fruit juices, jams, sauces, and sliced cold meats such as ham (Stewart & Cole, 2001). Chilling or refrigeration (0-5^{o}C) of food retards bacterial growth. However, once the food is removed from the low temperature environment the bacteria may recover and resume growth (Brock *et al*., 1994). Freezing food allows for long-term storage as bacteria are unable to grow at freezer temperature (-20^{o}C) but as

with chilling, freezing does not sterilise a food and bacteria may persist, recover and grow, once the food is thawed.

Alteration of the atmosphere of the food such as reducing oxygen levels through vacuum packaging or replacement of oxygen with a mix of carbon dioxide, oxygen, and nitrogen (modified atmosphere packaging) or 10% carbon dioxide (controlled atmosphere storage) can be used to prevent the growth of bacteria (Szabo & Coventry, 2001). However these techniques do not eliminate bacteria from the food and are generally used in conjunction with refrigerated or chilled storage.

The preservation of foods by reducing the water activity, using salt, sugar, and drying has been known for centuries. The reduction of water activity inhibits the growth of microorganisms and thus enables the ambient shelf life of an array of foods such as dried meats, cheeses, pasta, dried fruits, jams, and salted dried fish to be extended (Steele, 2001). However, as with a number of the standard food preservation techniques, prevention of bacterial contamination and bacterial growth in the raw material prior to preservation is vital as microorganisms may persist in the food product and multiply once favourable growth conditions are reinstated.

Chemical preservatives are defined as "substances capable of inhibiting, retarding or arresting the growth of microorganisms" (Adams & Moss, 1995). Chemical preservatives can be bactericidal, killing the target bacteria, or bacteriostatic, preventing the growth of the bacteria. A number of organic acids and their salts have antimicrobial activity and are widely used in the food processing industry (Cliver & Marth, 1990). Organic acids and their salts exert their antimicrobial action when they are in the undissociated form and are therefore more effective in acidic foods (Beuchat & Golden, 1989). Common antimicrobial organic acids used in the food processing industry are benzoic acid, acetic acid, lactic acid, citric acid, propionic acid, sorbic acid, *para*-hydroxybenzoic acid, and their salts (Giese, 1994). Nitrite is used to cure meats like bacon and is inhibitory against a wide range of bacteria. Importantly nitrite is able to inhibit the germination and growth of spores where other preservation techniques and chemicals do not (Adams & Moss, 1995). Sulphur dioxide is another common chemical preservative and is used widely in the wine industry and for fruit products (Giese, 1994). Medium-chain fatty acids (chain length 12-18 carbon atoms) have antimicrobial activity that is more effective against gram-positive microorganisms, including *L. monocytogenes*, and yeasts (Beuchat & Golden, 1989). The undissociated form of the fatty acid molecule is responsible for the antimicrobial effect.

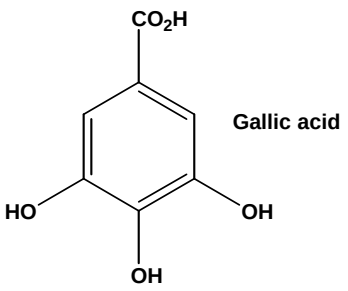
Consumer driven demand for foods free of "unnatural" preservatives such as chemicals has resulted in the restriction and subsequent decline of the use of chemical preservatives in the food processing industry. The demand for perceived "natural" preservatives in food is of growing interest and as a result the number of new natural antimicrobials that are derived from plants, bacterial, and animal sources continues to grow.

1.2 Antimicrobial Phenolic Compounds from Plant Sources

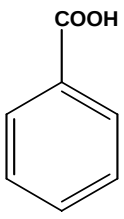
Phenolic compounds, whether simple or complex, are present in all plants and exhibit three major chemical properties. Firstly, they are acidic and partially dissociate in water. Secondly, the phenolic hydroxyl groups can form hydrogen bonds by intermolecular and/or intramolecular interaction. In the case of natural phenols intramolecular hydrogen bonding is common. The formation of hydrogen bonds between or within molecules affects the physical and chemical properties of the molecule. Thirdly, the phenolic hydroxyl group can form complexes with metal ions, in particularly iron and aluminium (Ribéreau-Gayon, 1972). In addition to hydrogen bonding, hydrophobic interactions play a significant role in the interaction of polyphenols with proteins and molecules such as polyvinylpolypyrrolidone (PVPP), and with each other (Haslam, 1998). There are a number of different types of phenolic compounds present in plants including hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and some phytoalexins.

1.2.1 Hydroxybenzoic acids and Hydroxycinnamic acid derivatives

Hydroxybenzoic acids and hydroxycinnamic acid derivatives are commonly found in plants. Phenolic acids are a range of substituted benzoic acid derivatives and present naturally in many plants and fruits. Phenolic acids have the general structure of C_6-C_1 (refer gallic acid structure **Figure 1.3**) and usually occur in conjugated or esterified forms (Ribéreau-Gayon, 1972).



Gallic acid

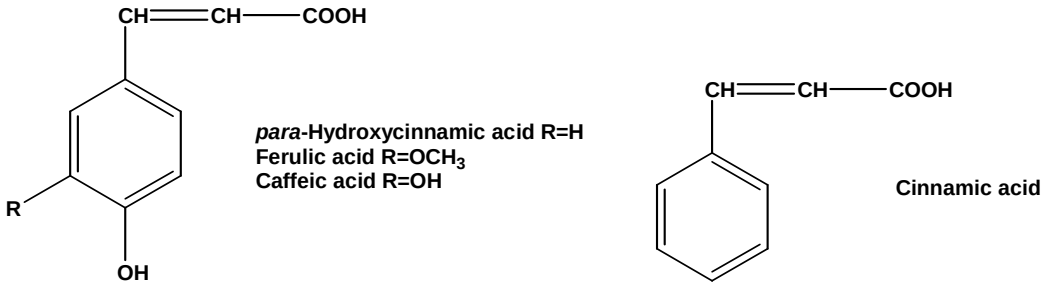


Benzoic acid

3.1 Figure 1.3 Structures of gallic acid and benzoic acid.

Tesaki *et al*., (1999) reported the inhibitory action of gallic acid (pK 4.41) against *Escherichia coli, Salmonella* Enteritidis, and *Staphylococcus aureus*. Panizzi *et al*., (2002) also found that gallic acid had antimicrobial activity against a range of bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and the fungus *Candida albicans*. Benzoic acid (pK 4.19) is one of the oldest and most commonly used food preservatives with the undissociated form of the acid responsible for the antimicrobial activity (Beuchat & Golden, 1989).

Hydroxycinnamic acids are distributed widely in the plant kingdom. The most common hydroxycinnamic acids present in plants are *para*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid, and practically all plants contain at least one of them (Ribéreau-Gayon, 1972). They have the general structure C_6-C_3 (**Figure 1.4**) and do not occur as free acids but as esters of quinic or shikimic acid or as sugar esters (Walker, 1975). Hydroxycinnamic acids contain a double bond and can therefore exist in two isomeric forms, *cis* and *trans*. Naturally occurring hydroxycinnamic acids exist in the more stable *trans* isomeric form. Derivatives of hydroxycinnamic acids including glycosides and sugar esters may be found covalently bonded to other phenolic compounds such as anthocyanins (Ribéreau-Gayon, 1972).

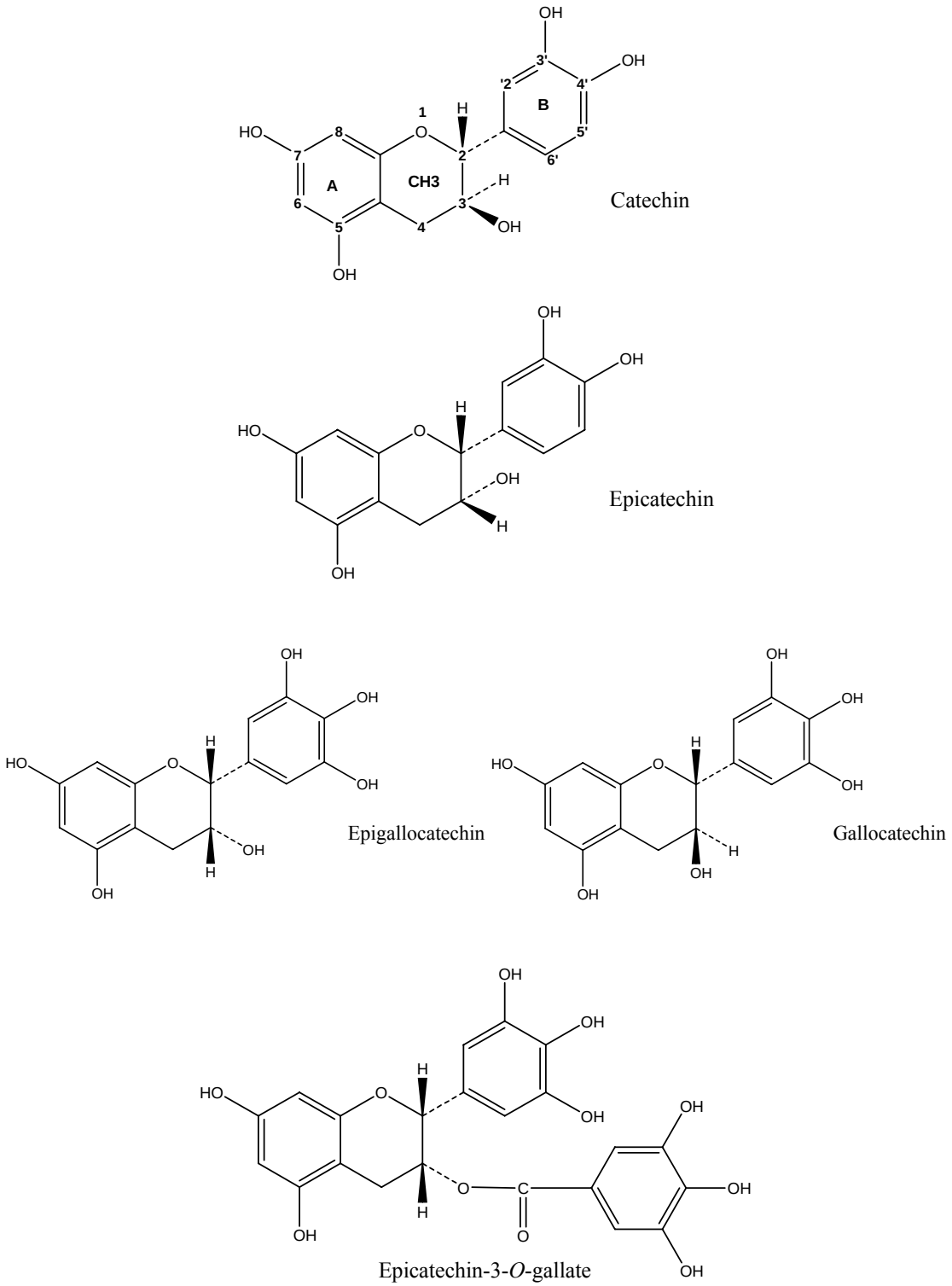


4.1 Figure 1.4 Structures of hydroxycinnamic acids, cinnamic acid.

The antimicrobial activity of a number of hydroxycinnamic acids has been reported. Wen *et al*., (2003) found that a number of hydroxycinnamic acids, had antilisterial activity. They found that cinnamic acid (*cis*-cinnamic acid pK 3.89, *trans*-cinnamic acid pK 4.44) had the strongest inhibitory activity and this activity was pH dependent, being bactericidal at pH 4.5 and bacteriostatic at pH 5.5. The order of increasing antilisterial activity of hydroxycinnamic acids was found to be cinnamic acid, *para*-coumaric acid, ferulic acid, and caffeic acid. Ferulic acid was also found to have antimicrobial activity against a range of bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* and the fungal isolates *Saccharomyces cerevisiae*, *Candida albicans* with slight activity against *Aspergillus niger* (Panizzi *et al*., 2002).

1.2.2 Monomeric Flavan-3-ols

Monomeric flavan-3-ols are flavonoid compounds frequently found in plant tissue where they can be found in monomeric or polymeric forms. The most important of these compounds are the isomers catechin and epicatechin (**Figure 1.5**). They have the structure C_6-C_3-C_6 and unlike other classes of flavonoids are not generally glycosylated or esterified (Ribéreau-Gayon, 1972), with the exception of the epicatechin-3-*O*-gallate identified in grapes (Su & Singleton, 1969). Monomeric flavan-3-ols with a trihydroxylated B ring exist as gallocatechin and epigallocatechin. Flavan-3-ols are frequently found in a polymerised form (e.g. condensed tannin).

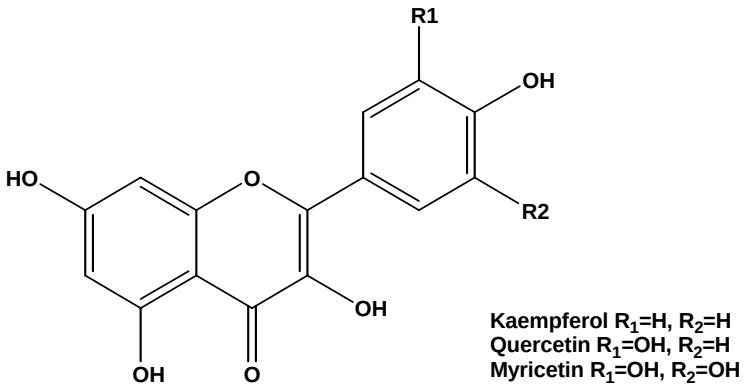


5.1 Figure 1.5 Structures of monomeric flavan-3-ols, catechin, epicatechin, gallocatechin, epigallocatechin, and epicatechin-3-*O*-gallate.

The antimicrobial activity of flavan-3-ols has been reported. Rauha *et al*., (2000) tested the antimicrobial activity of catechin and epicatechin on a range of microorganisms and found only the gram-positive bacterium *Staphylococcus aureus* to be slightly inhibited by both compounds and the mould *Aspergillus niger* slightly inhibited by epicatechin. A range of flavan-3-ols, found in green tea, were found to have an inhibitory action against the gram-negative bacterium *Helicobacter pylori*. Through structure-activity relationship studies the authors found that the antimicrobial activity was predominantly related to the gallic acid moiety and the number of hydroxyl groups with epigallocatechin gallate having stronger antimicrobial activity than epicatechin, epigallocatechin, and epicatechin-3-*O*-gallate. The authors also found that the antimicrobial activity of this compound was pH dependent with activity present at pH 7.0 but not at pH <5.0 (Mabe *et al*., 1999). Nakane & Ono (1990) found that the tea-derived flavan-3-ols, epicatechin gallate and epigallocatechin gallate, inhibited the activities of the enzymes reverse transcriptase and DNA and RNA polymerase of the HIV virus. They found that the monomeric units, epicatechin, epigallocatechin and gallic acid, of epicatechin gallate and epigallocatechin gallate, were not inhibitory to the enzymes assayed. Ikigai *et al*., (1993) investigated the antimicrobial effect of the flavan-3-ols epigallocatechin gallate and epicatechin. They found that epigallocatechin gallate was more inhibitory than epicatechin and that gram-positive bacteria were more susceptible than gram-negative bacteria. Their results showed that the mode of action of these flavan-3-ols was to cause damage to the bacterial cell membrane and they suggest that the outer membrane of gram-negative bacteria provides a protective barrier and prevents these flavan-3-ols from binding to the bacterial cell.

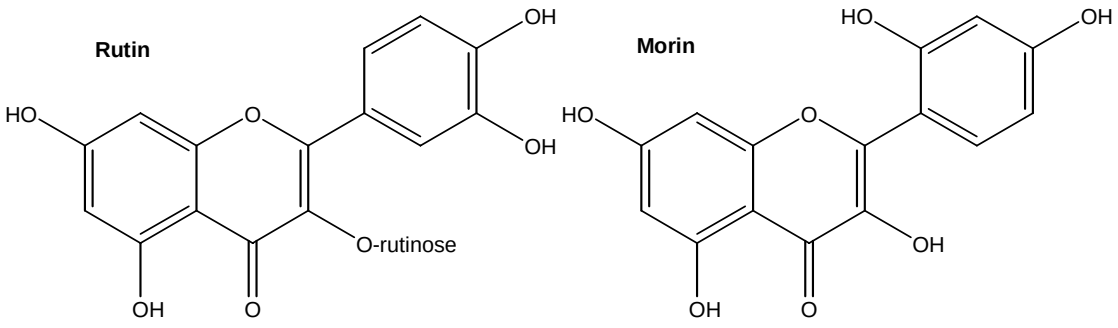
1.2.3 Flavonols

Flavonols are a class of flavonoids and are widely distributed in the plant kingdom. More than 200 flavonol have been identified in plants (Macheix *et al*., 1990). Flavonol aglycones have the general structure C_6-C_3-C_6 (**Figure 1.6**) but also exist as glycosides in which the C_6-C_3-C_6 aglycone part of the molecule is esterified with a number of different sugars. The three most common flavonols, kaempferol, myricetin, and quercetin, the latter is the most widely spread (Ribéreau-Gayon, 1972).



6.1 Figure 1.6 Structures of typical flavonols, kaempferol, quercetin, and myricetin.

Mori *et al*., (1987) reported the antimicrobial activity of a range of flavonols against the gram-positive bacterium *Staphylococcus aureus* and the gram-negative bacterium *Proteus vulgaris* and suggested that free hydroxyl groups on the A and B rings of the molecule were necessary for antimicrobial activity. They found that DNA synthesis inhibition was the predominant action against *P. vulgaris*, whereas RNA synthesis was inhibited in *S. aureus*. The antimicrobial activity of the extracts of several species of New Zealand Epacridaceae (Grass tree) plants, against multi-resistant *Staphylococcus auerus* (MRSA), has been reported. The anti-MRSA compound was identified as the flavonol kaempferol-diacyl-rhamnoside (Bloor, 1995). The flavonols, quercetin and its isomer morin, were found to have antimicrobial activity against a range of bacteria but not against moulds and yeasts. The glycosidic form of quercetin, rutin, did not have any antimicrobial activity (Rauha *et al*., 2000) (**Figure 1.7**).

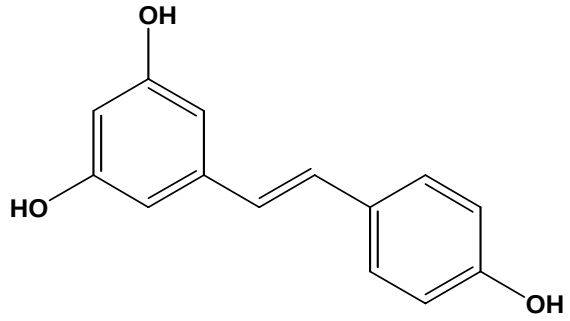


7.1 Figure 1.7 Structures of rutin and morin.

Puupponen-Pimiä *et al*., (2001) found that quercetin inhibited the growth of a gram-negative DNA repair mutant *Escherichia coli* CM 871 but did not inhibit the growth of *Escherichia coli* 50, which has a functioning DNA repair mechanism. They hypothesised that the main reason for the antimicrobial activity of quercetin was its interaction with the bacterial DNA although they did not report the mechanism of this reaction. Padmavati *et al*., (1997) reported the antimicrobial activity of the flavonol kaempferol against the rice pathogenic fungi *Pyricularia oryzae*. Kaempferol showed significant inhibition of spore germination of this fungus, but did not inhibit the spore germination of *Rhizoctonia solani*, another rice pathogenic fungus.

1.2.4 Phenolic Phytoalexins

Phytoalexins are compounds produced by plants as a defence mechanism against phytopathogenic microorganisms and other environmental stresses (Hart, 1981; O'Brien & Wood, 1973). Resveratrol, a stilbene phytoalexin found in grapes and red wine, has been widely studied (Siemann & Creasy, 1992; Goldberg *et al*., 1995b; Romero-Perez *et al*., 1996) because of its implications for phytopathology (Langcake & Pryce, 1976) and human health, in particular the "French Paradox" in which consumption of red wine has been linked to protection from heart disease (Goldberg *et al*., 1995a). This protection has been attributed to the antioxidant activity of resveratrol. Stilbenes have the general structure of two C_6 rings linked by an ethylene group (**Figure 1.8**), and in nature these compounds are generally in the *trans* form.



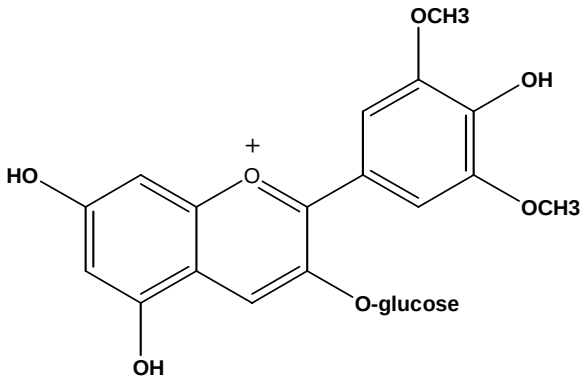
8.1 Figure 1.8 Structure of a phytoalexin, *trans*-resveratrol.

The antimicrobial activity of *trans*-resveratrol has been studied by a number of authors. *Trans*-resveratrol is present in red grape skin and therefore may confer antimicrobial action to red wine

and grape skin extracts. Docherty *et al*., (1999) found that *trans*-resveratrol inhibited the replication of the herpes simplex virus. Resveratrol was found to have antimicrobial activity against *Neisseria gonorrhoeae* and *Neisseria meningitidis* (both gram-negative) but had no inhibitory effect against the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* or the gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes,* and the yeast *Candida albicans* (Docherty *et al*., 2001). In contrast to this finding, Chan (2002) reported antibacterial activity of resveratrol against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and against a range of human skin pathogenic fungi. This difference may be explained by the low solubility of resveratrol in aqueous solutions. Docherty *et al*., (2001) dissolved resveratrol in ethanol and then diluted the ethanol to 0.5% whereas Chan (2002) dissolved the resveratrol in >1% dimethyl sulfoxide. Findings by Filip *et al*., (2003) showed that the solubility of synthetically prepared resveratrol was 40 mg/L in 10% ethanol, which could explain the conflicting antimicrobial spectrum of resveratrol reported by Chan (2002) and Docherty *et al*., (2001). Filip *et al*., (2003) also reported the antifungal activity of this synthetic resveratrol.

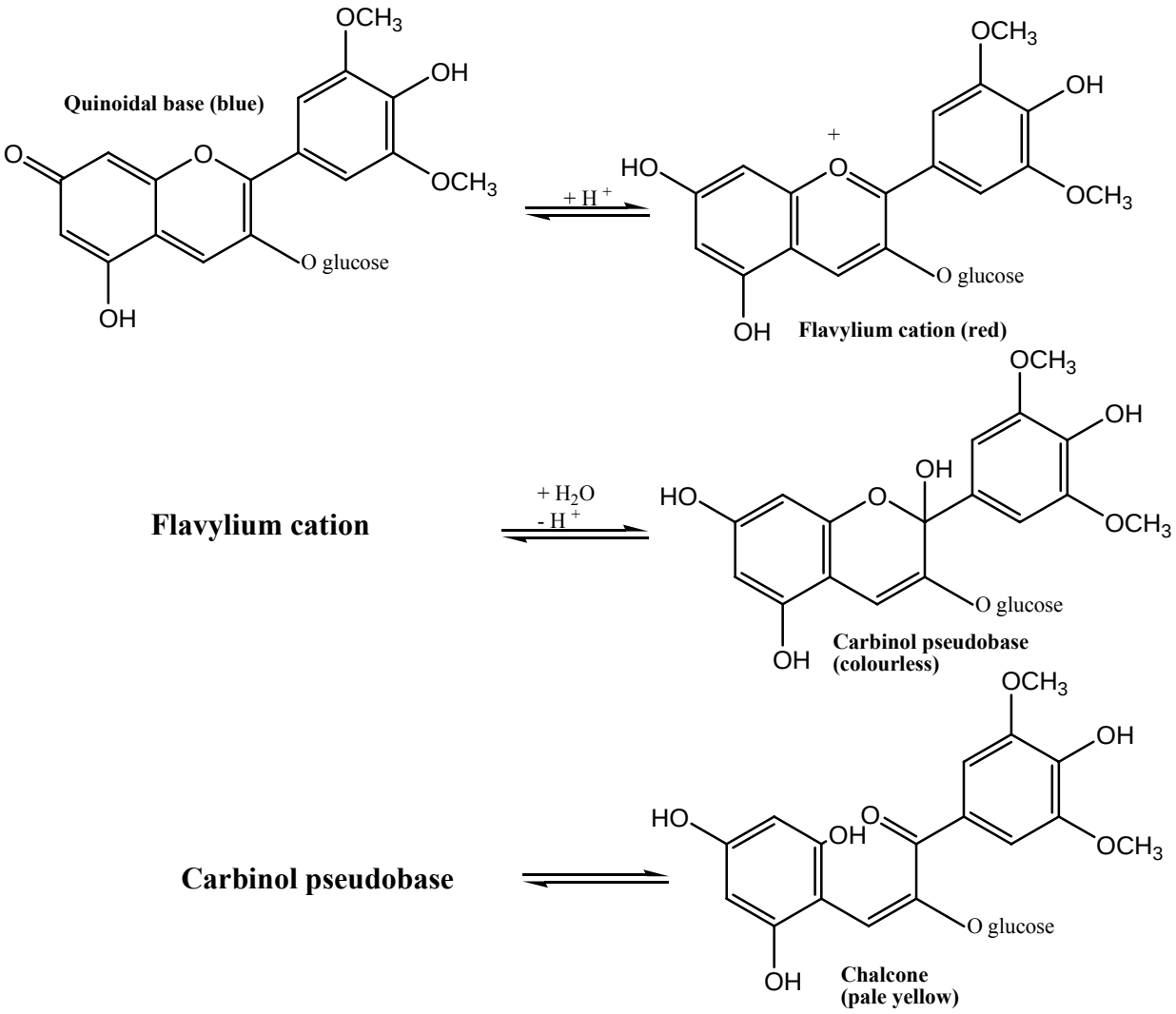
1.2.5 Anthocyanins

Anthocyanins are the universal plant colourants, with some exceptions (e.g. carotenoids and betalins), and are largely responsible for the colour of petals and fruits. Anthocyanins are generally found in solution in plant vacuoles and probably play a role in attracting animals and birds to act as pollinators or seed dispersers (Strack & Wray, 1994). There are five anthocyanidins in grapes: delphinidin, petunidin, malvidin, cyanidin, and peonidin. These anthocyanidin aglycones also exist as glycosides (anthocyanins): 3-monoglucosides and 3,5-diglucosides, and as acylated heterosides (Ribéreau-Gayon, 1974). Anthocyanins are a class of flavonoids and have the general structure C_6-C_3-C_6 (**Figure 1.9**).



9.1 Figure 1.9 Structure of a typical anthocyanin, malvidin-3-glucoside.

Anthocyanins occur naturally as glycosides with the most common sugar substitution being on the 3 position on the B ring. They can also be diglycosylated and acetylated, via an ester linkage to the sugar hydroxyl group, with acids such as *p*-coumaric acid, ferulic acid, and caffeic acid (Ribéreau-Gayon, 1972). Anthocyanins all have the same structure with the degree of hydroxylation and methylation of the benzene rings differentiating them. The coloured nature of anthocyanins is pH dependent. In acidic medium they are red, losing their colour as the pH increases with maximum colour loss observed at pH values of 3.2 to 3.5 (Ribéreau-Gayon *et al*., 1999). In strongly acidic media (pH<2.5), anthocyanins form stable flavylium cations whose colour is orange or red. When the acidicity of a solution of anthocyanins is reduced (pH 4-6) the blue quinoidal base is formed first, but is rapidly decolourised to form a carbinol pseudobase by hydration (Goto & Kondo, 1991). (**Figure 1.10**). At equilibrium and at any pH the quinoidal base is only present to a very small extent (Brouillard & Delaporte, 1977).



10.1 **Figure 1.10 pH equilibrium of malvidin-3-glucoside** (Brouillard & Delaporte, 1977).

The antimicrobial action of anthocyanins against a range of bacteria has been reported. Pratt *et al*., (1960) observed that anthocyanins, monoglucosides of cyanidin, pelargonidin, and delphinidin, isolated from frozen strawberries and commercially bottled Concord red grape juice decreased the maximum growth attained by *Escherichia coli* (gram-negative) and *Lactobacillus acidophilus* (gram-positive). Powers *et al*., (1960) found inhibitory and stimulatory growth effect of a number of anthocyanins. The anthocyanins pelargonium-3-glucoside and delphinidin-3-glucoside inhibited the growth of *Escherichia coli* whereas malvidin and delphinidin (unglycosylated) stimulated the growth of the microorganism. Hamdy *et al*., (1961) assessed the antimicrobial activity of pelargonidin-3-glucoside degraded by heat treatment and non-degraded, by disc diffusion assay and found that both the degraded and non-degraded anthocyanin inhibited the growth of *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive). Powers (1964) performed antimicrobial assays using a number of anthocyanins against a range of gram-negative and gram-positive bacteria, including *Salmonella* Typhosa (now *Salmonella* Typhi), *Salmonella* Enteritidis, *Shigella paradysenteriae, Staphylococcus aureus, Aerobacter aerogenes, Proteus vulgaris, Escherichia coli, Lactobacillus casei,* and *Lactobacillus acidophilus*. It was found that when glucose was present as a growth substrate most of the anthocyanins tested inhibited bacterial respiration and reproduction. In the absence of glucose bacterial cells metabolised the anthocyanins. The mode of antimicrobial action of anthocyanins against bacteria is not well understood. Somaatmadja *et al*., (1963) found that anthocyanin pigments were able to inhibit glucose oxidation of certain bacteria and that the inhibitory effect of malvidin-3-glucoside was reversed by the addition of magnesium or calcium to the growth media. They also reported the metal-complexing ability of malvidin-3-glucoside and suggest that this anthocyanin may exert its antimicrobial effect by chelating metal ions necessary for bacteria growth. Carpenter *et al*., (1967) reported the inhibitory effect of monoglucosides of delphinidin, malvidin and petunidin on a number of metal ion-requiring bacterial enzymes. The author found that the activities of glycerol dehydrogenase, malate dehydrogenase, and hexokinase decreased with the addition of greater than 0.01% anthocyanin.

1.2.6 Tannins

Tannins or polymeric phenols are a diverse and complex array of polymers that exist in plants. Swain & Bate-Smith (1962) defined tannins as phenolic compounds that have a molecular weight of between 500 to 3000 and are able to precipitate gelatine and other proteins. Tannins can be classified into two groups, the hydrolysable tannins and the condensed tannins.

1.2.6.1 Hydrolysable Tannins

Hydrolysable tannins are abundant in the leaves, fruit, pods, and galls of plants (Lewis & Yamamoto, 1989). Hydrolysable tannins include galloyl esters (gallotannins) and hexahydroxydiphenoyl esters (ellagitannins) (**Figure 1.11**). Depending on the hydrolysis products, distinctions are made between gallotannins, which release gallic acid, and ellagotannins, which release ellagic acid.

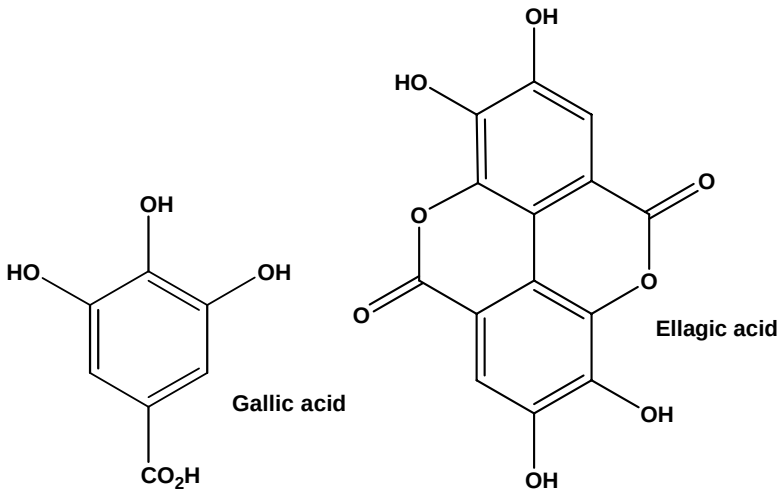
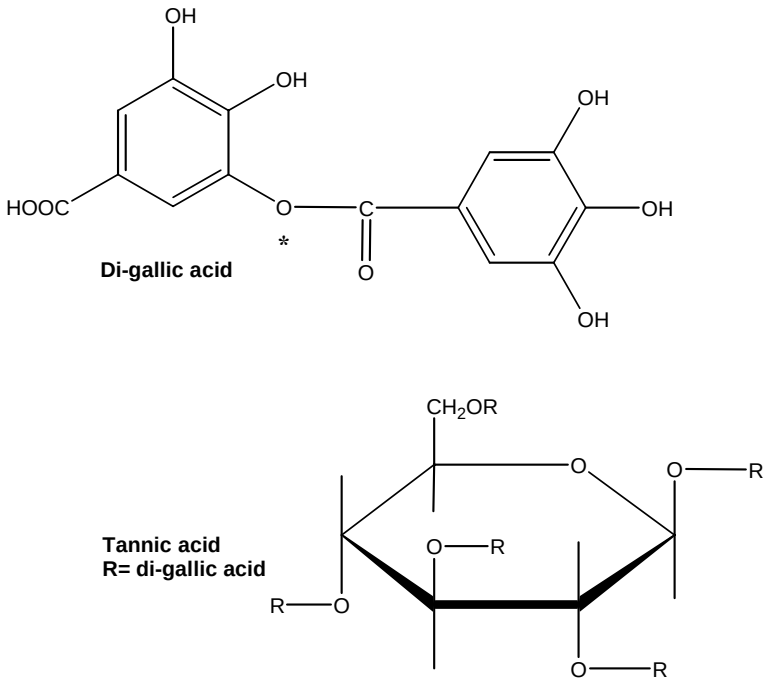


Figure 1.11 Gallic acid and ellagic acid.

Tannic acid (**Figure 1.12**), a hydrolysable tannin, but not its hydrolytic product gallic acid, was found to inhibit a range of human intestinal bacteria including the gram-positive *Clostridium* species and the gram-negative bacterial species *Escherichia coli*, *Bacteriodes fragilis*, *Enterobacter cloacae*, and *Salmonella* Typhimurium (Chung *et al*., 1998) and a range of food-borne bacterial pathogens including *Listeria monocytogenes* (gram-positive) (Chung *et al*., 1993). The authors suggested that tannic acid might act by chelating iron from the bacterial growth media thereby depriving bacteria of necessary growth factors and inhibiting bacterial growth.

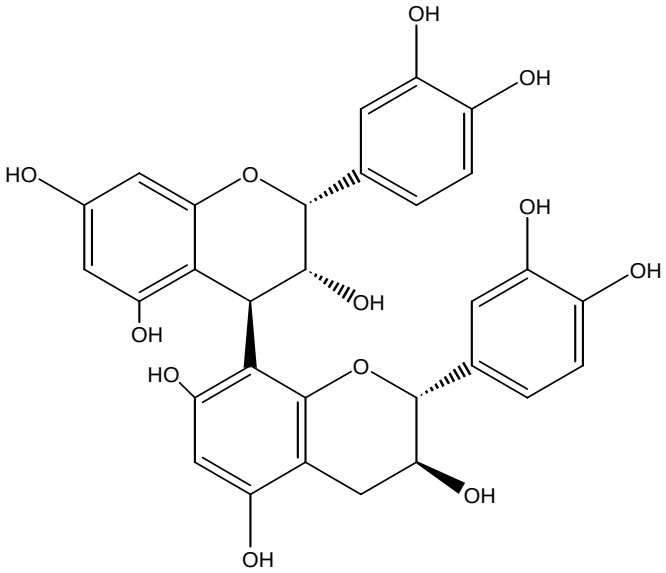


11.1 **Figure 1.12 Structure of a hydrolysable tannin, tannic acid.** *=linkage site.

1.2.6.2 Condensed Tannins

Condensed tannins or proanthocyanidins are a structurally complex and diverse group of molecules that are wide spread in the plant kingdom. Proanthocyanidins are present naturally in legume seeds, cereals, and are particularly abundant in some fruits and fruit juices (Santos-Buelga & Scalbert, 2000). Proanthocyanidins consist of two classes of polymers, procyanidins and prodelphinidins. Procyanidins consist of catechin and epicatechin and prodelphinidins consist of epigallocatechin and gallocatechin (**Figure 1.5**) (Cheynier *et al*., 1999). The flavan-3-ol units can bear various acyl or glycosyl substituents. Proanthocyanidins are formed by coupling at C-4 of an electrophilic flavanyl unit, generated from a flavan-3-ol or a flavan-3, 4-diol, to a nucleophilic flavanyl unit, generally a flavan-3-ol (De Bruyne *et al*., 1999). Most proanthocyanidins are linked between C_4 of the preceding monomer and C_6 or C_8 of the next flavan A-ring (**Figure 1.13**). The flavan-3-ol units are rarely glycosylated and if esterified are found predominantly as galloyl esters. The polymers that make up proanthocyanidins are polydisperse, the chains are irregular, sometimes branched, and can contain more than one type of monomer base unit (Porter, 1992). Oligomeric procyanidins correspond to polymers formed from three to ten flavanol units and condensed procyanidins have

more than ten flavanol units and a molecular weight of greater than 3000 (Ribéreau-Gayon *et al*., 1999).



12.1 Figure 1.13 Structure of a procyanidin dimer.

Recent investigations have suggested the estimated molecular weight, as determined by the mean degree of polymerisation (mDP), of proanthocyanidins derived from grape skin is as high as 11524 (Kennedy & Taylor, 2003). Souquet *et al*., (1996) reported tannins with a mean degree of polymerisation (mDP) in excess of 80 i.e. a molecular weight of greater than 24000 daltons. Phenolic compounds other than flavan-3-ols and flavan-3, 4-ols can be associated with condensed tannins. Of most recent interest is the association of anthocyanins with condensed tannins in the formation of pigmented polymers and ageing of red wines. The direct linkages between the C_6 or C_8 position on the anthocyanin and the C_4 of the catechin moiety has been reported (**Figure 1.14**) (Remy *et al*., 2000). However the exact linkage of anthocyanins to tannins varies and is not yet completely elucidated. Unlike monomeric anthocyanins whose colour is directly linked to pH (**Figure 1.10**), the red colour of anthocyanins linked with tannins is not very sensitive to variations in pH and the colour therefore remains stable and can even intensify (Ribéreau-Gayon *et al*., 1999).

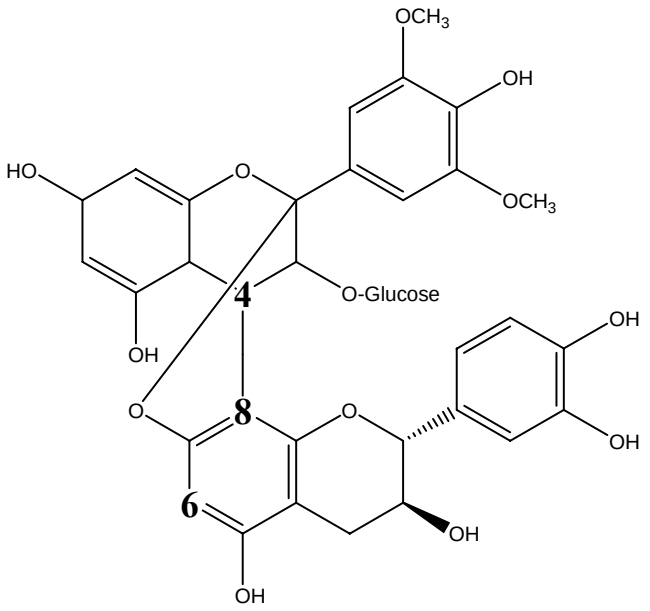


Figure 1.14 Structure of a bicyclic compound yielded by the reaction of an anthocyanin moiety (malvidin-3-glucoside) with a flavan-3-ol (taken from Remy *et al*., 2000).

The antimicrobial activity of proanthocyanidins has been widely recognised and tannins from a variety of plants have been found to have antimicrobial activity. Tannin-containing plants have a significant evolutionary advantage as the production of tannins by plants may protect them from predation by herbivores and can confer resistance against microbial infection (Scalbert, 1991). At least 43 different microorganisms, including yeasts, fungi, and bacteria, have been found to be susceptible to the toxic effects of tannins (Scalbert, 1991). The antimicrobial tannins conferring these toxic effects are derived from a wide variety of plants. The antimicrobial activity of tea tannins (polyphenols) and a number of flavanol monomers have been found to inhibit the growth of the gram-positive bacterial species *Clostridium difficile* and *Clostridium perfringens* (Ahn *et al*., 1991). Tea tannin had a marked inhibitory effect on a range of gram-negative phytopathogenic bacteria, including strains of *Erwinia, Pseudomonas, Clavibacter,* and *Xanthomonas* and the gram-positive *Agrobacterium* (Fukai *et al*., 1991). Antimicrobial polyphenols have been isolated from the wastewater of olive oil mills and were found to have bactericidal activity against the phytopathogenic bacteria *Pseudomonas syringae* (gram-negative) and *Corynebacterium michiganense* (gram-positive) (Capasso *et al*., 1995). Grape seed extracts are a rich source of tannin (polyphenols) and have antimicrobial activity against a wide range of gram-positive bacteria including *Bacillus cereus* and *Staphylococcus aureus*, and the gram-negative bacteria *Escherichia coli* and *Citrobacter freundii* (Palma & Taylor, 1999). Gram-positive bacteria, including *Bacillus*

cereus, Bacillus coagulans, Bacillus subtilis, and *Staphylococcus aureus* were more susceptible to grape seed tannins than the gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* and higher molecular weight polyphenols were more inhibitory than lower molecular weight polyphenols (Jayaprakasha *et al*., 2003). Polymeric phenolics with antimicrobial activity have been isolated from medicinal plants (Burapadaja & Bunchoo, 1995; Abram & Donko, 1999), coconut husk fibre (Esquenazi *et al*., 2002), apple (Yanagida *et al*., 2000), oak leaves (Makkar *et al*., 1988), and various berry fruits (Puupponen-Pimiä *et al*., 2001), including cranberries (Ho *et al*., 2000). The use of cranberry juice to prevent and treat urinary tract infection caused by *Escherichia coli* has been extensively studied. The antimicrobial factor in cranberry juice was found to be polyphenolic and was recently identified as A-type proanthocyanidin trimers (Foo *et al*., 2000b). The A-type proanthocyanidin trimers (**Figure 1.15**) from cranberry were found to prevent adherence of P-fimbriated *E. coli* isolates to urinary tract cell surfaces.

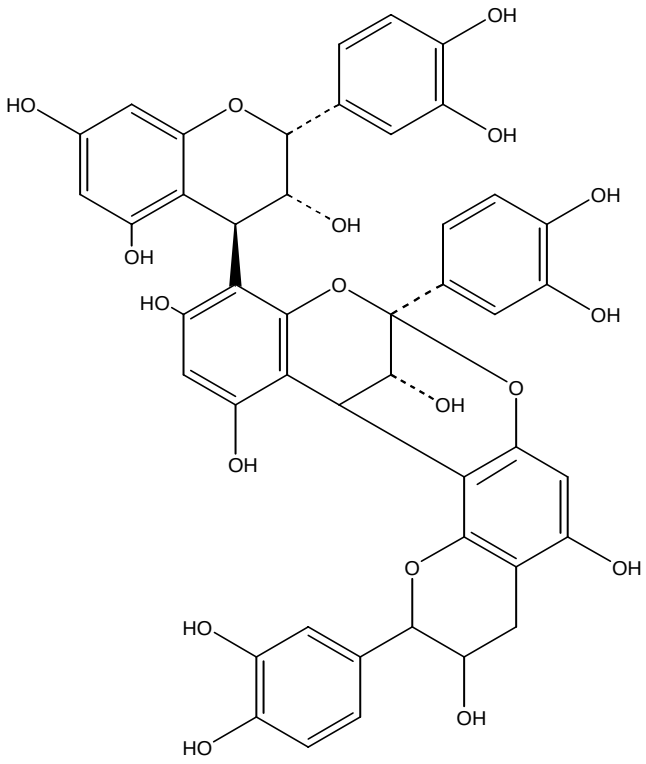


Figure 1.15 An example of an A-type proanthocyanidin trimer (Foo *et al*., 2000b).

Other modes of antimicrobial action of tannins (polyphenols) have been postulated including chelation of metal ions depriving bacteria from necessary growth constituents or inhibiting bacterial metallo-enzyme activity (Scalbert, 1991). The antimicrobial action of tannins has been attributed to the hydrogen bonding of tannins with vital microbial enzymes (Field & Lettinga, 1991) and/or the ability of tannins to complex with protein and carbohydrates (Scalbert, 1991).

1.2.7 Antimicrobials from Grapes and Wine

The antimicrobial action of wine has been known for centuries. The ancient Greeks would wash wounds with cold wine, warm wine, or vinegar and then apply bandages soaked with wine to the wound (Majno, 1975). Alois Pick published the first article on the antiseptic properties of wine in 1892 (cited in Majno, 1975). Cultures of cholera and typhoid bacilli were added to straight and diluted wine. In both undiluted and diluted wine all cholera vibrios (now *Vibrio cholerae*) were killed within 10-15 minutes and typhoid bacilli (now *Salmonella* Typhi) were eliminated after 24 hours (cited in Majno, 1975). Wine has a natural antimicrobial environment due to its relatively high alcohol content, low pH, and high amounts of organic acids (Just & Daeschel, 2003). However, the antimicrobial action of wine has been attributed to the phenolic compounds present in wine rather than the alcohol content (Masquelier, 1959). Masquelier (1959) assessed the antimicrobial action of red wine against a range of bacteria including the gram-negative species *Salmonella* Typhi, *Salmonella* Paratyphi, and *Escherichia coli* and the gram-positive bacteria *Staphylococcus pyogenes*, but not including *Listeria monocytogenes*. The author showed that decolourising the wine with charcoal resulted in the loss of antimicrobial activity suggesting it is not the alcohol or organic acids that contribute the antimicrobial effect to red wine. This author also showed that the antimicrobial activity of red wine steadily increases up to a certain wine age and then begins to decrease. The antiviral activity of grapes, grape juices, and red and white wine, was investigated by Konowalchuk & Speirs (1976). They reported that wines were less antiviral than grape juice and that white wine was less active than red wine. They also showed that black table grapes were more antiviral than green table grapes. The authors identified that the antiviral activity of red grapes resides in the skin of the grapes and has a wide range of molecular weights (i.e. <1000 to >30,000) and that the antiviral activity of grape juice was reduced with the addition of gelatine. Just & Daeschel (2003) evaluated the antimicrobial effects of red and white grape juice and wine on the gram-negative bacteria *Escherichia coli* O157:H7 and *Salmonella* Typhimurium. Both bacteria were inactivated after 60 minutes exposure to the wine but survived up to 16 days in the grape juice. The rapid bactericidal effect of red wine, against the gram-negative bacteria *Helicobacter pylori* was reported by Marimón *et al*., (1998). Their findings showed that the antimicrobial activity of red wine was very strong with *H. pylori* inhibited after 5 minutes in the wine and that this activity

was greater than ethanol at the same concentration and the same pH. They attributed the antimicrobial activity in red wine to substances other than alcohol. Marimón & Bujanda (1998) conducted experiments on the antimicrobial activity of red wine against *Salmonella* Enteritidis and their results suggested that the antimicrobial activity was due to the polyphenols present in the wine rather than any effect of low pH or alcohol. Sheth *et al*., (1988) showed the dramatic reduction in numbers of the gram-negative bacteria *Salmonella* Typhimurium, *Shigella sonnei,* and *Escherichia coli* after 4 hours exposure to red wine. Weisse *et al*., (1995) also reported the rapid antimicrobial effects of red wine against these same three bacteria with all bacteria eliminated after 20 minutes in wine. These authors suggested that the antimicrobial factor present in the wine was a polyphenol.

Extracts of grape seeds have been shown to have strong antimicrobial activity. Ahn *et al*., (2004) reported the inhibitory action of ActiVin, a commercial grape seed extract, against both gram-negative bacteria, *Escherichia coli,* and *Salmonella* Typhimurium, and against gram-positive bacteria including *Listeria monocytogenes*. Palma & Taylor (1999) reported the antimicrobial action of a grape seed extract against a range of human and plant pathogenic microorganisms. Jayaprakasha *et al*., (2003) reported the antimicrobial action of *Vitis vinifera* grape seed extract against *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus* (all gram-positive bacteria), but not including *Listeria monocytogenes*, and the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and found that gram-positive bacteria were more susceptible to the grape seed extract than gram-negative bacteria. A number of purified phenolic compounds present in grapes and wine, many of which have been described in the preceding sections, have been shown to possess antimicrobial activity (Pratt *et al*., 1960; Powers, *et al*, 1960; Hamdy *et al*., 1961; Somaatmadja *et al*., 1963; Powers, 1964; Carpenter *et al*., 1967; Mori *et al*., 1987; Makkar *et al*., 1988; Nakane & Ono, 1990; Ahn *et al*., 1991; Fukai *et al*., 1991; Chung *et al*., 1993; Ikigai *et al*., 1993; Bloor, 1995; Capasso *et al*., 1995; Burapadaja & Bunchoo, 1995; Padmavati *et al*., 1997; Chung *et al*., 1998; Abram & Donko, 1999; Docherty *et al*., 1999; Mabe *et al*., 1999; Palma & Taylor, 1999; Tesaki *et al*., 1999; Foo *et al*., 2000b; Ho *et al*., 2000; Rauha *et al*., 2000; Yanagidal *et al*., 2000; Docherty *et al*., 2001; Puupponen-Pimiä *et al*., 2001; Chan, 2002; Esquenazi *et al*., 2002; Panizzi *et al*., 2002; Jayaprakasha *et al*., 2003; Wen *et al*., 2003; Ahn *et al*., 2004). These include gallic acid, *para*-coumaric acid, ferulic acid, caffeic acid, catechin, epicatechin, epigallocatechin epicatechin gallate, epigallocatechin gallate, quercetin, kaempferol, morin, *trans*-resveratrol, pelargonium-3-glucoside, cyanidin-3-glucoside, delphinidin-3-glucoside and hydrolysable and condensed tannins or polymeric proanthocyanidins.

1.2.8 Antilisterial Factors Derived from Plant Sources

The antimicrobial activity, against *Listeria monocytogenes*, of a number of plant extracts and purified phenolic compounds have been reported. **Table 1.1** lists some of these extracts and phenolic compounds. A number of plants and plant extracts have been isolated that exhibit antilisterial activity.

A recent report by Ahn *et al*., (2004) found that a commercial grape seed extract (ActiVin) was more inhibitory against *Listeria monocytogenes* than *Salmonella* Typhimurium and *Escherichia coli*. The authors found that a concentration of ActiVin of 6 mg/ml or greater was required for inhibition of *Listeria monocytogenes* in both broth and agar cultures. To this researchers knowledge this is the first reporting of grape extract with antilisterial activity. The antilisterial activity of raw carrots has been investigated by a number of authors. *L. monocytogenes* numbers were found to decrease on contact with raw whole and shredded carrot but not cooked carrot (Beuchat & Brackett, 1990; Nguyen-the & Lund, 1991). Analyses of purified ethanolic extracts of peeled and shredded carrots showed that the antimicrobial activity was not linked to phenolic compounds but rather to free saturated fatty acids and methyl esters of fatty acids (Babic *et al*., 1994). Commercial plant extracts from clove and pimento leaves exhibited slight antilisterial activity (1 to 2-log reduction in cell numbers over 14 days) when added as an alcohol solution to *L. monocytogenes* contaminated chicken. However, in the same study a number of other commercial plant extracts, including angelica root, banana puree, bay, caraway seed, carrot root, marjoram, and thyme did not inhibit the growth of this organism (Hao *et al*., 1998). Cutter (2000) reported the antilisterial activity of two commercial blended herb extracts, Protecta One and Protecta Two, when applied to *L. monocytogenes* contaminated beef. She observed a 1.8-1.9-log reduction in cell numbers over a 7-day storage period. Kim *et al*., (2001) tested the antimicrobial effect of methanolic extracts of the petals of *Camellia japonica,* a member of the tea family. They identified fumaric acid as the inhibitory factor although when a pure standard of fumaric acid was assayed it did not exhibit the same degree of inhibition against *L. monocytogenes* as the petal extract. Gram-negative bacteria, *Salmonella* Typhimurium and *E. coli*, were more sensitive to the petal extract than *L. monocytogenes* (gram-positive).

13.1 Table 1.1 Some antilisterial compounds and extracts derived from plants.

Plant Source or Phenolic Compound	Type of compound	Reference
Tannic acid Quercetin	Hydrolysable tannin Flavonol	(Payne *et al*., 1989)
Citric acid	Organic acid	(Conner, 1990)
Carrot extract	possibly free saturated fatty acids	(Beuchat & Brackett, 1990; Nyugen-the & Lund, 1991; Babic *et al*., 1994)
Chinese medicinal plant extracts	Not determined	(Chung *et al*., 1990)
Raw and processed tomatoes	Acetic acid	(Beuchat & Brackett, 1991)
Tannic acid	Hydrolysable tannin	(Chung, 1993)
Rosemary oil	possibly α-pinene	(Pandit & Shelef, 1994)
Hop extract	β-acids	(Larson *et al*., 1996)
Commercial essential oils	Monoterpenes, eugenol, cinnamaldehyde, thymol	(Lis-Balchin & Deans, 1997)
Eugenol and pimento extracts	Not determined	(Hao *et al*., 1998)
Herb extracts	Not determined	(Cutter, 2000)
Capsicum annum extracts	Cinnamic acid, *meta*-Coumaric acid	(Dorantes *et al*., 2000)
Corni fructus (Dogwood fruit), Chinese chive, cinnamon combined extracts	Not determined	(Hsieh, *et al*., 2001)
Camellia japonica petal extract	Fumaric acid	(Kim *et al*., 2001)
Extracts of edible Asian plants	Not determined	(Alzoreky & Nakahara, 2002)
Cilantro extract	Long chain alcohols and aldehydes	(Delaquis *et al*., 2002)
Caffeic acid, *para*-Coumaric acid, Ferulic acid	Phenolic acids	(Wen *et al*., 2003)
Grape seed extract (ActiVin)	Polyphenolic compounds	(Ahn *et al*., 2004)

Capsicum annun (chilli peppers) were shown to be inhibitory against a number of bacteria. However *L. monocytogenes* was most sensitive to the inhibitory action of these chilli extracts (Dorantes *et al*., 2000). The authors concluded that cinnamic and *m*-coumaric acids contributed to the inhibitory effect of the chilli extracts.

Methanol and acetone extracts of ten plants commonly consumed in Asia (Alzoreky & Nakahara, 2002) and methanolic extracts of Chinese medicinal plants showed inhibitory action against *L. monocytogenes* (Chung *et al*., 1990). The inhibitory action of the Chinese medicinal plant extracts was suppressed by the addition of protein to the extracts but not by the addition of sodium chloride (Chung, *et al*., 1990).

A combination of ethanolic extracts of *Corni fructus* (the fruit of *Cornus officinalis,* common name Dogwood), Chinese chives, and cinnamon was found to inhibit the growth of *L. monocytogenes* in a pH dependant manner, with acid pH values being more inhibitory and inhibition decreasing as pH increased (Hsieh *et al*., 2001). A listericidal extract derived from hops was found to be more effective in acidic foods, such as coleslaw, than in foods with higher pH, such as milk. The authors suggested that β-acids might be the antilisterial factor in hops (Larson *et al*., 1996). The pH dependence of the antilisterial activity of various hydroxycinnamic acids was recently reported by Wen *et al*., (2003). They found that the hydroxycinnamic acids, caffeic acid, *p*-coumaric acid, and ferulic acid were all inhibitory and this inhibition was pH dependent with acidic hydroxycinnamic acid solutions exhibiting stronger antilisterial activity. They also suggested that the greater the degree of hydroxylation the greater the inhibitory action, with cinnamic acid showing the highest antilisterial activity. Payne *et al*., (1989) reported the antilisterial activity of tannic acid but limited activity of gallic acid, caffeic acid, ferulic acid, and coumaric acid. This finding is in agreement with Chung *et al*., (1993) who reported the antilisterial action of tannic acid but not gallic and ellagic acid, the hydrolysis products of tannic acid.

Organic acids have also been reported to show antilisterial activity. Beuchat & Brackett (1991) reported the inhibitory activity of raw tomatoes and processed tomato products against *L. monocytogenes*. This inhibition was attributed to acetic acid present in tomatoes. *L. monocytogenes* is more susceptible to organic acids than mineral acids with acetic acid being a more effective inhibitor compared to lactic, citric, or hydrochloric acid (Farber *et al*., 1989). Plant oils have also been reported to exhibit antilisterial activity. Pandit & Shelef (1994) screened 18 spices for listericidal activity and determined that only rosemary and clove oil were inhibitory. The antilisterial action of an aqueous extract of rosemary was considerably lower than that of whole

ground rosemary and an ethanolic extract of rosemary. The active component of rosemary oil was determined to be α-pinene. Other essential oils have been reported to have antilisterial activity. Lis-Balchin & Deans (1997) assessed the inhibitory action of 93 essential oils and found that strong antilisterial activity was often correlated with the oils containing a high percentage of monoterpenes, eugenol, cinnamaldehyde, and thymol. The antilisterial action of essential oil extracted from cilantro (leaves of immature *Coriandrum sativum* L.) was attributed to the presence of long-chain (C_6-C_{10}) alcohols and aldehydes in the oil (Delaquis *et al*., 2002).

1.3 Thesis Aims

The aims of this thesis are to

- Characterise the antimicrobial efficacy of a commercial grape juice.
- Compared and contrast the antilisterial action of extracts from different parts of the grape berry.
- Isolate and characterise the antimicrobial factor(s) present on the commercial grape juice.
- Assess the efficacy of grape-derived antimicrobial factor(s) as additives to food to eliminate *L. monocytogenes* contamination.

1.4 Thesis Outline

This thesis is comprised of six chapters including a general introduction to *Listeria monocytogenes*, listeriosis, and the importance of this food-borne pathogen in the food processing industry and natural antimicrobials derived from plant sources. The second chapter outlines the antimicrobial efficacy of a commercial grape juice that was found to have strong antilisterial activity. The third chapter investigates the origin of the antilisterial factor on the commercial grape juice by analysis of grape skin and seed extracts and an antilisterial fraction from commercial grape juice is identified. The fourth chapter describes the isolation and chemical characterisation procedures used to identify the antilisterial factor present in the commercial grape juice. The grape seed was also analysed for the presence of an antilisterial factor and the results were compared and contrasted with the commercial grape juice in terms of antimicrobial efficacy. The fifth chapter describes the antilisterial efficacy of the isolated and purified antilisterial factor and assesses its application as a food preservative. The sixth chapter is a general discussion reviewing findings, drawing conclusions, and identifying future research directions of this study.

2 CHARACTERISATION OF THE ANTIMICROBIAL ACTIVITY OF A COMMERCIAL GRAPE JUICE

2.1 Introduction

The antimicrobial activity of grapes, grape juice, and wine has been reported against a range of bacteria, fungi, and viruses (Masquelier, 1959; Konowalchuk & Spiers, 1976; Sheth *et al*., 1988; Weisse *et al*., 1995; Marimón & Bujanda, 1998; Marimón *et al*., 1998; Jayaprakasha *et al*., 2003; Just & Daeschel, 2003; Ahn *et al*., 2004).

The objective of this chapter was to assess the antimicrobial efficacy of a commercial red grape juice and to make a preliminary identification of the antimicrobial factor present in the grape juice. The aim of this investigation was to determine the antimicrobial spectrum of the commercial grape juice and to determine if this antimicrobial activity was unusually strong in comparison to the activity of other commercial red grape juices and New Zealand red wines. A comparison of the antilisterial activity of different batches of the commercial grape juice was performed and preliminary chemical composition analyses were conducted in order to identify a likely candidate antilisterial factor present in the juice. A number of antilisterial parameters were tested to gain an understanding of the parameters under which this juice was inhibitory to *Listeria monocytogenes* and to identify a candidate antilisterial factor (i.e. the minimum inhibitory concentration of the juice, the effect of pH on activity, the effect of removing phenolic compounds on activity, and the effect of the addition of ions on the antilisterial activity). An assessment was made as to whether the action of the commercial grape juice was bactericidal or bacteriostatic.

2.2 Materials

14.1 Table 2.1 List of materials used in Chapter Two.

Materials	Source
API Listeria test strips	BioMérieux, France
Bacto Peptone	Difco
Brain Heart Infusion Broth (BHI)	Difco
Citric acid (AR grade)	Ajax Chemicals
Di-sodium hydrogen orthophosphate (anhydrous)	BDH
Dorset Egg Slopes	Fort Richard Laboratories, New Zealand
Ferric chloride ($FeCl_3$)	BDH
Folin-Ciocalteu reagent	BDH
Gallic acid (LR grade)	Sigma
Glass microfibre filters (125 mm)	Whatman
Glycerol (AR grade)	BDH
Horse blood agar plates	Fort Richard Laboratories, New Zealand
Hydrochloric acid	BDH
Listeria Selective medium	Oxoid
Magnesium chloride ($MgCl_2.6H_2O$)	BDH
Model Grape Juice (MGJ)	
0.033 M L-tartaric acid, dextra-rotatory	BDH
Model Wine Solution (MWS)	
0.033 M L-tartaric acid, dextra-rotatory	BDH
99.95% ethanol (10% final concentration)	Merck
pH strips Panpeha plus pH 2.0-9.0	Schneder & Schuell
Polyvinylpolypyrrolidone (MW 40,000)	Sigma
Potassium meta-bisulphite	Sigma
Sodium carbonate (AR grade)	BDH
Sodium hydroxide (AR grade)	BDH
Tryptic Soy Agar (TSA)	Difco
Tryptic Soy Broth (TS)	Difco

2.2.1 Grape Juice

The commercial grape juice, brand name McCoys, used in this study was purchased from an Auckland, New Zealand, supermarket. The grape juice was made from *Vitis vinifera* var. Ribier black table grapes and was sourced from Chile as a concentrate, region and supplier unspecified

(Frucor, personal communication). The grapes were commercially processed by first crushing, then extracting the juice. The juice was then pre-concentrated, clarified, filtered, de-tartarated, refiltered, and concentrated. To reconstitute the grape juice for sale in New Zealand, the juice was homogenised with purified water, aroma compounds, and ascorbic acid. The single strength juice was pasteurised for 20 seconds at 95^{o}C and cooled to 27^{o}C prior to packaging (Frucor, personal communication). The packaged McCoys dark grape juice has a shelf life of 9 months, however the juice is stored in a warehouse for approximately 3 to 4 months prior to being supplied to supermarkets. The specifications of the single strength McCoys dark grape juice are as follows: brix 68-69, acidity 1.3-2.5 % (w/w) tartaric acid, colour greater than 350 optical density (520 nm pH 3.2) (Frucor, personal communication).

Individual lots of McCoys dark grape juice (1 litre) were purchased on 16 March 2001 (batch 1), 31 May 2002 (batch 2), and 4 July 2002 (batch 3). All batches of grape juice were stored as 100 ml aliquots decanted into sterile plastic containers and held at -20^{o}C.

2.3 Methods

2.3.1 Bacterial Cultivation Methods

All bacteria used in this thesis (**Table 2.2**) were obtained from the Institute of Environmental Science and Research (ESR) as pure cultures. Bacterial isolates were stored long-term at –80^{o}C in tryptic soy broth (TSB) containing 15% glycerol. Frequently used bacterial isolates were stored for up to 3 months on Dorset Egg slopes at 4^{o}C. For daily, use bacterial cultures were maintained on horse blood agar plates at 20^{o}C. Bacteria were stored on blood agar plates for up to two weeks before a fresh culture was prepared from Dorset Egg slope stock culture. Culture identity was reverified monthly using API test strips according to the manufacturer's instruction. A suspension of bacteria was prepared by adding a few bacterial colonies to a commercial suspension medium. An aliquot of suspension (100 µl) was added to a series of wells (API test strip) containing dehydrated growth substrates, which enables the performance of enzymatic tests and sugar fermentation. The API test strip was incubated and the resultant bacterial metabolism may produce colour changes to the growth substrates, which are read and then identified by consulting the API profile list.

15.1 Table 2.2 List of bacterial isolates used in this thesis.

Bacterial Isolate	Bacterial Isolate
Listeria monocytogenes ATCC 35152 serotype 1/2a	*Listeria grayi* ATCC 19120
Listeria monocytogenes AF 06283 serotype 1/2	*Listeria innocua* ATCC 33090 serotype 6a
Listeria monocytogenes AF 01403 serotype 1/2	*Listeria ivanovii* New Zealand Reference Culture Collection, Medical Section. Accession number 797 serotype 5
Listeria monocytogenes AE 9493 serotype not determined	*Brochothrix thermospacta* ATCC 11509
Listeria monocytogenes AD 4470 serotype 1/2	*Lactobacillus rhamnosus* ATCC 7469
Listeria monocytogenes AF 03320 serotype 1/2	*Escherichia coli* ATCC 25922
Listeria monocytogenes CDC KC18 serotype 4b	*Bacillus cereus* NCTC 8035
Listeria monocytogenes ATCC 19112 serotype 1/2c	*Staphylococcus aureus* ATCC 25923
Listeria monocytogenes ATCC 19113 serotype 3a	*Salmonella* Menston NCTC 7836
Listeria monocytogenes ATCC 19114 serotype 4a	*Escherichia coli* ATCC 25922
Listeria seeligeri ATCC 35967 serotype 1/2b	*Yersinia enterocolitica* ATCC 9610
Listeria welshimeri NCTC 11857	

2.3.1.1 Liquid Media

Broth cultures were prepared by inoculating 4-5 colonies of bacteria into 10 ml of brain heart infusion (BHI) broth. Broth cultures were grown for 16-20 hours at the appropriate incubation temperature (37^{o}C for all bacteria used in this thesis with the exception of *Bacillus cereus* and *Yersinia enterocolitica* which were grown at 30^{o}C and *Brochothrix thermospacta* which was grown at 22^{o}C).

2.3.1.2 Solid Media

The solid media employed for bacterial growth for all experimental work, with the exception of food assays, was tryptic soy agar (TSA). Agar plates were prepared and stored at 4°C for up to two weeks. For use the plates were warmed and excess moisture evaporated to 37°C in a warm air oven. Bacterial cultures were spread onto TSA plates using a sterile spreader and grown by incubation of plates for 24-48 hours. All bacterial isolates were grown at 37°C with the exception of *B. cereus* and *Y. enterocolitica*, which were grown at 30°C and *B. thermospacta* which was grown at 22°C.

2.3.1.3 Listeria Selective Media

For the food experiments (outlined in chapter 5 section 5.3.4) *Listeria monocytogenes* ATCC 35152 was grown on Listeria selective media. Listeria selective media was prepared according to the Oxoid instructions. Plates were stored at 4°C for up to two weeks.

2.3.2 Antimicrobial Assay

The antimicrobial assay used in this thesis followed the protocol outlined by Weisse *et al*., (1995). An overnight culture of bacteria was grown in 10 ml of BHI. The culture was diluted in 0.1% sterile peptone water to give a cell count of approximately 10^8 colony forming units (CFU)/ml. A 200 µl aliquot of dilute cells was inoculated into 3.8 ml of sterile commercial grape juice. The bacteria-grape juice solution was incubated at 20°C for the duration of the assay. At the following exposure times 0, 10, and 60 minutes, a 1 ml aliquot of bacteria containing juice was removed, serially diluted in 0.1% sterile peptone water, and duplicate 100 µl aliquots spread onto TSA plates. A positive control consisting of 200 µl dilute bacterial cells added to Model Grape Juice (MGJ) (0.033 M L-tartaric acid pH 3.5) was run in parallel. A control of 100 µl uninoculated grape juice was streaked onto a TSA plate to check for sterility. The plates were incubated at the appropriate temperature for 24 hours and colonies counted on a Quebec darkfield colony counter (American Optical). Plates containing between 25–250 colonies (Sperber *et al*., 2001) were counted and multiplied by the dilution factor and the mean and standard deviation of the duplicate plate counts were calculated. Results were expressed as either log CFU/ml or CFU/ml and plotted against grape juice exposure time.

2.3.3 Antimicrobial Spectrum of Grape Juice

The antimicrobial spectrum of commercial grape juice (batch 1 purchased 16 March 2001) was assessed for a range of food-borne bacterial pathogens (**Table 2.3**).

16.1 Table 2.3 Bacterial isolates used to determine the antimicrobial spectrum of commercial grape juice (batch 1).

Bacterial Isolate	Growth Temperature	Gram stain
Listeria monocytogenes ATCC 35152 serotype 1/2a	37°C	+
Listeria monocytogenes AF06283 serotype 1/2 isolated from salami	37°C	+
Listeria monocytogenes AF 01403 serotype 1/2 isolated from cooked pork	37°C	+
Listeria monocytogenes AE 9493 serotype not determined isolated from smoked fish	37°C	+
Listeria monocytogenes AD 4470 serotype 1/2 isolated from ice cream	37°C	+
Listeria monocytogenes AF 03320 serotype 1/2 isolated from sausages	37°C	+
Listeria monocytogenes serotype 4b CDC KC18	37°C	+
Listeria innocua ATCC 33090 serotype 6a	37°C	+
Listeria ivanovii New Zealand Reference Culture Collection, Medical Section. Accession number 797 serotype 5 (isolated from aborted animal foetus)	37°C	+
Listeria seeligeri ATCC 35967 serotype 1/2b	37°C	+
Bacillus cereus NCTC 8035	30°C	+
Staphylococcus aureus ATCC 25923	37°C	+
Salmonella Menston NCTC 7836	37°C	-
Escherichia coli ATCC 25922	37°C	-
Yersinia enterocolitica ATCC 9610	30°C	-

Five *Listeria monocytogenes* isolates, serotype 1/2, isolated from contaminated food by the Institute of Environmental Science and Research (ESR) Public Health Laboratory, were assayed to determine if the antilisterial spectrum was specific only to the reference isolate *L. monocytogenes*

ATCC 35152. Antimicrobial assays were performed according to the standard assay protocol described in section 2.3.2.

2.3.4 Minimum Inhibitory Concentration of Grape Juice

The minimum inhibitory concentration (MIC) was determined for grape juice (batch 1). A dilution series of grape juice was prepared with sterile reverse osmosis water to give the following concentrations of grape juice: 100%, 50%, 33.3%, 25%, 12.5%, and 10%. Each grape juice dilution was assayed against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2).

2.3.5 Variability of Antilisterial Activity of Different Batches of Grape Juice

Antimicrobial activity of different batches of the same brand of commercial grape juice was expected due to compositional variability of the initial grapes as a result of grape growing and harvesting conditions. The variability of the antilisterial activity of different batches, batches 1, 2, and 3 (section 2.2.1), of the undiluted commercial grape juice was assessed. Prior to storage at –20^{o}C each batch of grape juice was assayed for antilisterial activity against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2).

2.3.6 Chemical Analyses

2.3.6.1 Determination of Benzoic acid, Sorbic acid, and Sulphur dioxide

The commercial grape juice (batch 1) was tested for the presence of the preservatives benzoic acid, sorbic acid, and sulphur dioxide. Benzoic and sorbic acids were analysed by HPLC using standards according to the AOAC International Official Method of Analysis (1995) section 47.3.04. Sulphur dioxide was analysed according to the AOAC Official Methods of Analysis (1995) section 47.3.39, using the Monier-Williams method.

2.3.6.2 Total Polyphenol Level

The total polyphenol level of the grape juices, grape extracts, and grape fractions was measured using Folin-Ciocalteu reagent according to the method of Amerine and Ough (1980). A phenol

standard solution was prepared by dissolving 0.5 g of gallic acid in 100 ml distilled water. A calibration curve was generated by pipetting 0, 1, 2, 3, 5, and 10 ml of gallic acid solution into 100 ml volumetric flasks and diluting to 100 ml with water. The phenol concentrations of these solutions (gallic acid equivalents or GAE) were 0, 50, 100, 150, 250, and 500 mg/L. A 1 ml aliquot of each solution was transferred to separate 100 ml volumetric flasks and 60 ml of water was added. The solution was mixed and 5 ml of Folin-Ciocalteu reagent was added and mixed. After 30 seconds and before 8 minutes, 15 ml of 20% sodium carbonate solution was added and mixed. The volume was then adjusted to 100 ml with distilled water and the solutions reacted at 20^{o}C for 2 hours. The absorbance of each gallic acid standard was read at 765 nm using a Cary 50 Conc UV-visible spectrophotometer (Varian). A blank consisting of the reagents without the addition of gallic acid was used to zero the spectrophotometer prior to reading absorbencies. To determine the total phenolic level of grape derived samples, duplicate samples were diluted to within the calibration curve range with distilled water (generally 1:10) and reacted as described for the gallic acid standards. Total polyphenol levels were determined using the gallic acid calibration curve and results were expressed as mg/L gallic acid equivalents (GAE).

2.3.6.3 Percentage Tannin-Anthocyanin Polymers (% Polymeric Colour)

Percentage polymeric colour, a measure of polymerised anthocyanin-tannin complexes, of grape juices, grape extracts, and grape fractions was determined by their resistance to bleaching with bisulfite according to the method of Somers & Evans (1977). The polymerised coloured (anthocyanin-tannin complexes) are resistant to bleaching by bisulfite, whereas the monomeric anthocyanins will combine with bisulfite to form a colourless sulfonic acid adduct. The absorbance at 420 nm of the bisulfite treated samples gives a measure of the proportion of anthocyanin-tannin complexes in a sample that is resistant to bleaching. The absorbance at 520 nm gives a measure of the proportion monomeric anthocyanin in the sample. The absorbance at 700 nm gives a measure of the haze in the sample and allows correction for haze interference.

Prior to analysis the samples were diluted with distilled water to give absorbance readings within an acceptable range (absorbance of less than 1.0). Duplicate samples (2.8 ml) were prepared. To one sample a 200 µl volume of water was added. To the other sample a 200 µl volume of freshly prepared potassium meta-bisulphite (0.25 mg/ml dissolved in distilled water) was added. The solutions were equilibrated for 15 minutes at 20^{o}C and absorbencies read at the 420 nm, 520 nm and 700 nm using a Cary 50 Conc UV-visible spectrophotometer (Varian). Distilled water was used to zero the spectrophotometer at each wavelength. Percentage polymeric colour was calculated using equation 1.

17.1 Equation 1

Water treated = (Ab 420nm – Ab 700nm) + (Ab 520nm – Ab 700nm) x dilution factor

$K_2O_5S_2$ treated = (Ab 420nm – Ab 700nm) + (Ab 520nm – Ab 700nm) x dilution factor

Percentage polymeric colour = ($K_2O_5S_2$ treated / water treated) x 100

Results were expressed as percentage polymeric colour of juice, extract, or fraction.

2.3.6.4 Titratable Acidity

The titratable acidity of grape juices, grape extracts, and grape fractions was determined using the method of the AOAC Official Method of Analysis (1995) section 37.1.37. A 5 ml sample of grape juice was diluted 1:10 with distilled water. A 0.1 M sodium hydroxide solution was added drop wise into a continuously stirred 50 ml volume of sample. The pH was continuously monitored using a Meterlab pH meter (Radiometer) until the end point of pH 8.2 was reached. The titratable acidity was calculated using equation 2.

18.1 Equation 2

$$\text{Titratable Acidity} = 0.5 \times \text{molarity NAOH} \times \frac{V}{1000} \times \frac{1000}{v} \times MM$$

The molar ratio of tartaric acid to NaOH in the reaction is 0.5:1

Where 0.5 = number of moles of tartaric acid V = volume of NaOH used for titration, in ml

v = volume of grape juice, in ml MM = molar mass of tartaric acid (150.09)

Results were expressed as gram tartaric acid per litre of juice.

2.3.7 Antilisterial Activity of Various Commercial Red Grape Juices and New Zealand Red Wines

Various brands of commercial grape juice **(Table 2.4)** were purchased on the 4 July 2002, from an Auckland, New Zealand supermarket. New Zealand red wines were supplied by The Institute of Environmental Science and Research Ltd New Zealand (ESR).

19.1 Table 2.4 Red grape juices and red wines assayed for antilisterial activity.

Red Wine Assayed	Red Grape Juice Assayed
Cabernet Merlot (CJ Pask, Hawkes Bay 2000) Cabernet Sauvignon (Sacred Hill, Hawkes Bay 2000) Pinot Noir (Twin Islands, Malborough 2001) Merlot (Kemblefield, Hawkes Bay 2000) Syrah (Crossroads, Hawkes Bay 2000)	Grapetise sparkling red grape juice (Australian no batch number stated) Greenways grape juice (batch number 2301091-0944) Lloyds organic Shiraz grape juice (Australian best before 20Sept 04)

New Zealand white wines were not tested for antilisterial activity as the objective of the experiment was to make a comparison between red grape juices and red wines. In addition, white wines contain the known antimicrobial agent sulphur dioxide, which is added to the wine to prevent spoilage. All juices and wines were assayed for antilisterial activity against *L. monocytogenes* ATCC 35152 using an abbreviated version of the standard protocol (2.3.2). *L. monocytogenes* numbers were assessed at exposure times of 0 and 60 minutes only. A Model Wine Solution (MWS) (0.033 M L-tartaric acid, pH 3.5, 10% ethanol) (Kilmartin *et al*., 2001) was run as a control to account for any antimicrobial effects due to the alcohol in the wine.

2.3.8 Bactericidal or Bacteriostatic Antilisterial Action of Grape Juice

To determine whether the inhibitory action of the grape juice (batch 1) against *Listeria monocytogenes* ATCC 35152 was bacteriostatic or bactericidal a Most Probable Number (MPN) test was performed according to the protocol outlined by Swanson *et al*., (2001). An overnight culture of *L. monocytogenes* was grown in BHI. The culture was diluted to 10^8 CFU/ml in 0.1% peptone water and a 0.5 ml aliquot added to 9.5 ml of grape juice and incubated at 20^{o}C. At time 0, 10, 30, 60, and 180 minutes, a 1 ml aliquot was removed and serially diluted in 9 ml 0.1% peptone water to give final dilution's of 1:10, 1:100, and 1:1000. Triplicate 1 ml aliquots of each dilution were added to individual tubes of 9 ml BHI broth. The tubes were incubated for 48 hours at 37^{o}C. A negative control of BHI inoculated with 1 ml sterile undiluted grape juice was run in parallel. To

ensure sterility of BHI broth tubes two uninoculated tubes were run in parallel. Positive controls of each dilution of cells not exposed to the grape juice were run in parallel to check for bacterial viability. The tubes were check for turbidity after 24 and 48 hours and after 48 hours growth the turbid tubes were counted and the results determined using MPN statistical sheets (Swanson *et al*., 2001).

2.3.9 Effect of pH on the Antilisterial Activity of Grape Juice

The pH of grape juice (batch 1) was 3.4. To determine whether the antilisterial activity of this grape juice was pH dependent the pH of 10 ml aliquots of the juice was adjusted to pH 5.0, 6.4, 7.5, 8.4, and 9.3 with sterile 1 M NaOH. A 3.8 ml aliquot of each solution was retained for assay and the remainder was frozen at -20^{o}C and stored for 24 hours for use in a pH re-adjustment experiment. A 100 µl aliquot of each pH-adjusted solution was spread onto a TSA plate prior to inoculation with *L. monocytogenes* to check for juice sterility. The solutions were assayed for antilisterial activity as described in section 2.3.2. The pH of each solution was measured at the end of the assay time using pH strips. The unadjusted pH grape juice (pH 3.5) was assayed as a control. To determine whether the antilisterial activity of pH adjusted grape juice solutions was reversible the previously frozen grape juice solutions were thawed at 20^{o}C and the pH of each returned to pH 3.5 with sterile 1M HCl and the solutions were assayed for antilisterial activity (section 2.3.2). A 100 µl aliquot of each solution was spread plated onto TSA prior to inoculation to check for sterility.

2.3.10 Effect of PVPP on the Antilisterial Activity of Grape Juice

To gain an understanding of the nature of the antilisterial factor in the commercial grape juice (batch 1) the phenolic material was removed from the grape juice by treating it with polyvinylpolypyrrolidone (PVPP), a high molecular weight fining agent, using the method of the Commission Regulations (EEC) No 2676/90 (1990). The phenolic components of the commercial grape juice were removed by mixing 20 ml of juice with 500 mg of PVPP (MW 40,000) for 10 minutes. The solution was passed through a 125 mm glass microfibre filter. The pH of the decolourised juice was measured and the solution pasteurised at 63^{o}C for 30 minutes and assayed against *L. monocytogenes* ATCC 35152 using the standard protocol (section 2.3.2). The percentage loss of total phenolics of grape juice after PVPP treatment was determined using the total phenolic method described in section 2.3.6.2.

2.3.11 Effect of Iron and Magnesium on the Antilisterial Activity of Grape Juice

The effect of the addition of iron and magnesium on the antilisterial activity of commercial grape juice (batch 1) was assessed following the method of Hsieh *et al*., (2001). Solutions of 0.4 M magnesium chloride ($MgCl_2.6H_2O$) and 0.4 M ferric chloride ($FeCl_3$) were prepared with sterile distilled water. A 100 µl aliquot of each ion solution was added to separate 3.7 ml aliquots of grape juice and control solutions of model grape juice (MGJ) (0.033 M L-tartaric acid pH 3.5). All solutions were adjusted to pH 4.5 with sterile 10 mM HCl and made up to a final volume of 3.8 ml. The final concentration of Mg^{2+} or Fe^{3+} in each solution (grape juice and MGJ control) was 10 mM. Solutions were pasteurised at 63^{o}C for 30 minutes and assayed against *Listeria monocytogenes* ATCC 35152 according to the protocol outlined in section 2.3.2. Control solutions consisting of model grape juice (0.033 M L-tartaric acid pH 3.5) and commercial grape juice (batch 1) were run in parallel.

2.4 Results and Discussion

2.4.1 Preservatives

No benzoic acid, sulphur dioxide or sorbic acid was found in the commercial grape juice.

2.4.2 Antimicrobial Spectrum of Grape Juice

The antimicrobial activity of grape juice (batch 1) was specific to *Listeria* species. All *Listeria monocytogenes* isolates and all other *Listeria* species assayed were rapidly inhibited by the grape juice (**Table 2.5)**. *Listeria* isolates were reduced from 10^6-10^7 CFU/ml to no colonies detected on the plates within 10 minutes exposure to the juice. The grape juice inhibited *L. monocytogenes* serotypes 1/2a and 4b, which have been associated with food-borne disease, to the same extent. All the other bacterial isolates assayed were not inhibited by exposure to the grape juice with the exception of *B. cereus*, which was reduced by 1-log within 10 minutes exposure to grape juice although no further reduction in cell numbers was observed after 60 minutes. The positive control Model Grape Juice (MGJ) was not inhibitory to any of the bacteria assayed. The results of this experiment confirmed that *L. monocytogenes* ATCC 35152 is appropriate to use as a reference isolate when assessing antilisterial efficacy of the grape juice as it is not unusually susceptible to the antimicrobial activity of this grape juice. The remaining antimicrobial assays were performed using this bacterial isolate.

20.1 Table 2.5 Antimicrobial spectrum of grape juice (batch 1).

Microorganism	Exposure time (minutes) in grape juice		
	0 (<1)	10	60
	Colony Forming Unit/ml		
Listeria monocytogenes 1/2a ATCC 35152	$3x10^6$	<10*	<10
Listeria monocytogenes 1/2 AF06283	$3x10^6$	<10	<10
Listeria monocytogenes 1/2 AF 01403	$1x10^6$	<10	<10
Listeria monocytogenes serotype not determined AE 9493	$1x10^5$	<10	<10
Listeria monocytogenes 1/2 AD 4470	$2x10^5$	<10	<10
Listeria monocytogenes 1/2 AF 03320	$2x10^6$	<10	<10
Listeria monocytogenes 4b CDC KC18	$2x10^6$	<10	<10
Listeria innocua 6a ATCC 33090	$2x10^5$	<10	<10
Listeria ivanovii 5	$4x10^6$	<10	<10
Listeria seeligeri 1/2b ATCC 35967	$7x10^5$	<10	<10
Bacillus cereus NCTC 7836	$2x10^5$	$2x10^4$	$2x10^4$
Staphylococcus aureus ATCC 25923	$2x10^6$	$2x10^6$	$2x10^6$
Salmonella Menston NCTC 7836	$6x10^6$	$6x10^6$	$6x10^6$
Escherichia coli ATCC 9610	$2x10^6$	$2x10^6$	$2x10^6$
Yersinia enterocolitica	$6x10^6$	$6x10^6$	$6x10^6$

*The detection limit of the assay is 10 CFU/ml. A result of <10 indicates no colonies detected.

Wine is a well known antimicrobial solution but a review of the literature pertaining to the antimicrobial activity of wines, grape juices, and grape skin extracts found antimicrobial activity against a range of bacteria but no mention of antimicrobial assays performed on *Listeria monocytogenes* or other *Listeria* species. A recent report by Ahn *et al*., (2004) found that the commercial grape seed extract (ActiVin) had antimicrobial activity against *Escherichia coli, Salmonella* Typhimurium, and *Listeria monocytogenes*, with *L. monocytogenes* being more sensitive to the inhibitory effect than the other bacterial species assayed. The authors found that

exposure to concentrations of ≥ 6 mg/ml ActiVin reduced *L. monocytogenes* numbers from 4-log to no detectable cells after 16 hours. The authors reported that the grape seed extract (ActiVin) consisted of phenolic compounds, including epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, *p*-coumaric acid, resveratrol, kaempferol, quercetin, and myricetin. The proportion of each phenolic compound in the extract was not stated. Early studies on the bactericidal action of red wine and laboratory prepared black grape extracts by Masquelier (1959) showed that *Salmonella typhi, Salmonella paratyphi, Escherichia coli,* and *Staphylococcus pyogenes* were inhibited after 15 minutes exposure to undiluted Bordeaux red wine. The author tested the inhibitory effect of a black grape extract (grape variety not given) containing the whole of the colouring material and an extract consisting of the colourless must, against *E. coli,* and found no inhibitory effect after 60 minutes exposure. Masquelier (1959) reported that when Bordeaux wine was treated with charcoal and then centrifuged, the decolourised supernatant no longer had antimicrobial activity. The author stated that the decolourised wine still contained as much ethanol and organic acids as the whole wine and concluded that ethanol and organic acids were not responsible for the bactericidal action of Bordeaux red wine. When *Helicobacter pylori* was exposed to Spanish red wine (Campillo, La Rioja) made from Tempranillo grapes, cell numbers were reduced from 10^9 CFU/ml to no detectable colonies within 5 minutes (Marimón *et al*., 1998). This rapid and complete inhibition of bacterial cells is equivalent to the action of commercial grape juice (batch 1) against *L. monocytogenes*. Sheth *et al*., (1988) found that wine (colour or type not stated) was inhibitory to *Salmonella* Typhimurium, *Shigella sonnei*, and *Escherichia coli*, with a 7-log reduction in cell numbers after 4 hours exposure to the wine. The authors did not identify the inhibitory factor in the wine. Weisse *et al*., (1995) reported a 5 to 6-log reduction of the same three bacteria within 20 minutes exposure to red and white wine. Both red and white wines were found to be equally as inhibitory to these microorganisms (Weisse *et al*., 1995). The authors did not identify the inhibitory factor in the wine but suggested that the antimicrobial action of wine was not due to the alcohol content or the low pH of the wine. Marimón & Bujanda, (1998) suggested the rapid inhibitory action of red wine against *Salmonella* Enteritidis was due to polyphenols present in the wine rather than low pH or alcohol. In contrast to the inhibitory action of wines reported by Masquelier (1959), Sheth *et al*., (1988), Weisse *et al*., (1995), Marimón & Bujanda, (1998), and Marimón *et al*., (1998), this thesis research found that only *Listeria* species were inhibited by the commercial grape juice (batch 1). The lack of inhibition observed for other bacteria may be due to the concentration of the inhibitory factor in the commercial grape juice. It is feasible that *Listeria* species are particularly sensitive to this inhibitory factor and that this factor was present in the grape juice at sub-inhibitory levels for bacteria other than *Listeria* species. Just & Daeschel, (2003) found that exposure of *Escherichia coli* and *Salmonella* spp. to red and white wine, and red and white

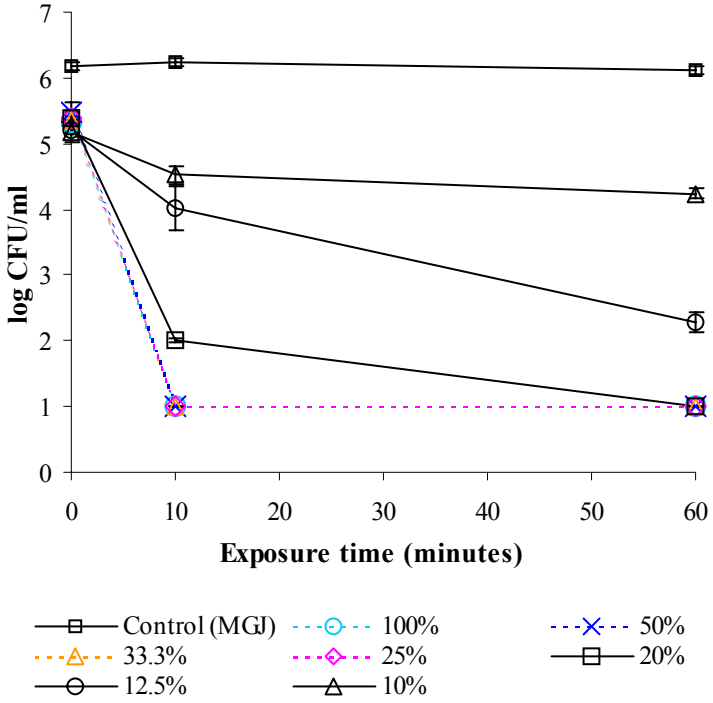
grape juice, made from Chardonnay and Pinot noir grapes obtained from Oregon vineyards, resulted in both bacteria being inhibited after 60 minute exposure to wine but surviving up to 16 days in the grape juice. This thesis researcher suggests that wine production processes may enhance the levels of antimicrobial factor in red and white wines and that this may increase the antimicrobial spectrum of wine. The commercial grape juice under investigation in this thesis was not processed in the same manner as wine and therefore may have had lower levels of the antimicrobial factor resulting in a narrow antimicrobial spectrum. In addition, the grape juice was made from black table grapes, which are a different variety than those used for the production of wine. The varietal differences between table grapes and wine grapes may in part, explain the differences in the antimicrobial spectrum reported for red wine compared to the commercial grape juice (batch 1).

Wine has been found to have antimicrobial activity against a range of microorganisms including gram-positive and gram-negative bacteria. However, the specificity of the commercial grape juice used in this study against *Listeria* species is curious. In general, *Listeria monocytogenes* is not unusually susceptible to biocides. However, it is well recognised that gram-positive bacteria are more susceptible to the action of biocides than gram-negative bacteria (Lambert, 2002). Gram–positive bacteria have a different cell envelope compared to gram-negative bacteria. Gram-positive bacteria have an inner cell membrane consisting of a lipid bilayer, and an outer cell wall consisting chiefly of peptideoglycan. Gram-negative bacteria have an inner cell membrane, an outer cell wall containing relatively little peptideoglycan, and an outer membrane composed of lipopolysaccharide, lipoprotein, and other macromolecules. The lack of an outer membrane and the permeable nature of the cell wall of gram-positive bacteria allows the penetration of biocides into the cell (Lambert, 2002). The impermeable outer membrane of gram-negative bacteria may provide a protective layer preventing the diffusion of hydrophobic compounds and large hydrophilic compounds (MW >600) (Ikigai *et al*., 1993) whereas the gram-positive cell wall allows access to antimicrobial molecules up to 30,000 to 57,000 Daltons, which includes most biocides (Lambert, 2002). The archetypal biocide, phenol, has a greater bactericidal efficiency against gram-positive bacteria than gram-negative bacteria (Clarke *et al*., 1963) because phenol and other compounds such as alcohols and aldehydes are small and can penetrate the gram-positive cell wall. The bactericidal activities of catechins were found to be more effective against gram-positive bacteria (Ikigai *et al*., 1993). The resistance of gram-negative bacteria was explained in part by the barrier function of the gram-negative outer membrane and the presence of negatively charged lipopolysaccharides at the exterior of the outer membrane, which reduced the binding of the biocide to the bacterial cell (Ikigai *et al*., 1993). Ahn *et al*., (2004) found that a commercial grape seed extract (ActiVin) had stronger inhibitory activity against *L. monocytogenes* than *E. coli* and *Salmonella* Typhimurium. The

antimicrobial action of grape seed extracts performed on a range of microorganisms showed that the extract was more effective against gram-positive bacteria than gram-negative bacteria (Jayaprakasha *et al*., 2003). It is well known that antimicrobial action of spices is more effective against gram-positive bacteria than gram-negative bacteria (Smith *et al*., 1998). The gram-positive bacteria *Bacillus cereus* was found to be more susceptible to cinnamic aldehyde derived from *Cinnamomum cassia* (cinnamon) than other gram-positive and gram-negative food-borne pathogens with inhibition of cell separation being a possible mechanism of action (Kwon *et al*., 2003). A range of berry extracts were found to inhibit the growth of gram-positive bacteria but not gram-negative bacteria and the authors suggest it is the difference in cell surface structure that attributes this difference in antimicrobial activity (Puupponen-Pimiä *et al*., 2001). *Listeria monocytogenes* is a gram-positive bacterium and this may in part explain the sensitivity of this bacterium to the inhibitory effects of the commercial grape juice under investigation.

2.4.3 Minimum Inhibitory Concentration of Grape Juice

The grape juice (batch 1) had very strong inhibitory activity against *L. monocytogenes*. The minimum inhibitory concentration (MIC) of grape juice (batch 1) against *L. monocytogenes* was 20% grape juice. After 60 minutes exposure to 20% grape juice *L. monocytogenes* numbers were reduced from 10^6 CFU/ml to no detectable colonies (the limit of detection of the assay is 10 colony forming units per ml or 1-log CFU/ml) (**Figure 2.1**). A 1-log reduction in cell numbers was observed at exposure time 0. Note that time 0 minutes does not refer to unexposed cells but rather to cells briefly exposed (less than 1 minute). This initial reduction of cell numbers at time 0 reflects the rapid inhibition that occurs in the short period of time taken to perform the serial dilution and spread plate the sample. The undiluted (100%) and 50%, 33.3%, and 25% dilution's of the grape juice were all strongly inhibitory with a 6-log reduction in cell numbers occurring after 10 minutes exposure to the juice. The most diluted grape juice solutions (12.5% and 10%) had slight inhibitory activity with a 4-log and 2-log reduction in cell numbers after 60 minutes, respectively. This result suggests that the antilisterial factor may be consumed in the assay possibly by binding to the bacterial cells and at a concentration of 12.5% and 10% grape juice the antilisterial factor may be depleted over the course of the assay. A consequence of this is that the inhibitory activity is reduced or halted after all the antilisterial factor has been "consumed".



21.1 Figure 2.1 Minimum Inhibitory Concentration (MIC) of grape juice (batch 1) against *Listeria monocytogenes* ATCC 35152. Error bars are the ± standard deviation of mean log CFU/ml.

2.4.4 Assessment of the Variability of Antilisterial Activity of Different Batches of Grape Juice

Being a commercial product, different batches of the grape juice used in this research might be expected to show variability in antilisterial activity. To assess the variability of antilisterial activity, two further batches (batch 2 purchased 31 May 2002, and batch 3 purchased 4 July 2002) of grape juice, undiluted, were assayed for antilisterial activity and compared to the activity of batch 1 grape juice. Variability in the antilisterial activity of three different batches (1, 2, and 3) of the same commercial grape juice was observed (**Figure 2.2**). Batch 1 grape juice had the strongest antilisterial activity (6-log reduction within 10 minutes). However, batches 2 and 3 grape juice only reduced *L. monocytogenes* numbers by 3-log within 10 minutes. After 60 minutes exposure to batch 2 grape juice, cell numbers were reduced further to give a total reduction of 4-log. Batch 3 grape juice had the weakest antilisterial activity with a total reduction of 3-log over 60 minutes. The control Model Grape Juice (MGJ) solution had no inhibitory effect.

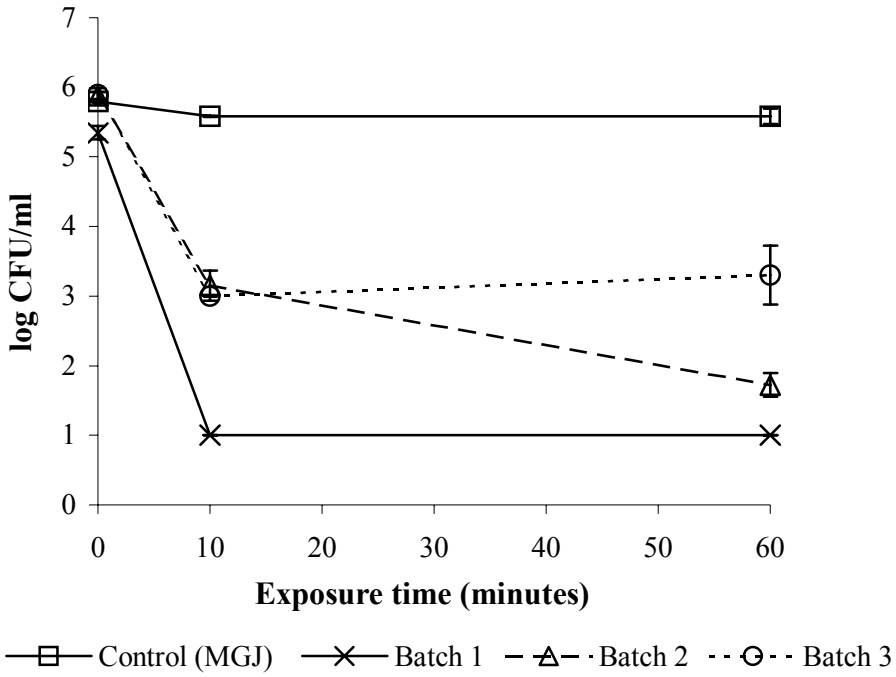


Figure 2.2 Survival of *Listeria monocytogenes* ATCC 35152 in three different batches of the same commercial grape juice. Error bars are the ± standard deviation of mean log CFU/ml.

The commercial grape juice was made from a grape juice concentrate supplied from Chile. The typical processing of grapes for juice involves heating, depectinisation with pectic enzymes, extraction, and concentration by evaporation of water. The length of time and temperature used in the pressing process determines the amount and nature of phenolic material including colour extracted into the final grape juice (McLellan & Race, 1990). Therefore, processing of grapes for juice can result in different chemical profiles of juice batches. This may be a reason for the variability in antilisterial activity of the three batches of commercial grape juice used in this thesis. The commercial grape juice used in this thesis is shelf stable with nine-month shelf life (Frucor, personal communication). It is known that the concentration and composition of compounds in natural grape concentrate can alter during storage. Buglione & Lozano (2002) found that colour deterioration of red grape juice occurred over time and increased storage temperatures (up to 30^{o}C) exacerbated this deterioration. Given that the commercial juice in this study is stored on supermarket shelves at room temperature (20^{o}C) it is possible that chemical changes will occur over time and that the longer the storage time on the shelf the greater the resultant chemical changes. This may also be a reason for the observed variability in antilisterial activity.

The process of concentrating grape juice can result in reduced solubility of some juice components and as the grape juice concentrate ages solids can settle out resulting in the reconstituted concentrate differing in chemical composition and physical parameters from the original juice (Haight & Gump, 1995). Differences in chemical composition of the three batches of commercial grape juice (section 2.4.5) and differences in storage time may have contributed to chemical changes in the grape juices, which in turn affected the antilisterial activity.

2.4.5 Chemical Analyses of Various Batches of Commercial Grape Juice

The pH, titratable acidity, total polyphenol level, and percentage polymeric colour of the three batches of commercial grape juice were measured in order to determine a reason for the variation in antilisterial activity and to attempt to identify a candidate antilisterial factor.

Preliminary chemical composition analyses performed on the three batches of commercial grape juice showed that the total polyphenol level and percentage polymeric colour only differed slightly in concentration from batch to batch and some were within experimental error (i.e. total phenolic level of batch 1 and 2 (**Table 2.6**)).

22.1 Table 2.6 Chemical composition of 1 ml samples of three batches of commercial grape juice.

	Decreasing antilisterial activity →		
Chemical Parameter	Batch 1	Batch 2	Batch 3
pH	3.40	3.47	3.43
Titratable acidity g/L tartaric acid	4.9	4.6	4.7
Total polyphenol mg/L GAE	3260 ± (309)	3155 ± (60)	2535 ± (14)

% polymeric colour (tannin-anthocyanin complexes)	65	58	62

Numbers in parentheses are standard deviations of the mean of two measurements.

The pH of the three samples was also similar as were the levels of titratable acidity. The most active batch (batch 1) had the highest total polyphenol level, titratable acidity, and percentage polymeric colour and the lowest pH. It is possible that in combination these chemical parameters could have conferred the highest level of antilisterial activity to this batch of grape juice. However, further detailed analyses were needed to identify the antilisterial factor.

Although the different batches (1, 2, 3) of grape juice were made from the same variety of grapes (*Vitis vinifera* var. Ribier) it was not known whether the grower of the grapes or the region the grapes were grown in was the same. Differences in growing conditions (soil conditions, plant root stock, prevalence of disease), environmental conditions (sunlight, temperature, presence of disease), harvest time, and processing of the grapes could all result in changes to the chemical composition of the commercial grape juice produced. As a result, any one variable or a combination of variables could alter the composition or level of the antilisterial factor in the grape juice.

The levels of total phenolic in the batches of grape juice were in the range of grape juices and wines reported in the literature. Amerine & Ough (1980) reported that red wine has an average total phenolic content of 1400 mg/L GAE with a total phenolics ranging between 190-3800 mg/L GAE. The total phenolic level of the three batches of commercial grape juice ranged from 2535-3260 mg/L GAE, which was within the ranges reported by the previous authors. The titratable acidity level of the three grape juices was 4.6-4.9 g/L, which was slightly below levels reported for grape juice and red wine. This may be because the grape juice was de-tartarated during processing. Amerine & Ough (1980) reported grape juice acidity between 6-9 g/L tartaric acid. The percentage polymeric colour (the percentage of colour in the sample that is contributed by pigmented compounds (anthocyanins) incorporated into polymeric structures) was greater than 50% in all batches of juice with the most active batch (batch 1) having the highest percentage of polymeric colour. Timberlake & Bridle (1976) reported that older red wines showed a decline in the amount of detectable monomeric anthocyanins with an increase in the level of polymeric colour. The high level of polymeric colour in the commercial grape juices could indicate that juice processing and

storage conditions might have induced the formation of polymeric colour. This could account for the high level of polymeric colour present in these juices, which in turn could confer the antilisterial activity. However, these gross chemical measurements did not give an overall indication of the nature of the antilisterial factor present in the commercial grape juice and further detailed chemical analyses were need to identify the antilisterial factor.

2.4.6 Antilisterial Activity of Various Commercial Red Grape Juices and New Zealand Red Wines

A number of different preservative-free commercial red grape juices and New Zealand red wines were assayed for antilisterial activity. None of these grape juices had an inhibitory action as potent as McCoys dark grape juice (batch 1) (**Table 2.7**).

23.1 Table 2.7 Antilisterial activities of various commercial red wines and red grape juices.

	Exposure time (minutes) in grape juice or wine	
Solutions Assayed	0	60
Red Wines	Colony Forming Units/ml	
Control (Model Wine Solution)	6.1×10^5	5.9×10^5
Cabernet Sauvignon	5.7×10^5	<10
Pinot Noir	5.7×10^5	<10
Cabernet Merlot	5.4×10^5	<10

Syrah	$5.9x10^5$	<10
Merlot	$5.9x10^5$	$5.8x10^5$
Grape Juices		
Control (Model Grape Juice)	$6.0x10^5$	$6.0x10^5$
McCoys Grape Juice (batch 1)	$6.4x10^5$	<10
Grapetise	$5.5x10^5$	$3.1x10^5$
Greenways	$3.4x10^5$	$1.7x10^4$
Organic Shiraz	$6.0x10^5$	$5.2x10^5$

After 60 minutes exposure time only Greenways red grape juice had an antilisterial effect. This juice reduced *L. monocytogenes* numbers by 1-log over 60 minutes. When compared to the 6-log reduction achieved by the McCoys grape juice (batch 1) the antilisterial activity of Greenways juice was much lower. New Zealand red wines showed much higher antilisterial activity than all the commercial red grape juices, excluding McCoys grape juice (batch 1). With the exception of Merlot, all the red wines reduced *L. monocytogenes* numbers from 10^6 CFU/ml to no detectable colonies after 60 minutes exposure. Merlot reduced *L. monocytogenes* number by 1-log after 60 minutes exposure to the wine. The reason for the reduced inhibitory action of Merlot was not elucidated. The positive control Model Wine Solution (MWS) (0.033 M L-tartaric acid, pH 3.5, 10% ethanol) had no antilisterial effect thereby discounting any antimicrobial effect of the alcohol and acidic pH present in the wine.

It is not surprising that red wines were inhibitory to *Listeria monocytogenes* given the rapid and strong antimicrobial action of red wines against other bacteria reported by others (Masquelier, 1959; Sheth *et al*., 1988; Weisse *et al*., 1995; Marimón & Bujanda, 1998; Marimón *et al*., 1998). Of interest was the lack of antilisterial action of all other preservative-free commercial red grape juices (excluding McCoys grape juice) currently available in New Zealand. The result indicated that McCoys grape juice contains an antilisterial factor not present or present but at sub-inhibitory levels in other grape juices. The similar strength of antilisterial activity present in New Zealand red wines

assayed indicated that the antilisterial factor present in the McCoys grape juice might be similar in concentration and/or chemical composition to red wine. Masquelier (1959) found that grape juice had a lower antimicrobial action compared to red wine but the author discounted the effect of ethanol and organic acids. This thesis research suggests that it might be the grape variety and/or grape juice processing and storage methods that determine whether a grape beverage will have antimicrobial activity.

2.4.7 Bactericidal or Bacteriostatic Antilisterial Action of Grape Juice

A Most Probable Number (MPN) test can be used to determine whether an antimicrobial compound has a bactericidal or a bacteriostatic effect. Bactericidal antimicrobial compounds kill the organism being tested (i.e. removal of the factor from the assay system does not result in the organism being revived). A bacteriostatic effect means that once the inhibitory factor is removed from the assay system or the bacterium's surrounding the bacteria is able to resume growth.

The MPN test results revealed that the commercial grape juice (batch 1) was bactericidal. Once exposed to the grape juice, *L. monocytogenes* was killed and diluting the antilisterial factor in BHI broth to below inhibitory levels (dilution's 1:10, 1:100, and 1:1000 (minimum inhibitory concentration grape juice 20% see section 2.4.3)) did not result in the regrowth of the bacteria. At 0 minutes exposure to the grape juice (batch 1) *L. monocytogenes* numbers were greater than 1100 cells/ml (**Table 2.8)**. After 10 minutes exposure cell numbers were reduced to 460 cells/ml with 95% confidence that the number of live bacteria is between 100 and 2400 cells/ml. After 30 minutes cell numbers were further reduced to 9 cells/ml with 95% confidence numbers ranging between 2 and 38 cells/ml. After 60 and 180 minutes exposure to grape juice the number of viable bacterial cells was reduced to 3 cells/ml. After exposure to grape juice for 60 minutes or longer and dilution of the antilisterial factor to below an inhibitory level, recovery and growth of *L. monocytogenes* did not resume.

24.1 Table 2.8 Statistical analyses of Most Probable Number tubes performed on *Listeria monocytogenes* ATCC 35152 exposed to undiluted commercial grape juice (batch 1).

Exposure time of bacteria in undiluted grape juice	Dilution				
	1:10	1:100	1:1000	Most Probable Number	95% CI*

Minutes	Number of positive growth tubes[#]			CFU/ml	
0	3	3	3	>1100	---
10	3	3	1	460	100, 2400
30	2	0	0	9	2, 38
60	0	0	0	<3	---
180	0	0	0	<3	---

*95% confidence interval refers to estimated mean of the surviving population of *L. monocytogenes* after exposure to the undiluted grape juice.

The number of positive growth tubes refers to the number of tubes of BHI broth inoculated with *L. monocytogenes* after exposure to the grape juice that are positive for bacterial growth. For example after *L. monocytogenes* is exposed to grape juice for 60 minutes none of the three tubes of each dilution are positive for bacterial growth, indicating that all *L. monocytogenes* cells are dead. This results gives an MPN of <3 which statistically is the lowest number of viable cells.

A bacteriostatic effect would have resulted in the regrowth of the bacteria once the exposed cells were placed into fresh growth media. This did not occur and as such the result of this experiment confirmed that the grape juice (batch 1) had bactericidal activity against *L. monocytogenes*. After 48 hours incubation the uninoculated control tubes showed no turbidity indicating that sterility of the media was intact and that positive growth tubes were not due to media contamination.

The bactericidal and bacteriostatic action of biocides tends to be on a continuum and the nature of the action depends on biocide concentration as well as bacterial exposure time (Denyer, 1995). Many antimicrobial agents are bacteriostatic at low concentration where they can cause reversible damage to the cell such as permeability changes to the cell membrane causing inhibition of nutrient transport and energy generation, reversible interaction with nucleic acids, and reversible enzyme inhibition. Once the biocide is removed the bacteria is able to repair cellular damage and begin growing again. In many cases the biocide can cause irreparable cell damage such as cell leakage, autolysis, and cytoplasmic coagulation. In this case the biocide is bactericidal (Denyer, 1995). As *L. monocytogenes* did not recover and grow in the growth media the commercial grape juice was classified as bactericidal.

This result also confirmed that acid shock (the acid induced cessation of bacterial growth) was not responsible for the observed decrease in *L. monocytogenes* numbers after exposure to the

commercial grape juice. Many bacteria will cease to grow below pH 4.5 but remain viable (Hill *et al*., 1995). *L. monocytogenes* cells grown at pH 7.0 overnight (16 hours) are tolerant to challenge at pH 3.5 with growth beginning within 60 minutes (Hill, *et al*., 1995). Most Probable Number results indicated that acid shock was not responsible for the inhibition of *L. monocytogenes* exposed to the grape juice as the cells do not begin to grow once removed from the acid stress (grape juice) by inoculation into fresh growth media at pH 7.0.

2.4.8 Effect of pH on the Antilisterial Activity of Grape Juice

The antilisterial activity of the grape juice (batch 1) was pH dependent. When the pH of the grape juice was raised to pH 6.4 or above, with 1 M NaOH, the antilisterial activity was lost (**Table 2.9**). The pH of the juice samples was confirmed at the end of the assay and found to be the same pH as they had been adjusted to. Exposure of *L. monocytogenes* to grape juice at pH 6.4, 7.5, 8.4, and 9.3 resulted in no reduction in cell numbers after 60 minutes exposure to the grape juice. At acidic pH (3.5 and 5.0) the grape juice had antilisterial activity with *L. monocytogenes* cell numbers being reduced from 10^6 CFU/ml to no detectable colonies after 10 minutes. The level of *L. monocytogenes* inhibition at pH 5.0 was equivalent to the inhibitory activity observed at the natural pH of the grape juice (pH 3.5).

25.1 Table 2.9 Effect of increased pH of commercial grape juice (batch 1) on the antilisterial activity.

		Exposure time (minutes) in grape juice		
		0	10	60
pH of Grape Juice	Colour of Grape Juice	Colony Forming Units/ml		
pH 3.5	Red	$1.8x10^6$	<10	<10
pH 5.0	Red	$1.7x10^6$	<10	<10
pH 6.4	Black	$1.9x10^6$	$2.1x10^6$	$1.3x10^6$

pH 7.5	Black	$1.4x10^6$	$1.3x10^6$	$1.3x10^6$
pH 8.4	Black	$2.3x10^6$	$1.9x10^6$	$1.9x10^6$
pH 9.3	Black	$2.2x10^6$	$2.0x10^6$	$1.9x10^6$

Adjusting the pH of the grape juice samples (pH 6.4, 7.5, 8.4, 9.3) back to pH 3.5 with 10 mM HCl resulted in the complete reinstatement of the antilisterial activity of these four grape juice samples (**Table 2.10)**. The pH readjusted grape juice samples reduced *L. monocytogenes* to the same extent (10^6 CFU/ml to no detectable colonies after 60 minutes exposure to the juice) as the natural pH 3.5 grape juice. The antilisterial factor in the grape juice was not destroyed by altering the pH, as it was reinstated once the pH was returned to acidic levels (pH 3.5).

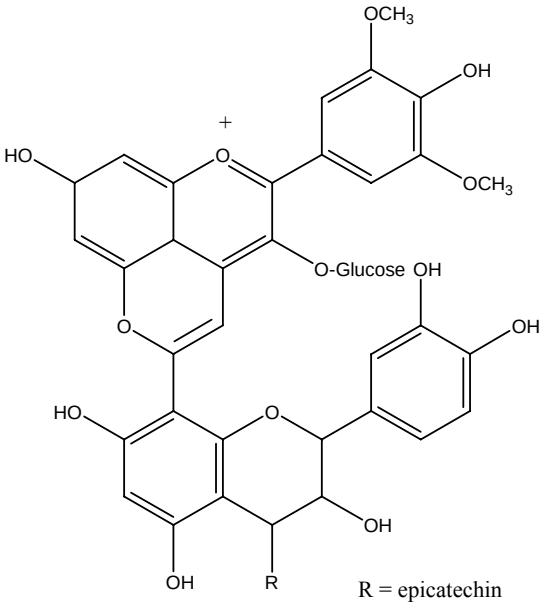
Table 2.10 Effect on the antilisterial activity of re-adjusting the pH of commercial grape juice samples back to 3.5.

		Exposure time (minutes) in grape juice		
		0	10	60
pH of Grape Juice	Colour of Grape Juice	Colony Forming Units/ml		
pH 5.0 to 3.5	Red	$1.1x10^6$	$5.8x10^2$	<10
pH 6.4 to 3.5	Red	$3.0x10^5$	<10	<10
pH 7.5 to 3.5	Red	$4.8x10^5$	<10	<10
pH 8.4 to 3.5	Red	$6.3x10^5$	<10	<10

pH 9.3 to 3.5	Red	$4.0x10^5$	<10	<10

Concomitant with an increase in pH (pH 6.4 and above) and the loss of antilisterial activity was a change in colour of the grape juice from red to opaque black. When the grape juice samples were adjusted back to pH 3.5 the colour of the juice turned from black back to red concomitant with the recovery of antilisterial activity. Many of the phenolic compounds in grape juice, including the coloured anthocyanins have different structures at acidic pH compared to neutral or alkaline pH. Monomeric anthocyanin pigments undergo reversible structural transformations with change in pH (**Figure 1.10**). Asenstorfer *et al*., (2003) reported that at pH less than 3.6, malvidin-3-glucoside was cationic (red) to varying degrees depending on the pH of the solution. The authors also reported that at pH values of greater than 5 two major anionic species existed, the blue-coloured quinonoidal anion and the yellow-coloured chalcone anion. It is feasible that when the commercial grape juice was at pH ≥6.4 the antilisterial activity was lost because the majority of the anthocyanins were in the quinonoidal base and chalcone forms rather than the cationic flavylium form. When the grape juice was at pH ≤5.0 (coloured red) it was inhibitory to *L. monocytogenes* and this may be due to the anthocyanin moiety being in the mono ionised quinonoidal base and the flavylium cation forms, with the mono ionised quinonoidal base form predominating. However, given that the grape juice was red at pH 3.5 and 5.0 it appears that some of the anthocyanins may have been in the flavylium cationic form. Somers (1971) proposed the presence of the flavylium form of anthocyanins in the polymer structures of red wine. He reported that the equilibrium of red wine polymeric pigments was more complex than monomeric anthocyanins and reported the colour stability of the wine polymeric pigments, which retained more than 50% of the maximum colour in the pH range of 0.75 to 4.76. It is therefore feasible that anthocyanins incorporated into polymeric structures in the commercial grape juice are in the cationic (red) form. The structure of the anthocyanin moieties incorporated into polymers is unknown. However, a number of researchers have proposed different linkages and structures of anthocyanins in tannin polymers. The structure proposed by Francia-Aricha *et al*., (1997) showed that the anthocyanin moiety was in the flavylium (cationic) form (**Figure 2.3**), however other species in the anthocyanin equilibrium may also be present. This researcher speculates that the positively charged anthocyanin moiety of the polymer may be the initial interaction point of the condensed tannin with the negatively charged surface of *L. monocytogenes* resulting in cell death. Given that the antilisterial activity of the commercial grape juice was pH dependent and reversible (i.e. the antilisterial activity was lost at pH 7.0 but reinstated once the pH of the juice is returned to pH 3.5) and that anthocyanins undergo reversible

structural transformations with a change in pH, it is possible that anthocyanin moiety in the polymeric structure may be important in conferring antilisterial activity to the grape juice.



26.1 **Figure 2.3 Proposed structure and linkage of malvidin-3-glucoside and a flavan-3-ol.** (taken from Francia-Aricha *et al*., 1997)

27.1

Many phenolic compounds are weak acids and will partially dissociate, depending on the structure of the molecule, and form phenolates in alkaline (pH 10) solutions (Ribéreau-Gayon, 1972). The pKa of natural phenols is pH 9.0 to 10 (pK phenol 9.89) and at or below this pH the protonated form of phenol predominates (Singleton, 1987). Organic acids are known to be antimicrobial only under acidic conditions. The undissociated (protonated) form of benzoic acid is responsible for the antimicrobial activity (Beuchat & Golden, 1989). *L. monocytogenes* is know to be susceptible to the antimicrobial action of organic acids such as acetic acid, lactic acid, citric acid (Farber *et al*., 1989; Lake *et al*., 2002), and propionic acid (Conner *et al*., 1990). Given the naturally acidic environment of wine and this commercial grape juice, most of the carboxylic acids, including all the phenolic compounds such as those described in Chapter One, will be in a predominantly undissociated form. This state could confer the antimicrobial activity of the grape juice and wines. However, previous authors (Masquelier, 1959; Marimón & Bujanda, 1988) have discounted organic acids as the major antimicrobial factor in red wines.

2.4.9 Effect of PVPP Treatment on the Antilisterial Activity of Grape Juice

Treating the grape juice (batch 1) with PVPP (25 mg/ml w/v) (section 2.3.10) to remove phenolic compounds significantly reduced the antilisterial activity of the juice. After 60 minutes exposure to the PVPP treated grape juice only a 0.5-log decrease in *L. monocytogenes* numbers was observed compared to a 6-log reduction in the untreated juice over the same exposure time (**Figure 2.4).**

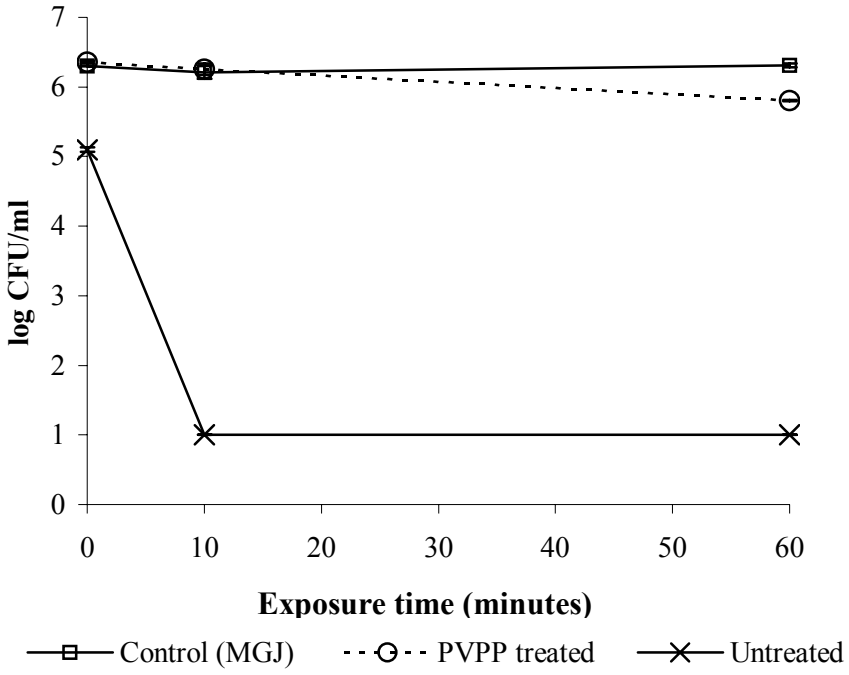


Figure 2.4 Effect of PVPP treatment on the antilisterial activity of grape juice (batch 1). Error bars are the ± standard deviation of mean log CFU/ml.

Observation of the PVPP treated grape juice compared to untreated juice showed the treated juice was partially decolourised (**Figure 2.5**).

28.1 Figure 2.5 PVPP treated (left) and non-treated (right) commercial grape juice samples.

The percentage loss of total polyphenols from the PVPP treated grape juice, as measured by the total phenolic method using the Folin-Ciocalteu reagent, was 65% (**Table 2.11**). This result indicated that the antilisterial factor could be a phenolic compound.

Table 2.11 pH and total polyphenol level of untreated and PVPP treated commercial grape juice (batch 1).

Sample	pH	Total polyphenol level mg/L GAE
Untreated grape juice	3.40	3240
PVPP treated grape juice	3.42	1134
% loss total polyphenols after PVPP treatment mg/L GAE		65%

PVPP has an affinity for phenolic compounds and forms hydrogen bonds between the carbonyl group on the PVPP and the phenolic hydrogens (Zoecklein *et al*., 1995a). PVPP has poor affinity for condensed tannins but moderate to high affinity for low molecular weight flavan-3-ols (Rossi & Singleton, 1966). In addition, PVPP has a cross-linked structure with cavities within the polymer matrix which gives rise to its specificity for smaller polyphenol molecules and is used specifically

for removing low molecular weight phenolics from white grape juice and wine (G. Jones, personal communication). Only partial decolourisation of the grape juice was achieved with PVPP as seen in **Figure 2.5**. The pH of the PVPP treated juice (3.42) was not altered from the original juice pH of 3.4 (batch 1). This indicated that the majority of the organic acids remained in the decolourised juice and therefore did not contribute the majority of the antilisterial activity to the commercial grape juice. This result is in agreement with the findings by Masquelier (1959) who found that decolourisation of red wine using charcoal resulted in the loss of bactericidal action of the decolourised supernatant against *Escherichia coli*. The bactericidal action of wine against *E. coli* was reinstated when the charcoal-eluted material was added back to the decolourised wine. Masquelier (1959) postulated that the bactericidal action of wine is due to colouring material as the decolourised wine still contained the ethanol and organic acid components. Konowalchuk & Spiers (1976) found that the antiviral effect of grape juice was destroyed by the addition of 0.5% PVPP. The results of our experiment are in agreement with the findings of Masquelier (1959) and Konowalchuk & Spiers (1976) as the antimicrobial factor present in grape juice was removed from the solution by PVPP. Because PVPP binds phenolic compounds, the result of this experiment suggested that the antilisterial factor present in the grape juice is likely to be a phenolic compound or group of phenolic compounds rather than organic acids.

2.4.10 Effect of Iron and Magnesium on the Antilisterial Activity of Grape Juice

Many macronutrients and micronutrients are required for biosynthesis of bacterial cell components. Iron plays a major role in cellular respiration and is a key component of cytochromes and iron-sulphur proteins are involved in electron transport (Brock *et al*., 1994). Magnesium is needed to stabilise the bacterial cell membrane, stabilise ribosomes, and is required for phosphate transfer enzymes (Brock *et al*., 1994). The addition of Mg^{2+} or Fe^{3+} (final concentration 10 mM $MgCl_2.6H_2O$ and $FeCl_3$, respectively) to the commercial grape juice (batch 1) reduced the antilisterial activity (**Figure 2.6**).

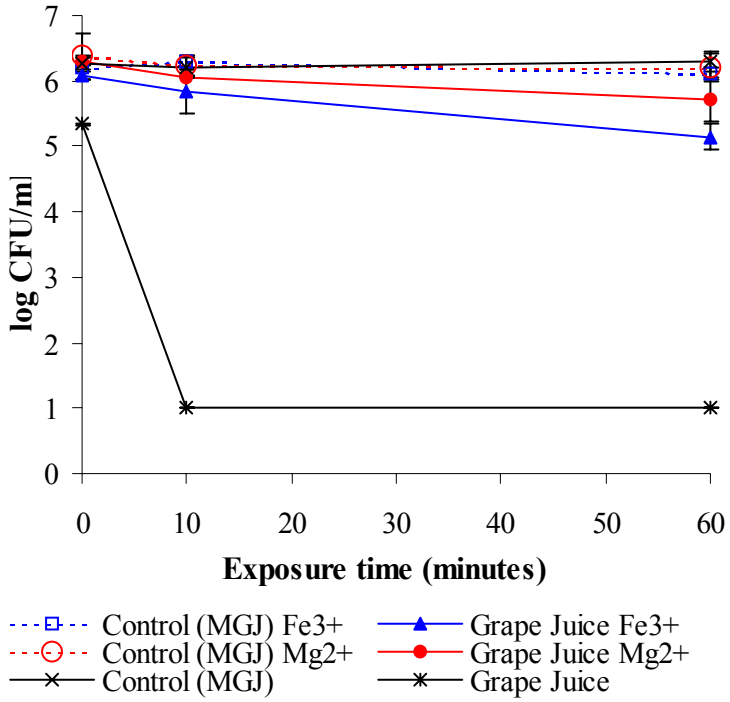


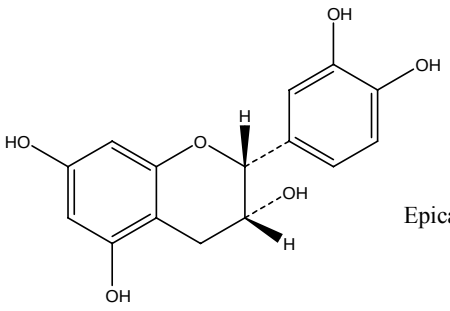
Figure 2.6 Effect of the addition of iron and magnesium on the activity of grape juice (batch 1) against *Listeria monocytogenes* ATCC 35152. Error bars are the ± standard deviation of mean log CFU/ml.

$Mg^{2+}$was slightly more effective than Fe^{3+} in reducing the antilisterial activity of the juice. Less than 1-log reduction in *L. monocytogenes* numbers was observed after 60 minutes exposure to the juice containing iron or magnesium, a significant reduction in activity compared to the 6-log reduction observed in juice without the addition of these ions. After the addition of the ions to the grape juice the pH of the grape juice was adjusted to pH 4.5, which is within the antilisterial range (section 2.4.8), indicating that a change in pH was not responsible for the loss of antilisterial activity.

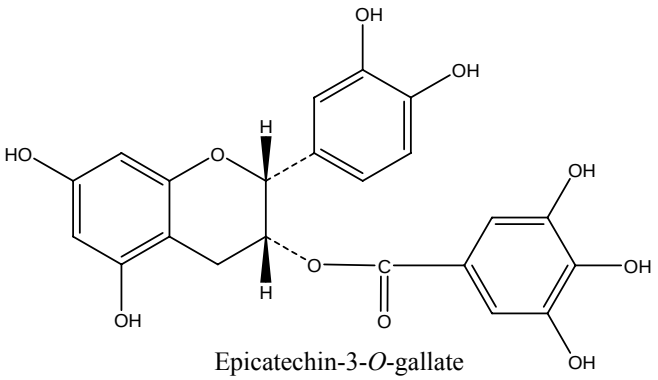
The mechanism by which condensed tannins inhibit bacteria has not been clearly elucidated (Smith *et al*., 2003). However, the antimicrobial action of condensed tannins and anthocyanins has been suggested to be due to the ability of these compounds to chelate metal ions necessary for microbial growth (Scalbert, 1991). Somaatmadja *et al*., (1963) demonstrated that anthocyanins could form chelates with copper ions in a pH dependent manner. The addition of $MgCl_2$ to the anthocyanin malvidin-3-glucoside reduced the inhibitory effect of this anthocyanin against *S. aureus* (Powers, 1964). The authors suggested that the antimicrobial mechanism of the anthocyanin might be

chelation of metals essential for microbial nutrition or a particular enzyme system. Chung *et al*., (1998) found that the antimicrobial activity of tannic acid against a range of bacteria was reduced by the addition of 2 μM $FeSO_4.7H_2O$ to the growth media. They suggested that tannic acid chelates iron in the bacterial growth medium making iron unavailable to the bacteria. Hsieh *et al*., (2001) found the addition of 1 mM and 10 mM Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{3+} to various plant extracts decreased the antimicrobial action of these extracts. The authors found that Fe^{3+} was less effective than the other ions at reducing the antimicrobial action. They suggested that the metal ions might interact with the plant extract thereby eliminating the antimicrobial effect. The authors did not suggest a reason for more pronounced effect of Mg^{2+} and other ions compared to Fe^{3+}. Khokher & Owusu Apenten (2003) reported that the presence of *ortho*-dihydroxyphenol moieties of polyphenols were important for iron binding. This author suggests that the addition of metal ions (Mg^{2+} and Fe^{3+}) to the grape juice may result in metal ion binding to the *ortho*-dihydroxyphenol groups in the grape juice thereby reducing the ability of these groups to bind to the bacterial cells resulting in a reduction in the inhibitory effect of the polyphenols.

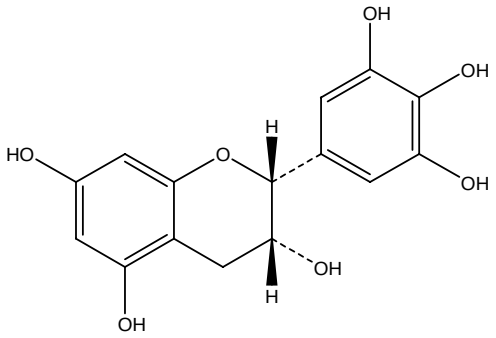
The antimicrobial activity of tannins (polymeric phenolics) is probably determined by the molar content and spatial configuration of the *ortho*-dihydroxyphenolic groups (Schofield *et al*., 2001). These *ortho*-dihydroxyphenol groups can form chelates with ferric and cupric ions (Scalbert, 1991; Santos-Buelga & Scalbert, 2000). Khokher & Owusu Apenten (2003) determined the iron binding efficiencies (*in vitro*) of a number of pure phenolic compounds including gallic acid, tannic acid, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin, and epigallocatechin-gallate. The authors found that the presence of a 3,4 dihydroxy group on the flavonoid B ring (i.e. epicatechin) was associated with high iron binding efficiency, whereas the presence of a 3,4,5 trihydroxyl group of ring B (i.e. epigallocatechin) and gallate moiety (i.e. epicatechin-3-*O*-gallate) was associated with reduced iron binding (**Figure 2.7**). The authors concluded that the presence of *ortho*-dihydroxyl groups and a large number of OH groups were important for iron binding.



Epicatechin



Epicatechin-3-*O*-gallate



Epigallocatechin

Figure 2.7 Structures of epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin.

The results of this thesis experiment suggested that the antilisterial factor in the commercial grape juice was able to chelate metal ions and thus may have chemical or physical properties similar to or the same as condensed tannins and/or anthocyanins, both of which are present in the grape juice.

2.5 Summary

The commercial grape juice used in this study was made from *Vitis vinifera* var. Ribier grapes and was found to contain no common preservatives such as benzoic acid, sorbic acid, or sulphur dioxide. Preliminary screening of the antimicrobial spectrum of the grape juice showed the juice to be highly inhibitory to all *Listeria* spp. assayed including the *L. monocytogenes* serotype 1/2a and 4b, which have been associated with food-borne disease and listeriosis. The antilisterial activity of the grape juice was comparable in lethality to New Zealand red wines. Other commercial red grape juices were found to have little or no antilisterial activity. The inhibitory action of the commercial juice was found to be bactericidal not bacteriostatic. The lethality of this juice was found to be comparable to the results of previous studies on the antimicrobial action of red wine (Masquelier, 1959, Sheth *et al*., 1988; Weisse *et al*., 1995; Marimón & Bujanda, 1998; Marimón *et al*., 1998; Sugita-Konishi *et al*., 2001). In contrast to these studies on wine the commercial grape juice was

only inhibitory against *Listeria* species and this narrow antimicrobial spectrum may indicate the presence of a specific antilisterial factor in this juice that differs from that responsible for the effects seen in red wine. This is conceivable given varietal differences between wine and table grapes and the fact that wine has been through a process of fermentation that alters the chemical composition of the initial grape juice. As a commercial product, the grape juice might be expected to show variability in chemical composition as well as antimicrobial activity. There was variability in the antilisterial activity of three different batches (1, 2, 3) of the same commercial grape juice as well as variability in chemical composition. Grape juice (batch 1) had the strongest antilisterial activity with a total reduction of 6-log over 60 minutes whereas grape juice (batch 3) had the weakest antilisterial activity with a total reduction of 3-log for the same time period. Chemical analyses performed on the three batches of grape juice suggested a relationship between antilisterial activity and total phenolic level but no other chemical parameter analysed. It is possible that it is not the actual level of phenolic compounds present in the commercial grape juices but rather a specific type, or combination of phenolic compounds that may impart the antilisterial activity. Decolourisation of the juice with PVPP resulted in the loss of inhibitory activity and the addition of iron and magnesium to the juice prior to assay also resulted in the suppression of antilisterial activity. Both these results suggested the organic acids were not the major antilisterial factor and that polymeric phenolics (condensed tannins) were implicated as being the inhibitory factor. This thesis researcher speculates that *ortho*-dihydroxyphenol groups of the grape juice condensed tannin might be responsible, in part, for the antilisterial activity. The antilisterial activity of the grape juice was found to be pH dependent. Increasing the pH of the grape juice from pH 3.5 to above pH 6.4 had a significant effect on the antilisterial activity with the loss of activity occurring at neutral to alkaline pH. This pH effect was reversible with antilisterial activity reinstated after the pH of the juice was returned to 3.5.

These investigations suggest that polymeric phenolics (condensed tannin) were involved in the antilisterial activity of the commercial red grape juice. Condensed tannin is present in the skin and seed of grapes. Fractionation and analysis of the phenolic material was performed on the skin, seed, and juice/pulp of Ribier grapes to identify the source of the antilisterial compound(s) present in the commercial grape juice.

3 ORIGIN AND ISOLATION OF AN ANTILISTERIAL FRACTION FROM COMMERCIAL GRAPE JUICE

3.1 Introduction

The commercial grape juice investigated in this thesis is made from *Vitis vinifera* variety Ribier black table grapes. The objective of Chapter Three was to firstly identify the type of phenolic compound that imparts the antilisterial activity of the commercial grape juice. Investigations outlined in Chapter 2 suggest that phenolic material, in particular polymeric phenolic material (condensed tannin) might be the antilisterial factor. In order to identify the antilisterial factor, the commercial grape juice was fractionated into polymeric and monomeric phenolic fractions and the fractions assessed for antilisterial activity. McCoys grape juice batch 2 was selected for fractionation due to the lack of availability of batch 1 and because it had stronger antilisterial activity when compared to batch 3. Polymeric phenolic material (condensed tannin) is known to complex with proteins, carbohydrates, and polysaccharides (Bate-Smith, 1973; Haslam, 1974; Scalbert, 1991; Mueller-Harvey & McAllen, 1992). In order to further characterise the type of

phenolic compound responsible for the antilisterial activity of the commercial grape juice the polymeric phenolic fraction was treated with protein and the effect on antilisterial activity assessed. The second objective of Chapter three was to determine the origin of the antilisterial factor in the commercial grape juice by assessing the inhibitory activity of the components (skin, seed, juice/pulp) of the Ribier black table grapes. The distribution of total phenolics in red grapes has been reported as seed 62.6%, skin 33.3%, and juice/pulp 4.1% (Amerine & Ough, 1980). The antilisterial activity of Ribier skin, seed, and juice/pulp fractions of Ribier grapes was assessed. To further identify the origin of the antilisterial factor in the commercial grape juice polymeric phenolic fractions of the Ribier grape skin and seed extracts were assessed for antimicrobial spectrum, the effect of pH on the inhibitory action, and the effect of dialysis on the inhibitory action and compared to the effects of these treatments on the commercial grape juice polymeric phenolic fraction.

3.2 Materials

29.1 **Table 3.1 List of materials used in Chapter Three.**

Materials and Instruments	**Source**
Acetic acid (HPLC grade)	BDH
Acetone (AR grade)	BDH
Acetonitrile (HPLC grade)	BDH
Bovine Serum Albumin	Sigma
Caffeic acid	Sigma
Catechin	Sigma
Dialysis tubing (12-14 kDa MWCO)	Visking, Medicell
Gallic acid	Sigma
Hydrochloric acid	BDH
Malvidin-3-glucoside (HPLC grade)	Extrasynthase, France (info@extrasynthase.com)
Methanol (HPLC grade)	Merck
para-Courmaric acid	Sigma
pH meter (Meterlab)	Radiometer
trans-Piceid	Polyphenols, Norway (mail@polyphenols.com)
Sephadex LH20	Amersham Pharmacia Biotech AB, Sweden
Sodium hydroxide (AR grade)	BDH
Syringic acid	Sigma

All other materials used have been listed in the Materials list in Chapter 2.

3.3 Methods

3.3.1 Fractionation of Monomeric and Polymeric Phenolics from Grape Juice

Preliminary investigations from Chapter 2 suggested that polymeric phenolic material (condensed tannin) may confer the antilisterial activity to McCoys red grape juice. The monomeric phenolic compounds and polymeric phenolic component of the commercial grape juice (batch 2) were separated according to the method of Kantz and Singleton (1990) with some modifications.

Sephadex LH20 was swelled according to manufacturer's instructions. A column of 1 cm diameter was packed with the Sephadex matrix to a height of 15 cm. The matrix volume was 12 ml. The column matrix was pre-equilibrated with acidified water (0.1% HCl). A 5 ml aliquot of grape juice was added slowly added to the top of the matrix and allowed to diffuse into the matrix. Acidified water (0.1% HCl) was run through the column to wash the juice into the matrix. The monomeric phenolics and sugars were eluted (gravity flow rate 0.7 ml/min) in succession with acidified water (120 ml), 20% aqueous acidified methanol (0.1% HCl) (20 ml), and then 40% aqueous acidified methanol (0.1% HCl) (20 ml). These fractions were pooled. The polymeric phenolic material was eluted with 50% aqueous acidified acetone (0.1% HCl) (50 ml). The monomeric and polymeric phenolic fractions were rotary evaporated (Büchi, Switzerland) at 60^{o}C to remove solvent and water to reduce the volume. The fractions were made up to 4.8 ml with sterile distilled water. The pH of the fractions was adjusted to 3.5 with sterile 10 mM NaOH and made up to a final volume of 5 ml (the original volume of fractionated grape juice) with sterile distilled water. Prior to pH adjustment, a 500 µl volume of 99.95% ethanol was added to the polymeric fraction, as it was not completely soluble in the aqueous solution. The monomeric and polymeric fractions were analysed for total phenolics (section 2.3.6.2) and assayed for antilisterial activity as described in section 2.3.2. A control of 50% aqueous acidified acetone (0.1% HCl), rotary evaporated under the same conditions and made up to the same volume and pH, was run in parallel to account for any antilisterial effects due to any remaining HCl or residual acetone.

3.3.2 HPLC Analysis of Monomeric and Polymeric Phenolic Fraction of Grape Juice.

Monomeric and polymeric grape juice fractions of commercial grape juice (batch 2) were analysed for phenolic compounds by HPLC using the method of Kilmartin *et al*., (2002).

The fractions were analysed on a Hewlett Packard 1100 Series HPLC with an 1100 diode-array detector. Samples were chromatographically separated using a reverse phase C18 column (250 mm 4.6 mm i.d, 5µm particle size, 100 Å pore size) (Phenomenex) heated to 25^{o}C. All solvents used were HPLC grade. Injection volume 20µl, elution conditions were as follows: flow rate 0.8 ml per minute, solvent A milli-Q water, solvent B 5% aqueous acetic acid solvent C 100% acetonitrile. A wash and re-equilibration phase was performed at the end of the gradient (115-125 minutes). The gradient used is presented in **Table 3.2**.

Table 3.2 Solvent gradient used to chromatograph the monomeric and polymeric fractions of commercial grape juice (batch 2).

Time (minutes)	**Percentage Solvent used**		
	A (milli-Q-water)	**B** (5% acetic acid)	**C** (100% acetonitrile)
0	45	55	0
10	25	75	0
20	0	100	0
30	0	100	0
50	0	90	10
70	0	85	15
80	0	80	20
95	0	60	40
105	0	55	45
115	45	55	0
125	45	55	0

Detection of peaks was at the following wavelengths: 520 nm, 280 nm, 305 nm, 320 nm, and 365 nm. The phenolic compounds gallic acid, catechin, malvidin-3-glucoside, vanillic acid, syringic

acid, *para*-courmaric acid, caffeic acid, and *trans*-piceid were identified using commercial standard retention times. Caftaric acid was identified using the retention time of Zou *et al*., (2002) who conducted their research using the same HPLC instrument, column, and solvent system as reported in this thesis.

3.3.3 Effect of Protein (Bovine Serum Albumin) Addition on the Antilisterial Activity of Polymeric Phenolic Fraction of Grape Juice

Polymeric phenolics (condensed tannin) can bind to proteins and precipitate from solution (Swain & Bate-Smith, 1962). To ascertain whether the antilisterial factor isolated from the commercial grape juice (batch 2) was able to bind to protein, and therefore exhibits typical tannin chemistry the fraction was subjected to protein precipitation using the method described by Harbertson *et al*., (2002). The polymeric phenolic fraction was treated with increasing concentrations of bovine serum albumin (BSA), the precipitated material was removed and the supernatant was assessed for antilisterial activity.

A 1 mg/ml solution of bovine serum albumin (BSA) was prepared using sterile water. A 4 mg/ml solution of the polymeric phenolic material was prepared by dissolving the material in 0.5 ml 99.95% ethanol and diluting to a final volume of 5 ml with sterile water. The pH of the solution was adjusted to 3.5 with sterile 10 mM HCl and the solution pasteurised at 63^{o}C for 30 minutes. Duplicate solutions consisting of 1 ml BSA at the following concentrations, 0.5, 0.25, 0.1, 0.01, and 0.001 mg/ml, and 0.5 ml of polymeric phenolic material were prepared in sterile Eppendorf tubes. The two solutions were gently mixed for 15 minutes. The resulting protein-polyphenol precipitate was pelleted by centrifugation for 5 minutes at 10,500 rpm (Heraeus). A pellet was visible in each tube. Duplicate supernatant samples were pooled in sterile bottles and diluted with sterile water to a final volume of 3.8 ml and assayed against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2).

3.3.4 Fractionation of Ribier Grapes

Extracts of *Vitis vinifera* var. Ribier grape skin, seed, and juice/pulp were prepared. Seven kilograms of Chilean Ribier grapes were purchased (12 September 2001) from an Auckland, New Zealand supermarket. On the day of purchase the grapes were surface sterilised by washing with 70% (v/v) ethanol followed by sterile reverse osmosis water and then air-dried at 20^{o}C. The grape skins and seeds were manually dissected from the grape pulp using a sterile scalpel and tweezers. The skin, seed, and juice/pulp fractions were weighed. The weight of the grape skin was 1.06 kg,

seed 0.89 kg, and juice/pulp 4.3 kg. A volume of 1 ml of 10% (v/v) ethanol was added per gram of skin and juice/pulp and 2 ml of 10% (v/v) ethanol per gram of seed. The fractions were macerated using a commercial blender (Waring) and stored in the dark for 72 hours at 4°C to extract the grape compounds. The skin and seed extracts were clarified by centrifugation at 3000 rpm for 10 minutes using a Centra GP8 centrifuge (International Equipment Company) and the supernatant decanted and frozen in 100 ml aliquots at -20°C. Prior to freezing an aliquot of each extract (skin, seed and juice/pulp) was adjusted to pH 3.5 with sterile 10 mM HCl, pasteurised at 63°C for 30 minutes, and assayed against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2).

3.3.5 Large Scale Preparation of Commercial Grape Juice, Grape Skin and Grape Seed Polymeric Phenolics

Large-scale preparation of grape juice, skin and seed polymeric phenolic material was achieved using the method described in section 3.3.1 with the following modifications. Six columns (matrix volume 16 ml) were run in parallel. A total volume of 60 ml of grape juice (batch 2) or skin and seed extract was fractionated (10 ml per column). The monomeric phenolic fraction was discarded and polymeric material was eluted with 50% acetone (acidified with 0.1% HCl). The acetone was removed from the polymeric fraction by rotary evaporation at 30°C and the polymeric material was then freeze-dried (Virtis). Polymeric fractions were analysed for total phenolics (section 2.3.6.2). The freeze-dried polymer powders were stored at 20°C under desiccation for up to 6 months and used for a number of experiments.

3.3.6 Antimicrobial Spectrum of Polymeric Phenolics from Commercial Grape Juice, Grape Skin and Seed Extracts

The commercial grape juice used in this study had antimicrobial activity specifically against *Listeria* species (section 2.4.2). The antimicrobial spectrum of the polymeric phenolics isolated from the commercial grape juice and grape skin and seed was investigated. The polymeric phenolic fractions were assayed against *Listeria monocytogenes* ATCC 35152 and two other common food-borne bacterial pathogens, *Staphylococcus aureus* ATCC 25923 (gram-positive) and *Escherichia coli* ATCC 25922 (gram-negative). Freeze-dried polymers were made to a final concentration of 250-280 mg/L GAE, as determined by the total phenolic method (section 2.3.6.2) (0.4 mg/ml skin and juice polymers, 0.25 mg/ml seed polymers), by dissolving in 400 µl 99.95% ethanol and dilution to 3 ml with sterile water. The pH was adjusted to 3.5 with sterile 10 mM HCl and the solution diluted to a final volume of 3.8 ml with sterile water. The solution was then pasteurised at 63°C for 30 minutes and assayed against *L. monocytogenes, S. aureus,* and *E. coli* according to the

standard protocol (section 2.3.2). A positive control of Model Wine Solution (MWS) (0.033 M L-tartaric acid, 10% ethanol, pH 3.5) was run in parallel for each bacterial isolate assayed.

3.3.7 Effect of pH on the Antimicrobial Activity of Commercial Grape Juice, Skin, and Seed Polymeric Phenolics

The commercial grape juice was found to be antimicrobial only at acidic pH (section 2.4.8). To determine whether the antilisterial activity of the polymeric phenolics isolated from commercial grape juice, skin, and seed extracts was also pH dependent the extracts were assayed according to the method described in section 3.3.6. The pH of the three polymer solutions was adjusted to pH 7.0 with sterile 10 mM NaOH. The solutions were assayed against *L. monocytogenes, S. aureus*, and *E. coli* according to the standard protocol (section 2.3.2).

3.3.8 Effect of Dialysis on the Antilisterial Activity of Commercial Grape Juice, Skin, and Seed Polymeric Phenolics

To gain further insight in to the physiochemical nature of the juice, skin, and seed polymeric phenolic extracts, an aliquot of each sample was dialysed against water and the retentate assayed for antilisterial activity. Polymeric phenolic material isolated from commercial grape juice (batch 2) (10.8 mg), Ribier grape skin (7.9 mg), and Ribier grape seed (10 mg) were each dissolved in 10 ml methanol and placed in a 10 cm long piece of 12-14 kDa MWCO dialysis tubing. The solutions were dialysed in individual vessels against distilled water (5 L) in the dark. The solutions were dialysed for 48 hours at 4^{o}C with a change of water every 12 hours. Each retentate was freeze-dried (Virtis) and the weight loss calculated. Retentates (1 mg) were analysed for total phenolics (section 2.3.6.2), then polymer solutions were prepared (0.4 mg/ml juice and skin polymers, 0.25 mg/ml seed polymers) as described in section 3.3.6 and assayed against *L. monocytogenes* according to the standard protocol (section 2.3.2).

3.4 Results and Discussion

3.4.1 Fractionation of Monomeric and Polymeric Phenolic Fractions of Commercial Grape Juice

The phenolic compounds of commercial grape juice (batch 2) were separated into monomeric and polymeric fraction using Sephadex LH20 (section 3.3.1). The fractions were then analysed for

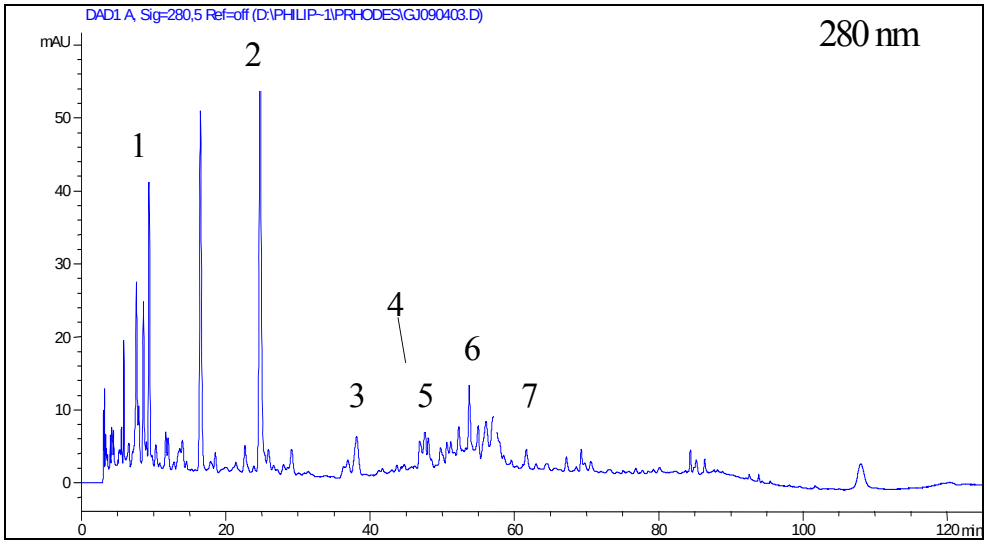
composition by reverse phase HPLC (section 3.3.2) and assayed for antilisterial activity (section 2.3.2).

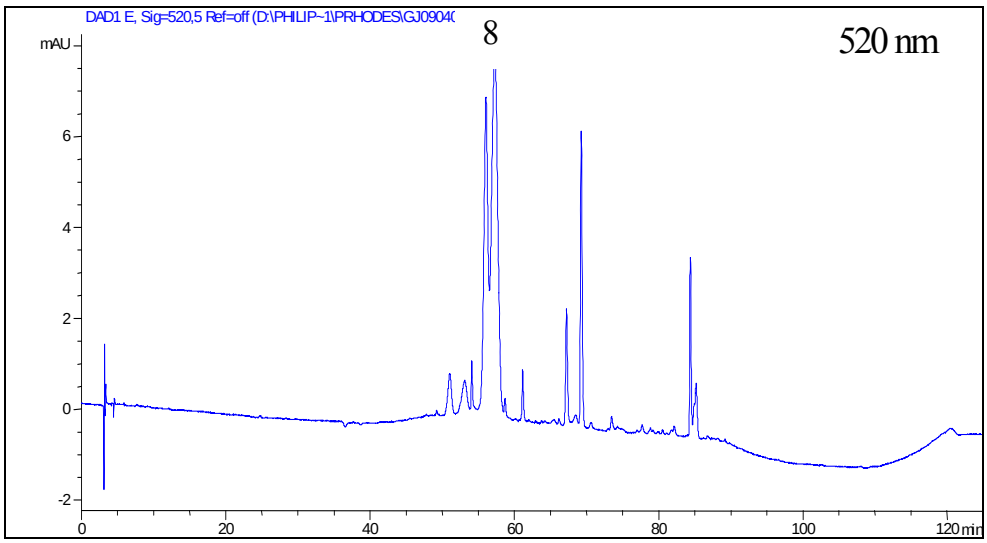
3.4.1.1 HPLC Analysis of Monomeric and Polymeric Phenolic Fractions of Commercial Grape Juice

Analysis of the composition of the monomeric and polymeric phenolic fractions was achieved by separation of the phenolic compounds by HPLC using a reverse phase C18 column and an elution gradient of increasing acidified acetonitrile. Phenolic compounds were identified by comparison to standard retention times.

3.4.1.1.1 Monomeric Fraction

The monomeric phenolic compounds contained within the monomeric fraction of the commercial grape juice were separated by HPLC on a reverse phase C18 column. The HPLC chromatographs of the monomeric phenolic fraction are shown in **Figure 3.1.**





30.1 Figure 3.1 HPLC chromatographs of the monomeric fraction of commercial grape juice (batch 2).

The monomeric fraction contained a number of monomeric phenolic compounds typically found in red wines (Amerine & Ough, 1980). These compounds included gallic acid, caftaric acid, catechin, vanillic acid, caffeic acid, syringic acid, *para*-coumaric acid, and the anthocyanin malvidin-3-glucoside (**Table 3.3**).

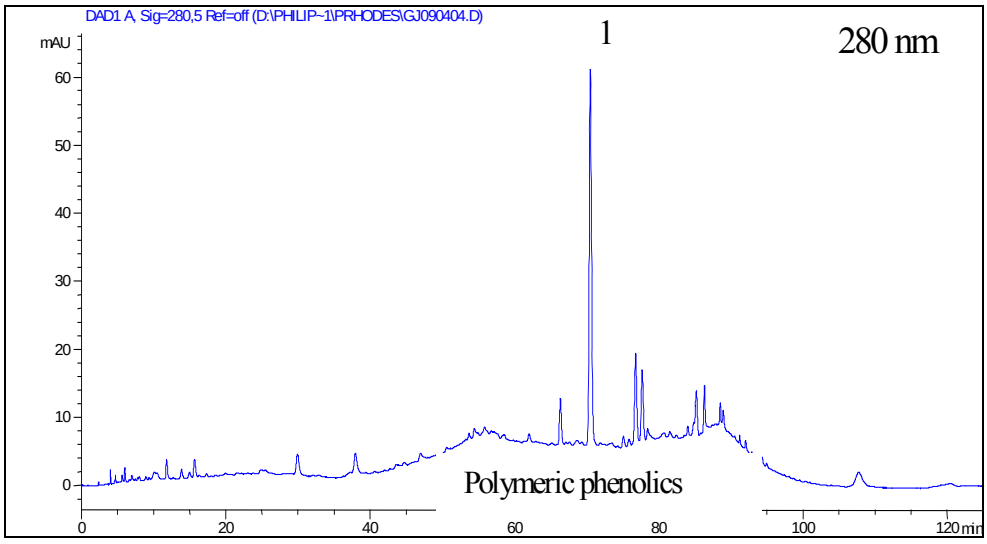
31.1 Table 3.3 Monomeric phenolic compounds identified by HPLC using standard retention times.

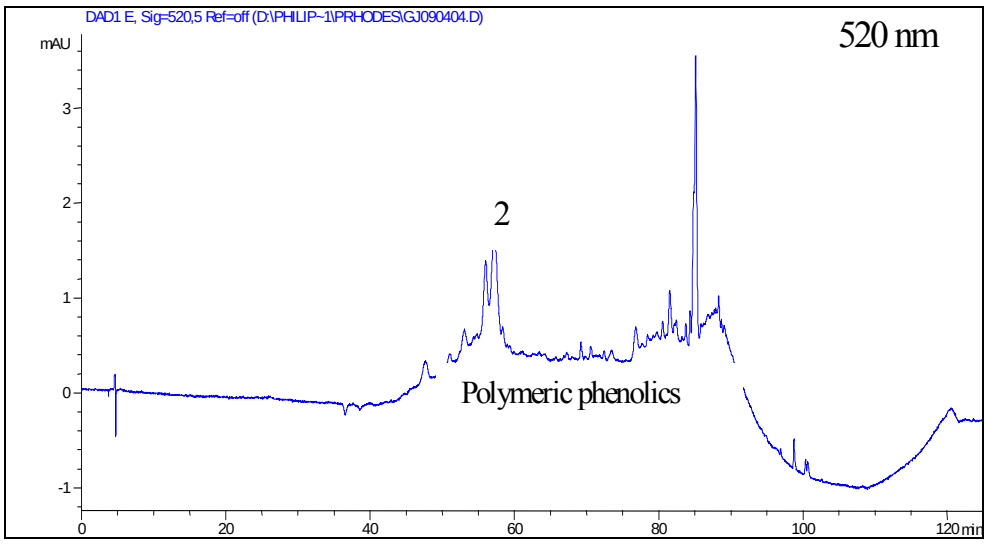
Peak Number	Compound Identified	Retention Time
1	Gallic acid	9.3
2	Caftaric acid*	24.7
3	Catechin	38.1
4	Vanillic acid	46.8
5	Caffeic acid	47.6
6	Syringic acid	53.7
7	*para*-Coumaric acid	61.7
8	Malvidin-3-glucoside	57.3

*Identified from retention time published by Zou *et al*., (2002).

3.4.1.1.2 Polymeric Fraction

The monomeric and the polymeric phenolic separation method did not result in complete separation of monomers and polymers. Two monomeric phenolic compounds were identified in the HPLC spectrum of the polymeric phenolic fraction of the commercial grape juice (**Figure 3.2**). Peak 1 was identified as *trans*-piceid by comparison to the retention time of a commercial standard of *trans*-piceid. The second peak (peak 2) was identified as the anthocyanin malvidin-3-glucoside by comparison to the retention time of a commercial standard of malvidin-3-glucoside. A large peak with a retention time of 85 minutes was not identified but was likely to be malvidin-3-(6-O-*p*-courmaryl) glucose, as it was observed at 520 nm, which is the wavelength used to detect red pigments (G Jones, personal communication).





32.1 Figure 3.2 HPLC chromatographs of the polymeric phenolic fraction of commercial grape juice (batch 2).

These HPLC results indicated there was some carry over of monomeric phenolic compounds into the polymeric fraction. Although the polymeric phenolic fraction contained some monomeric phenolic compounds the majority of the material in the fraction eluted as a broad hump over the solvent gradient. Kantz and Singleton (1990) showed the chromatographic separation of polymeric phenolic fraction of Semillon grape seed where the polymeric material eluted as a hump from a C18 column, over a gradient of 20-80% acidified acetonitrile. Remy *et al*., (2000) also reported the elution of a red wine polymeric phenolic fraction as a hump from C18 column with an increasing concentration, 15-60%, of acidified acetonitrile. The HPLC chromatograph of the polymeric phenolic fraction (isolated from commercial grape juice batch 2) was similar to those reported by Kantz & Singleton, (1990) and Remy *et al*., (2000) as the polymeric phenolic material eluted from a C18 column as a broad hump with increasing concentration of acidified acetonitrile. The polymers began eluting at 10% acetonitrile (retention time 50 minutes) and continued eluting throughout the gradient of acetonitrile to 40% acetonitrile (retention time 95 minutes). The HPLC results confirmed that the majority of the monomeric and polymeric compounds present in the commercial grape juice (batch 2) were separated.

3.4.1.2 Antilisterial Activity of Monomeric Phenolics and Polymeric Phenolics Isolated from Commercial Grape Juice

The commercial grape juice (batch 2) was separated into monomeric phenolics and polymeric phenolics on Sephadex LH20 using increasing non-polar solvent (section 3.3.1). The fractions were made up to the original volume of grape juice (5 ml), fractionated, and assayed for antilisterial activity. When assayed against *L. monocytogenes* the polymeric phenolic fraction showed the greatest antilisterial activity with a reduction in cell numbers from 10^6 CFU/ml to no colonies detectable within 10 minutes (**Figure 3.3**). The polymeric fraction of the commercial grape juice (batch 2) had a similar degree of antilisterial activity to that of commercial grape juice (batch 1) (section 2.4.4) and the whole grape seed extract, and grape skin extracts (section 3.4.3). A significant reduction in *L. monocytogenes* numbers (2-log) was observed at 0 minutes (equivalent to <1 minute) exposure to the polymeric fraction. This initial reduction in cell numbers showed the rapid nature of the antilisterial activity of the polymeric phenolic fraction. The monomeric phenolic fraction exhibited some antilisterial activity, with a 3-log reduction in cell numbers after 60 minutes exposure. The acetone control was also slightly inhibitory with a 2-log reduction in *L. monocytogenes* numbers after 60 minutes exposure. The acetone control was assayed to take into account any inhibitory effects from residual solvent and HCl. The limited inhibitory activity of this control suggests that the presence of residual solvent or residual acid may have contributed some but not all of the antilisterial activity observed in both the monomeric and polymeric fractions. Both the monomeric and polymeric fractions reduced cell numbers to a greater extent than the acetone control indicating that these fractions had antilisterial activity over and above any inhibitory activity contributed by residual solvent or acid. A Model Wine Solution (MWS) containing 10% ethanol was used as a control to take into account any inhibitory effects due to the 10% ethanol present in the polymeric phenolic fraction (section 3.3.6). The control model wine solution (MWS) was not inhibitory to *L. monocytogenes*.

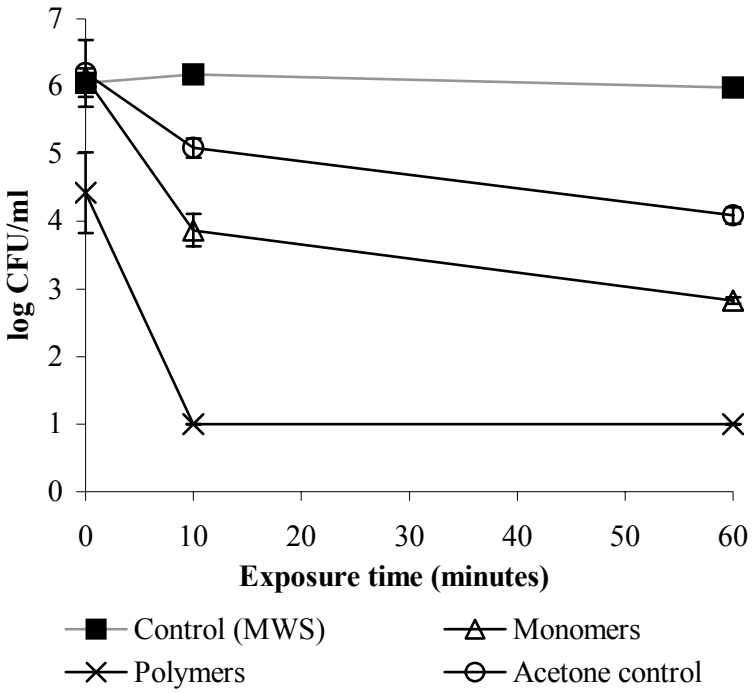


Figure 3.3 Antilisterial activity of monomeric and polymeric grape juice phenolics. Error bars are the ± standard deviation of mean log CFU/ml.

The total phenolic level of 1 ml aliquots of the polymeric and monomeric fractions was measured using Folin-Ciocalteu reagent (section 2.3.6.2). The polymeric fraction contained 1347 mg/L GAE which was twice the level of that in the monomeric fraction, 617 mg/L GAE. The higher level of phenolic compounds present in the polymeric fraction compared to the monomeric fraction may have contributed to the higher antilisterial activity of the polymeric fraction. However, all three batches of commercial grape juice had phenolic levels of greater than 3000 mg/L GAE (section 2.4.5 **Table 2.6**) but lower antilisterial activity (batches 2 and 3) (section 2.4.4 **Figure 2.2**) than the polymeric fraction. Grape juice contains high sugar levels, which are known to provide a protective effect to bacterial cells against external stresses, and the higher antimicrobial activity of the fractionated polyphenols compared to the original grape juice may be due to the presence of sugars in the grape juice (G. Jones, personal communication). It appears that it is not the total phenolic

level that is the inhibitory factor but more likely a specific type, or combination of phenolic compounds that imparts the antilisterial activity.

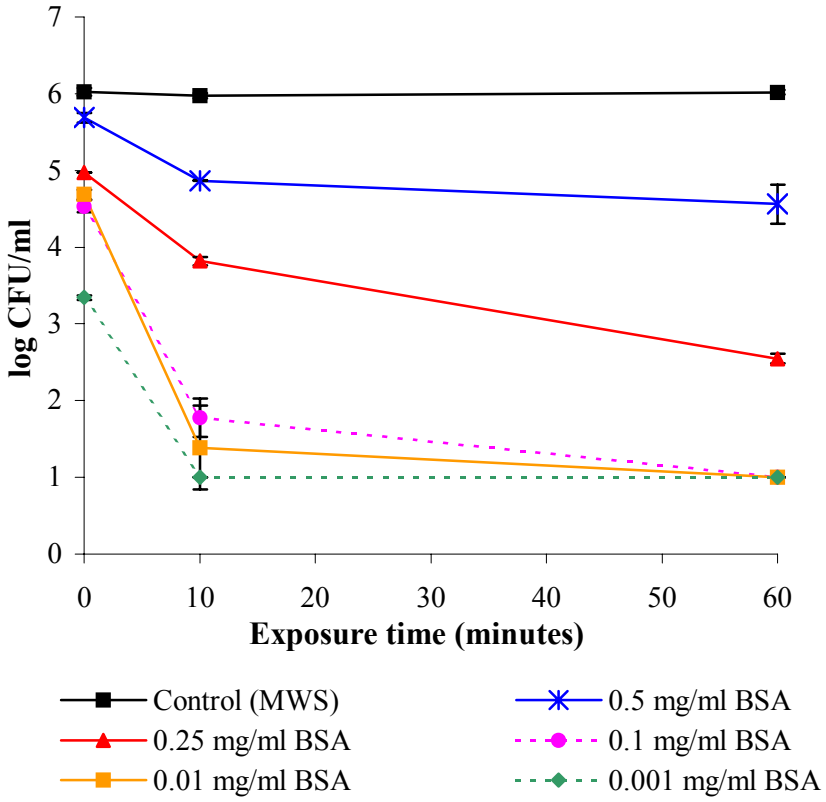
The polymeric phenolic fraction made up 68% of the total phenolic content of the commercial grape juice (batch 2) which was higher than that reported for red wines. Zoecklein *et al*., (1995b) reported that polymeric phenolics (condensed tannins) made up an estimated 54% of the total phenolic content of a typical *Vitis vinifera* red wine with monomeric phenolics comprising the remaining 46%. Singleton (1988) reported the estimated percentage of polymeric phenolics in a young red table wine as 42% with 58% being monomeric phenolics. The antilisterial polymeric phenolic fraction contributed 68% of the total phenolics of the whole grape juice. The high percentage of polymeric material (68%) present in the commercial grape juice (batch 2) suggested that polymeric phenolics might be responsible for the antilisterial activity of the grape juice. Additionally, the high antilisterial activity of the polymeric fraction compared to the monomeric fraction also indicated that polymeric phenolics (condensed tannins) might confer the inhibitory action of this grape juice.

Polymeric phenols are formed during the ageing process of red wine (Singleton, 1988). Masquelier (1959) reported increased bactericidal activity of red wine concomitant with an increase in wine age (up to 10 years). The antimicrobial activity then slowly declined as the wine aged further. It is known that as wine ages phenolic compounds continue to polymerise until the polymers eventually precipitate from solution (Bungard, 2001). Processing methods and storage conditions of the commercial grape juice used in this thesis could have resulted in chemical ageing of the juice generating increased levels of polymerised phenolics in the juice, and thus resulting in the high antilisterial activity observed.

3.4.2 Effect of Addition of Protein (Bovine Serum Albumin) on the Antilisterial Activity of Polymeric Phenolic Fraction of Commercial Grape Juice

Polymeric phenolics (condensed tannin) can bind to protein, carbohydrates, and polysaccharides (Bate-Smith, 1973; Haslam, 1974; Scalbert, 1991; Mueller-Harvey & McAllen, 1992). In this experiment solutions of protein (BSA) (0.5, 0.25, 0.1, 0.01, 0.001 mg/ml) were mixed with aliquots of the antilisterial polymeric phenolic fraction. The protein binds to the polymeric material and the resultant protein-tannin complexes then precipitate. The protein-polyphenol complexes are then removed from the solution by centrifugation and the remaining supernatant assayed for antilisterial activity.

When increasing concentrations of protein (BSA) was added to the polymeric phenolic fraction a decrease in antilisterial activity was observed (**Figure 3.4**). At the highest concentration of BSA (0.5 mg/ml) the polymeric phenolic supernatant showed little antilisterial activity with a 1-log reduction in *L. monocytogenes* numbers after 60 minutes exposure. As the concentration of BSA added to the polymeric phenolic fraction decreased, the antilisterial activity of the remaining supernatant increased. When the polymeric phenolic fraction was treated with a concentration of 0.25 mg/ml BSA, the resulting supernatant reduced *L. monocytogenes* numbers from 10^6 CFU/ml to 10^3 CFU/ml within 60 minutes. When the polymeric phenolic fraction was treated with lower concentrations of BSA (0.1, 0.01, and 0.001 mg/ml) the resulting supernatants had strong antilisterial activity.



33.1 **Figure 3.4 Effect of protein (bovine serum albumin) addition on the antilisterial activity of polymeric phenolic fraction of commercial grape juice (batch 2).** Error bars are the ± standard deviation of mean log CFU/ml.

The result of this experiment suggested that the antilisterial factor in polymeric phenolic fraction exhibited typical condensed tannin chemistry in that the tannin was precipitated from solution in the presence of protein (BSA). As the concentration of protein added to the polymeric phenolic solution increased the antilisterial activity of the resulting supernatant solutions decreased which indicated that the antilisterial factor in the commercial grape juice was likely to be condensed tannin

Konowalchuk & Spiers (1976) found that the addition of gelatine to a poliovirus-grape juice complex resulted in the restoration of virus activity. They suggested that the gelatine acted by releasing the virus from the grape juice (antiviral factor not stated) allowing the virus to multiply. Nguyen-the & Lund, (1992) found that the antilisterial activity of carrot extract was suppressed by the addition of 0.05% bovine serum albumin. Chung *et al*., (1990) showed that the addition of protein (1% Bacto-peptone) suppressed the antilisterial activity of a Chinese plant (Siu Mao Heung) extract. This thesis research finding is in agreement with the findings of Chung *et al*., (1990) and Nguyen-the & Lund, (1992) as the addition of protein resulted in the precipitation of the antilisterial factor and the loss of antilisterial activity.

3.4.3 Antilisterial Activity of Extracts of Ribier Grapes

Fractionation of *Vitis vinifera* var. Ribier black table grapes into skin, seed, and juice/pulp fractions and then assay of the fractions (250-280 mg/L GAE final concentration) against *L. monocytogenes* revealed that both the skin and seed extracts had antilisterial activity but the juice/pulp fraction was not active (**Figure 3.5**). The grape skin and seed extracts had a similar degree of antilisterial activity to that of batch 1 of the commercial grape juice (section 2.4.4). Grape skin and seed extracts decreased *L. monocytogenes* numbers from 10^6-10^7 CFU/ml to no detectable colonies within 10 minutes. The juice/pulp extract did not inhibit *L. monocytogenes* as the number of colony forming units were not reduced from the initial inoculum of 10^6-10^7 CFU/ml. The positive control Model Wine Solution (MWS) was not inhibitory to *L. monocytogenes*.

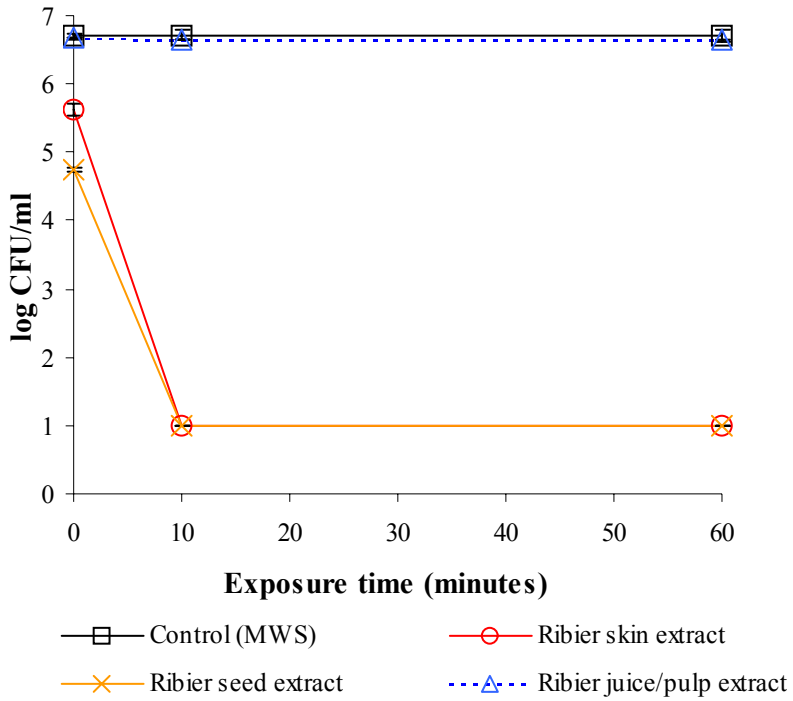


Figure 3.5 Antilisterial activity of skin, seed, and juice/pulp extracts of Ribier grapes. Error bars are the ± standard deviation of mean log CFU/ml.

The antimicrobial property of wine has been attributed to phenolic compounds (Masquelier, 1959). Phenolic compounds in grapes are mainly found in the seeds and skins of the fruit with lower levels in the juice and pulp. The distribution of total phenolics in red grapes has been reported as seeds 62.6%, skins 33.3%, juice 3.4%, and pulp 0.7% (Amerine and Ough, 1980). This thesis research found a no antilisterial activity of the juice/pulp fraction of Ribier grapes and but high activity of the skin and seed fractions which suggested that phenolic compounds were responsible for the antilisterial activity of the skin and seed fractions. This result also suggested that the antilisterial factor in the commercial grape juice was derived from the skin and/or seed of Ribier grapes but not the juice or pulp of these grapes. Konowalchuk and Spiers (1976) found an antiviral factor in Concord grape skin extract but not the grape pulp extract. Our results are in agreement with Konowalchuk and Spiers (1976) in that the grape skin but not the grape pulp had antilisterial activity. The antimicrobial activity of grape seed extracts has been reported previously (Palma and Taylor, 1999, Jayaprakasha *et al*., 2003; Ahn *et al*., 2004). However, to this researcher's knowledge this is the first reporting of an antilisterial factor present in black grape skin.

3.4.4 Antimicrobial Spectrum of Polymeric Phenolics derived from Commercial Grape Juice, Ribier Skin, and Seed Extracts

The antimicrobial spectrum of the polymeric phenolic material isolated from Ribier grape skin and seed and the commercial grape juice (batch 2) were assessed against *Listeria monocytogenes* and two common food-borne pathogens, *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) at a concentration of 250-280 mg/L GAE. The antimicrobial activity of polymeric phenolics isolated from the commercial grape juice and the skin and seed of Ribier grapes was specific to *L. monocytogenes*. All polymeric fractions reduced *L. monocytogenes* numbers from 10^6 CFU/ml to no detectable colonies within 10 minutes (**Table 3.4**).

34.1 Table 3.4 Antimicrobial activity of commercial grape juice, skin, and seed polymeric phenolics (pH 3.5) against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli*.

		Juice Polymers	Skin Polymers	Seed Polymers
	Exposure time (minutes)	Colony Forming Units/ml		
L. monocytogenes	0	$4x10^4$	$4x10^5$	$5x10^3$
	10	<10	<10	<10
	60	<10	<10	<10
S. aureus	0	$6x10^5$	$6x10^4$	$1x10^5$
	10	$3x10^5$	$9x10^4$	$6x10^4$
	60	$1x10^5$	$7x10^4$	$3x10^4$
E. coli	0	$4x10^5$	$6x10^5$	$7x10^4$
	10	$3x10^5$	$5x10^4$	$5x10^4$
	60	$3x10^5$	$6x10^4$	$5x10^4$

All of the polymeric phenolic fractions, with the exception of skin polymers against *S. aureus*, were very slightly inhibitory to *S. aureus* and *E. coli*. However, a less than 1-log reduction in cell numbers was observed after 60 minutes exposure of these bacterial isolates to all of the polymeric

phenolic fractions (juice, skin, seed), with the exception of a 1-log reduction in *E. coli* exposed to skin polymer. When compared to the reduction in *L. monocytogenes* numbers exposed to the same polymeric phenolic fractions the reduction in *S. aureus* and *E. coli* numbers was very small. The narrow spectrum of antimicrobial activity of the polymeric fractions of juice, skin and seed reflects the narrow antimicrobial spectrum of the unmodified commercial grape juice (section 2.4.2).

Ahn *et al*., (2004) reported the inhibitory activity of a commercial grape seed extract against *L. monocytogenes*, *E. coli*, and *Salmonella* Typhimurium. The authors showed that the lowest inhibitory concentration of grape seed extract was 6 mg/ml, which caused the complete inhibition of all three pathogens after 6 days exposure. Jayaprakasha *et al*., (2003) reported the antimicrobial action of grape (*Vitis vinifera*) seed extracts against a range of bacteria including *S. aureus* and *E. coli.* They found that the gram-positive bacteria assayed, including *S. aureus*, were completely inhibited by 0.85-1 mg/ml extract while gram-negative bacteria, including *E. coli*, were inhibited at 1.25-1.5 mg/ml concentration. In this thesis experiment the assay concentration of the grape seed polymers was 0.25 mg/ml which was below that inhibitory concentration used by Ahn *et al*., (2004) and Jayaprakasha *et al*., (2003). This might explain the lack of inhibitory action of this thesis grape seed polymeric phenolic fraction against *E. coli* and *S. aureus*. Further this thesis result indicated that *L. monocytogenes* was more sensitive than other bacteria to the inhibitory action of grape seed polymeric phenolics and other grape derived tannin. This thesis result agrees with the findings of Ahn *et al*., (2004) that *L. monocytogenes* was more sensitive to the inhibitory action of grape seed phenolics than other bacterial species.

Jayaprakasha *et al*., (2003) extracted grape seed phenolics with 90% methanol. Standard methods of extracting phenolic material included extraction with 75-90% methanol, ethanol, or acetone (Arnold & Noble, 1978; Harbertson *et al*., 2002). These high concentrations of solvents extract high levels of phenolic compounds. However, commercial juicing processes do not generally use solvents to extract phenolic compounds and other chemicals from grapes, so in order to achieve a reasonable representation of the phenolic profile of the commercial grape juice this researcher chose an extraction process that utilised lower levels of solvent (10% ethanol). This was deemed to be a reasonable compromise between phenolic extraction methods reported in the literature and juice industry processing methods. The difference in extraction solvent concentration, and therefore the concentration of phenolic compounds extracted, might also explain the wide antimicrobial spectrum reported by Jayaprakasha *et al*., (2003) and the narrow antimicrobial spectrum observed in this thesis experiment. Possibly, the polymeric phenolic extracts derived from Ribier grape skin and

seeds as well as the commercial grape juice would be inhibitory to a wider range of bacteria if a higher concentration of polymeric material was used in the antimicrobial assay.

3.4.5 Effect of pH on the Antilisterial Activity of Commercial Grape Juice, Skin and Seed Polymeric Phenolics

Previous experimental work showed that the antilisterial activity of the commercial grape juice was pH dependent (section 2.4.8). This researcher postulates that the origin of the antilisterial factor, whether grape skin or seed, would also have pH dependent inhibitory activity. Increasing the pH of the commercial grape juice, skin, and seed polymeric phenolic fractions from pH 3.5 to 7.0 with 10 mM NaOH had a significant effect on the antilisterial activity of the grape juice and skin polymers but not the seed polymers (**Table 3.5**).

35.1 Table 3.5 Antimicrobial activity of commercial grape juice, skin, and seed polymeric phenolics at pH 3.5 and 7.0, against *Listeria monocytogenes*.

		Exposure time (minutes)		
		<1	10	60
Solution assayed	pH	Colony Forming Unit/ml		
Control (Model Wine Solution)	pH 3.5	$8x10^5$	$9x10^5$	$9x10^5$
	pH 7.0	$9x10^5$	$8x10^5$	$8x10^5$
Juice polymers	pH 3.5	$4x10^4$	<10	<10
	pH 7.0	$6x10^5$	$5x10^5$	$5x10^5$
Skin polymers	pH 3.5	$7x10^5$	<10	<10
	pH 7.0	$8x10^5$	$6x10^5$	$2x10^4$
Seed polymers	pH 3.5	$1x10^4$	<10	<10
	pH 7.0	$3x10^5$	$1.5x10^1$	<10

At pH 3.5 all polymeric phenolic fractions reduced *L. monocytogenes* numbers from 10^5-10^6 CFU/ml to no detectable colonies. However, when adjusted to pH 7.0 the juice and the skin polymers showed a reduction in antilisterial activity. At pH 7.0 the juice polymeric phenolic fraction did not reduce *L. monocytogenes* numbers after 60 minutes exposure. At pH 7.0 the skin

fraction reduced *L. monocytogenes* numbers by 2-log reduction after 60 minutes exposure. In contrast, the seed fraction at pH 7.0 had a similar antilisterial activity (6-log reduction after 60 minutes exposure) to that at pH 3.5. The seed polymers showed a slightly reduced inhibitory action at pH 7.0 compared to pH 3.5. However, after 60 minutes exposure both seed solutions (pH 3.5 and pH 7.0) reduced *L. monocytogenes* numbers to no detectable colonies. Controls of Model Wine Solution (MWS) at pH 3.5 and 7.0 did not reduce *L. monocytogenes* numbers over the 60 minute assay time.

Previous experimental work in this thesis showed that the commercial grape juice had pH dependent antilisterial activity. At acidic pH the grape juice was inhibitory to *L. monocytogenes* but at pH 6.4 and higher this inhibitory activity was lost (section 2.4.8). The polymeric phenolic grape juice and skin fractions showed the same trend, with antilisterial activity present at pH 3.5 but not at pH 7.0. In contrast, the grape seed fraction had antilisterial activity at both pH 3.5 and pH 7.0. The dependence on pH for antilisterial activity of the commercial grape juice and Ribier skin polymers, but not Ribier grape seed polymers, suggested that the antilisterial activity present in the commercial grape juice was derived from the skin and not the seed of Ribier grapes.

3.4.6 Effect of Dialysis on the Antilisterial Activity of Commercial Grape Juice, Skin and Seed Polymeric Phenolics

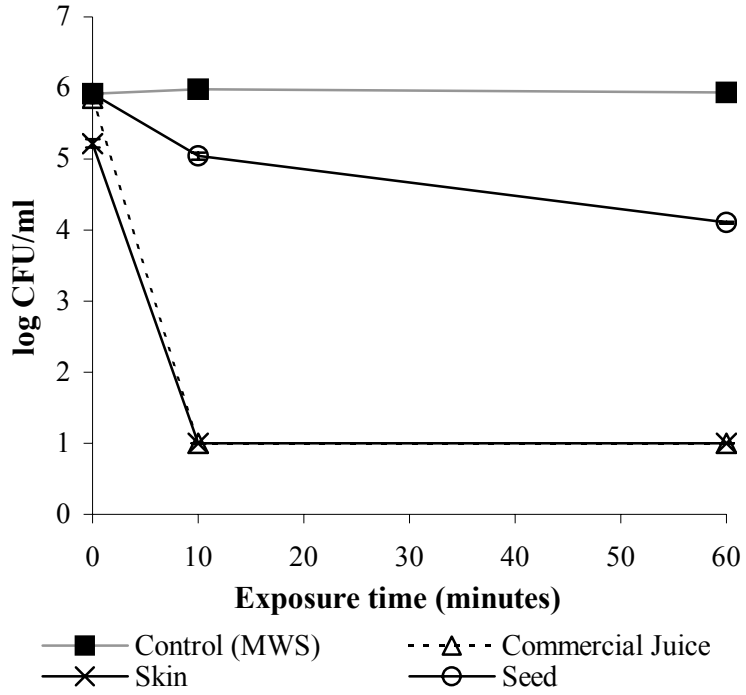
Souquet *et al*., (1996) reported tannins from grape skin (*Vitis vinifera* var. Merlot) with a mean degree of polymerisation (mDP) in excess of 80 i.e. a molecular weight of greater than 24000 daltons. Kennedy & Taylor, (2003) reported a mass spectrum peak of 11524 corresponding to a flavanol polymer present in grape skin. The molecular weight of grape derived polymeric phenolics (condensed tannins) ranges from 400 to as high as 24000 (Kennedy & Taylor, 2003). The juice, skin, and seed polymers were dialysed (12-14 kDa MWCO) and the retentates freeze-dried and analysed for weight loss, total phenolic level (section 2.3.6.2), and antilisterial activity (section 2.3.2). Dialysis of these three polymeric samples resulted in a significant loss of material from all three polymeric samples (**Table 3.6**). The grape seed fraction had the greatest loss of material with 90% (w/w) being removed by dialysis. The commercial grape juice and skin fractions showed similar levels of weight loss, 73 (w/w) and 72% (w/w), respectively. The total phenolic level (mg/L GAE) of the grape juice polymers was reduced by 50% following dialysis and the skin polymer sample was reduced by 62%. The total phenolic level of seed polymers was not determined due to insufficient sample remaining after antimicrobial assay.

Table 3.6 Effect of dialysis on commercial juice, skin, and seed polymers.

Polymer	Dialysed Weight (mg)	Retentate Weight (mg)	% Weight Loss	Total phenolic mg/L GAE	% Total phenolic loss mg/L GAE
Grape Juice	10.8	2.9	73%	276 ± 13	62%
Grape Skin	7.9	2.2	72%	322 ± 28	50%
Grape Seed	10	1.0	90%	ND*	ND*

*Not determined due to insufficient sample remaining.

When assayed for antilisterial activity the juice and skin retentates showed no loss in inhibitory activity even though a loss of greater than 70% weight and 50% total phenolics of both polymers resulted from dialysis. After exposure to both these polymeric samples, *L. monocytogenes* cell numbers were reduced from 10^6 to no detectable colonies within 10 minutes (**Figure 3.6**). The antilisterial activity of the grape seed polymeric fraction was reduced by dialysis. After 60 minutes exposure to the seed retentate a 2-log reduction in cell numbers was observed compared to a 6-log reduction with undialysed seed polymers (section 3.4.3). The retention of the antilisterial factor in the grape juice and skin retentate but not the seed retentate suggested that the antilisterial factor in the juice and skin were similar whereas the antilisterial factor in the seed differed from both the juice and skin. This result indicated that the antilisterial factor in the commercial grape juice was likely derived from the skin of Ribier grapes.



36.1 **Figure 3.6 Antilisterial activity of dialysis retentates of polymeric phenolic fractions of commercial grape juice, Ribier grape skin, and seed.** Error bars are the ± standard deviation of mean log CFU/ml.

Brownlee *et al*., (1990) reported that the antifungal activity of a fraction isolated from cocoa shoot tissue was retained within dialysis tubing when dialysed against water. They did not report on the molecular weight cut off of the dialysis tubing but suggested that polymeric procyanidin (condensed tannin) was responsible for the antifungal activity.

This research finding that the loss of activity of the seed polymer retentate but not the juice and skin retentates suggested that the antilisterial factor present in the skin and the commercial grape juice had a higher molecular weight than that present in the seed. Polymeric phenolics isolated from the skin of grapes (*Vitis vinifera* var. Merlot) have been found to have molecular weights as high as MW 24000 (Souquet *et al*., 1996). The fact that the antilisterial factor present in the juice and skin polymeric fractions remained in the dialysis tubing (12-14 kDa molecular weight cut off) suggests that the molecular weight of the antilisterial polymeric phenolics could be higher than MW 12000. Grape skin polymers have been found to have a higher estimated molecular weight (up to MW 24000) (Souquet *et al*., 1996) than grape seed polymers (up to MW 7570) (Kennedy & Taylor,

2003). This could explain the greater loss of seed polymers via dialysis, compared to the skin and juice polymers observed in this research.

Kennedy & Taylor (2003) also suggested that grape seed polymers might have a more extended conformation compared to grape skin polymers. The extended conformation of the seed polymers might also explain this thesis research observation of greater loss of seed polymers compared to the skin and juice polymers. If the seed polymers were more extended they may be more likely to diffuse through the dialysis membrane whereas the less extended or more bunched skin and juice polymers may be trapped within the dialysis membrane. It is possible that the structure of the polymers could physically prevent the polymers from moving out of the dialysis tube. Polymeric phenolics (condensed tannin) are able to bind to proteins, carbohydrates, and polysaccharides (Bate-Smith, 1973; Haslam, 1974; Scalbert, 1991; Mueller-Harvey & McAllen, 1992; Siebert *et al*., 1996; Sarni-Manchando *et al*., 1999; Maury *et al*., 2001; Charlton *et al*., 2002). Given this, it is possible that some of the polymers may be less than 12-14 kDa in molecular weight but through physical interactions have bound to the dialysis tube surface, thus retaining them in the tube. The loss of 90% of the grape seed polymers removed by dialysis, compared to 70% of the juice and skin polymers indicated that the juice and skin polymers had a higher molecular weight than the seed polymers and/or that the skin and juice polymers had a less extended conformation or a different chemical structure, than the seed polymers, resulting in a greater proportion of these polymers being retained within the dialysis tube.

The nature of the antilisterial factor present in the grape seed differed in size and/or chemical composition to that of the juice and skin, which were similar in both size and/or chemical properties. This result indicated that the antilisterial factor in the commercial grape juice was likely derived from the skin and not the seed of Ribier grapes.

3.5 Summary

The commercial grape juice used in this study was made from *Vitis vinifera* var. Ribier black table grapes. Fractionation of these grapes into skin, seed, and juice/pulp fractions revealed the skin and seed extracts were inhibitory to *Listeria monocytogenes* but the juice/pulp extract was not. The antimicrobial action of the Ribier skin and seed extracts were found to be specific to *L. monocytogenes* with no inhibitory action against other gram-positive or gram-negative bacteria observed at the concentrations assayed. The result was analogous to the narrow antimicrobial spectrum of the commercial grape juice (batch 1). Investigations in Chapter Two suggested that

polymeric phenolics (condensed tannin) were involved in the antilisterial activity of the commercial grape juice. Separation and assay of the monomeric and polymeric phenolic components of the commercial grape juice revealed the polymeric fraction had the stronger antilisterial activity than the monomeric fraction. Assay of the polymeric phenolic fraction of Ribier grape skin and seed revealed that these polymeric fractions were strongly inhibitory to *L. monocytogenes*. All polymeric phenolic fractions (juice, skin, and seed) reduced *L. monocytogenes* numbers by 6-log within 60 minutes. Assay of the polymeric phenolic fractions of juice, skin and seed at pH 3.5 and pH 7.0 revealed that the seed polymers were active at both pHs but neither the juice nor skin polymers were inhibitory at pH 7.0. This result was analogous to the pH dependent activity of the unmodified grape juice (Chapter Two section 2.4.8). The juice and skin, but not the seed polymers, dependence on acidic pH for activity indicated that the skin and juice antilisterial factors were similar in nature. A further understanding of the nature of the antilisterial factor of the juice, skin, and seed polymers was achieved by dialysis of the samples. Dialysis of the juice and skin polymers and assay of the retentates resulted in no reduction in antilisterial activity of the retentate even though a 70% weight loss of material occurred. Dialysis of the seed polymers resulted in a 90% weight loss of the sample and a significant reduction in antilisterial activity. This result suggested that the grape seed polymers were significantly different in size, chemical composition, and/or structure to the polymeric phenolic isolated from the commercial grape juice and Ribier grape skin. Together, the pH effect and dialysis effect results suggested that the commercial grape juice had antilisterial parameters similar to those of the Ribier grape skin polymers but significantly different than those of the grape seed polymers. It was concluded that although both Ribier grape skin and seed have antilisterial activity the antilisterial factor present in the commercial grape juice was likely derived from the skin of *Vitis vinifera* var. Ribier grapes.

Addition of protein (BSA) to the grape juice polymeric phenolic material resulted in the loss of antilisterial activity suggesting that the antilisterial factor was condensed tannin. The antilisterial factor was identified as polymeric phenolic (condensed tannin) material. A more detailed fractionation and analyses were performed on the polymeric phenolic material isolated from the commercial grape juice to characterise the antilisterial factor.

4 ISOLATION AND CHARACTERISATION OF THE ANTILISTERIAL FACTOR IN COMMERCIAL GRAPE JUICE

4.1 Introduction

Investigations in Chapter Three indicated that polymeric phenolic material (condensed tannin) was responsible for the antilisterial activity of the commercial grape juice. The aim of this Chapter was to characterise the whole polymeric phenolic (condensed tannin) fraction of commercial grape juice (batch 2) by analysing the elements that make up the fraction and by comparing the spectra of the fraction generated by FTIR to that of published proanthocyanidins (condensed tannin) spectra.

Condensed tannins (also termed proanthocyanidins) consist of chains of flavan-3-ol units. The flavan-3-ol subunits can be linked through both C_4-C_8 and C_4-C_6 linkages. The flavan-3-ol subunits can also be gallated. Proanthocyanidins consist of two classes polymers, procyanidins and prodelphinidins. Procyanidins consist of catechin and epicatechin. Prodelphinidins consist of epigallocatechin and gallocatechin (Cheynier *et al*., 1999). *Vitis vinifera* grape seed condensed tannin is comprised of procyanidin polymers (Prieur *et al*., 1994; Saucier *et al*., 2001; Kennedy & Taylor, 2003), whereas *Vitis vinifera* grape skin and red wine condensed tannins consist of both procyanidin and prodelphinidin polymers (Souquet *et al*., 1996; Sun *et al*., 1998; Labarbe *et al*., 1999; Sarni-Manchando *et al*., 1999). Red wine and red grape skin condensed tannin also contain anthocyanin (pigmented) moieties (Remy *et al*., 2000). As red wine ages the percentage of anthocyanin moieties incorporated into the condensed tannin polymeric structure increases. This increase in pigment condensed tannin results in a wine that is more red/brown in colour compared to the typical bright red colour of a young red wine (Somers, 1971). Although anthocyanins are known to be associated with the condensed tannin in red wine and grape skin the exact linkage of these moieties is essentially unknown. Remy *et al*., (2000) was the first to report the confirmation of red wine products resulting from direct anthocyanin-tannin reactions. To this researcher's knowledge this is the only report of a direct reaction of an anthocyanin and condensed tannin in red wine.

The method of choice for the analysis of condensed tannin (also called proanthocyanidins or polymeric phenolics) is thiolysis (Rigaud *et al*., 1991; Prieur *et al*., 1994; Souquet *et al*., 1996; Sun *et al*., 1998; Labarbe *et al*., 1999; Sarni-Manchado *et al*., 1999; Kennedy *et al*., 2000; Remy *et al*., 2000; Santos-Buelga & Scalbert, 2000). Thiolysis is the acid-catalysed depolymerisation of condensed tannin (proanthocyanidin) in the presence of the nucleophile benzylmercaptan. The reaction results in the release of a terminal unit from the polymer chain and the bonding of the nucleophile to the electrophilic free end of the polymer chain (extension unit). The reaction products are separated by HPLC and the mean polymer size, reported as mean degree of polymerisation (mDP), is determined by calculating the ratio between total units (terminal plus extension) and terminal unit. The optimal thiolysis reaction time was determined for the polymeric phenolic fraction. In order to identify the antilisterial factor in this polymeric phenolic fraction the fraction was subjected to further fractionated by molecular weight and the fractions generated assessed for antilisterial activity and for mean degree of polymerisation using the thiolysis reaction. The fractions were also assessed for the percentage of polymeric colour. The percentage of polymeric colour is the percentage of coloured material (anthocyanin) in the fraction that is incorporated into a polymeric structure. The composition of the most inhibitory polymeric fraction was determined by mass spectroscopy analysis of the thiolysis products of the fraction to determine the monomeric phenolic compounds that make up the oligomer. The fraction was also analysed by Infusion Electrospray Spectroscopy Mass Spectroscopy (IES-MS) in order to identify some of the oligomers present in the fraction. Finally a number of monomeric phenolic compounds that make up the oligomer were assessed for antilisterial activity. The antilisterial Ribier grape seed fraction isolated in Chapter Three was also fractionated by molecular weight and the fractions assessed for antilisterial activity and characterised by thiolysis.

4.2 Materials

37.1 Table 4.1 List of materials used in Chapter Four.

Materials and Instruments	Source
Acetic acid (HPLC grade)	BDH
Acetone (AR grade)	BDH
Acetonitrile (HPLC grade)	BDH
Benzylmercaptan	Fluka
Butan-1-ol (AR grade)	BDH
Catechin	Sigma
Epicatechin	Sigma
Filters Minisart RC4 (0.45 μm)	Sartorius
Fourier Transform Infrared Spectrophotometer (FTS40)	BioRad
Freeze drier	Virtis
Gallic acid	Sigma
Hydrochloric acid (HCl)	BDH
Malvidin-3-glucoside (HPLC grade)	Extrasynthase, France
Methanol (HPLC grade)	Merck
Potassium bromide (KBr)	BDH Spectrosol
Rotary evaporator	Büchi, Switzerland
Sephadex LH20	Amersham Pharmacia Biotech AB, Sweden
Sodium hydroxide (AR)	BDH
Thin Layer Chromatography plates (silica gel 60)	Merck

All other materials used in this chapter are outlined in previous Chapters materials lists.

4.3 Methods

4.3.1 Chemical Analyses of Polymeric Phenolic Fraction Isolated from Commercial Grape Juice

A range of analyses was performed on the polymeric phenolic fraction, isolated from commercial grape juice (batch 2), in order to characterise the chemical composition and identify the antilisterial factor.

4.3.1.1 Elemental Analysis

Elemental analysis of the polymeric phenolic fraction was performed by the Microanalysis Unit at Otago University, New Zealand, using a Carlo Erba Elemental Analyser EA 1108. The sample was analysed for total carbon, hydrogen, oxygen and nitrogen. The analytical method is based on the complete and instantaneous oxidation of the sample by "flash combustion" which converts all organic and inorganic substances into combustible products. The sample was held in a tin capsule and dropped into a vertical quartz tube, containing catalyst (tungstic oxide) and copper, which was maintained at a temperature of 1020^{o}C. The helium carrier gas was temporarily enriched with pure oxygen as the sample was dropped into the tube. The sample and its tin container melted with the tin promoting a violent reaction. Under these conditions even the thermally resistant substances are completely oxidised. Quantitative combustion was then achieved by passing the mixture of gases over a catalytic layer (tungstic oxide) and then through copper to remove excess oxygen and reduce nitrogen oxides to nitrogen. The resulting mixture was directed to a chromatographic column where the components were separated and detected by thermal conductivity detector. This gave an output signal proportional to the concentration of the individual components of the mixture. Percentages of elements were calculated using the original weight of the sample.

4.3.1.2 Fourier Transform Infrared (FTIR) Spectroscopy Analysis

Fourier Transform Infrared (FTIR) Spectroscopy analysis of polymeric phenolic fraction was performed using a FTIR Spectrometer FTS40 Instrument (BioRad). The sample was prepared by grinding 0.1-0.2 mg of sample with 0.5 g KBr and a disc prepared. Sixteen scans of each sample discs were run using a resolution of eight. The KBr background was automatically subtracted and the results were analysed using WinIR software (BioRad).

4.3.1.3 Optimisation of Polymeric Phenolic Fraction Thiolysis Conditions

Thiolysis reaction time was optimised for the polymeric phenolic fraction derived from commercial grape juice batch 2 by modifying the thiolysis protocol of Souquet *et al*., (1996).

Thiolysis reactions were performed as follows. A solution of 1 mg/ml polymeric phenolic in 10% ethanol was prepared. The solution was pasteurised for 30 minutes at 63°C to replicate the conditions used in the antilisterial assay. A series of triplicate samples (400µl) of polymeric phenolic were placed into glass ampoules. A 400 µl aliquot of thiolysis reaction mix (5% benzylmercaptan, 0.2 M HCl in methanol) was added to each sample. The ampoules were sealed with an acetylene/oxygen flame and reacted in a heating block held at 60°C for 0, 0.5, 2, 5, 16, 24, and 48 hours. After the specified reaction times the samples were removed from the heating block and cooled in a freezer for 5 minutes to stop the thiolysis reaction. The thiolysed and control polymeric phenolic samples (0 hour thiolysis reaction time) were filtered through 0.45 µm regenerated cellulose filters into amber HPLC vials. Samples were analysed for the presence of monomers (terminal units) and benzylthioether adducts (extension units) using the HPLC method of Rigaud *et al*., (1991) with some modifications. The samples were analysed on a Hewlett Packard 1100 Series HPLC with an 1100 diode-array detector. Samples were separated on a Phenomenex reverse phase C18 column (250 mm 4.6 mm i.d, 5µm particle size, 100 Å pore size) heated to 30°C. Elution conditions were as follows: injection volume 20µl, flow rate 0.8 ml per minute, solvent A 2.5% aqueous acetic acid, solvent B 80% acetonitrile 20% solvent A. Elution was performed using a linear gradient from 5% to 50% solvent B in 35 minutes and from 50% solvent B to 60% solvent B in 5 minutes and from 60% solvent B to 100% solvent B in 5 minutes. The column was then equilibrated with 5% solvent B for 10 minutes. Peak detection was at the following wavelengths: 280 nm, 305 nm, 320 nm, 365 nm, and 520 nm. Identification of flavanol monomers and the corresponding benzylthioether products was based on standard retention times, observed at 280 nm, in the case of catechin and epicatechin and by Liquid-Chromatography Mass Spectroscopy (LC-MS) for other peaks (section 4.3.5.1).

4.3.2 Fractionation of the Polymeric Phenolic Fraction by Molecular Weight

The polymeric phenolic (pigmented condensed tannin) fraction, isolated from commercial grape juice batch 2, was fractionated by molecular weight using the method of Kennedy & Taylor (2003).

A Sephadex LH20 column of 1 cm width 23 cm height (column volume 18 ml) was prepared and equilibrated with methanol:water:concentrated (12M) HCl (498:498:2). A 165 mg sample of polymeric phenolic freeze-dried powder was dissolved in 14 ml of methanol:water:HCl (498:498:2) and run via gravity into the matrix. The polymers were eluted from the column (gravity flow rate 0.7ml/min) using the following solvent system (all solvents contained 0.2% HCl): 60% methanol (70 ml), 75% methanol (70 ml), 90% methanol (80 ml), 10% acetone:80% methanol (130 ml), 20%

acetone:65% methanol (80 ml), 30% acetone:40% methanol (80 ml), and 60% acetone (130 ml). Fractions (5 ml) were collected over the elution series, sealed in screw top vials, and stored at -20^{o}C. To pool the fractions, 10-15 µl spots of each fraction was spotted onto silica thin layer chromatography plates and the plates chromatographed using a mixture of butan-1-ol:acetic acid:water (BAW 40:10:22) (Walker, 1975). The 99 fractions were pooled into eight fractions based on fraction colour (i.e. the first 10 fractions were pink rather than red and showed an extra spot on TLC) and Rf values generated by thin layer chromatography. The eight pooled fractions (designated A, B, C, D, E, F, G, and H) were rotary evaporated at 30^{o}C to remove solvent and freeze-dried (Virtis). The dry weight of each fraction was measured and the percentage dry weight recovery calculated.

4.3.3 Antilisterial Activity of the Eight Polymeric Phenolic Fractions

The eight powdered polymeric phenolic fractions (A, B, C, D, E, F, G, H) were assayed for antilisterial activity at decreasing concentrations (0.5 mg/ml, 0.1 mg/ml, 0.05 mg/ml final concentration) until the most inhibitory fraction was identified. The fractions were prepared for assay by first dissolving the appropriate weight of each fraction in 400 µl 99.95% ethanol and diluting it to 3.6 ml with sterile water. The pH of each solution was adjusted to 3.5 with sterile 10 mM HCl and the solutions made up to a final volume of 3.8 ml with sterile water. This gave solutions with a final concentration of 0.5 mg/ml, 0.1 mg/ml, and 0.05 mg/ml in 10% ethanol. The assay solutions were pasteurised at 63^{o}C for 30 minutes and assayed against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2). Due to low sample weight, the freeze-dried powders of fractions A and B were pooled and then assayed for antilisterial activity. A positive control of Model Wine Solution (MWS) (0.033 M L-tartaric acid, 10% ethanol pH 3.5) was run in parallel.

4.3.4 Chemical Characterisation of the Eight Polymeric Phenolic Fractions

The eight polymeric phenolic fractions (1 mg/ml) were analysed by thiolysis (section 4.3.1.3) using an optimal reaction time of 16 hours (section 4.4.1.3) and for percentage polymeric colour (section 2.3.6.3).

4.3.5 Mass Spectroscopy Analysis of the Thiolysis Products of Fraction G

The most inhibitory fraction (G) (section 4.4.3) was thiolysed for 16 hours according to the standard protocol (section 4.3.1.3) and the thiolysis products analysed via Liquid Chromatography Mass Spectroscopy (LC-MS) by this researcher (section 4.3.5.1). The unthiolysed fraction G was also

analysed by Infusion Electrospray Spectroscopy Mass Spectroscopy (IES-MS) (section 4.3.5.2) and by Liquid Chromatography Mass Spectroscopy (LC-MS) (section 4.3.5.3) by the Australian Wine Research Institute (AWRI) (Adelaide, Australia).

4.3.5.1 Liquid Chromatography-Mass Spectroscopy of Thiolysed Fraction G

A 1 mg/ml sample of fraction G was thiolysed according to the protocol outlined in section 4.3.1.3 using the optimal thiolysis reaction time of 16 hours. The thiolysed sample was chromatographed according to the conditions outlined in section 4.3.1.3 and analysed by Ionising Electrospray Mass Spectroscopy (Mariner, Perseptive Biosystems, Chemistry Department, University of Auckland). Data was generated in positive ion mode using the following conditions; 0.55 L/min nebuliser (nitrogen gas), 1.9 L/min nozzle plate, 9.5 L/min turbo ion source, spray tip potential 5.492 kilo-volts, nozzle temperature 195^{o}C, and mass range 100-4000.

4.3.5.2 Infusion Electrospray Spectroscopy-Mass Spectroscopy of Fraction G

The AWRI analysed the polymeric phenolic fraction G by IES-MS. Data was generated as follows: flow rate 5 µl/minute, mass range 100-3000 m/z, and solvent 72% acetonitrile:27.5% water:0.5% formic acid. The sample concentration was 0.5 mg/ml and the positive ion mode spectra were recorded from the accumulation of 50 scans.

4.3.5.3 Liquid Chromatography-Mass Spectroscopy of Fraction G

The AWRI also analysed the polymeric phenolic fraction G by LC-MS. The sample was chromatographed on a reverse phase C18 column (2mm x 150mm). Solvents were as follows: solvent A 5% formic acid, solvent B 5% formic acid:80% acetonitrile. The flow rate was 0.2 ml per minute. The gradient used was 20-60% B from 0-30 minutes, 60-90% B from 30-45 minutes, and 90% B from 45-60 minutes. Positive ion mode spectra were recorded.

4.3.6 Antilisterial Activity of Monomeric Phenolic Units of Polymeric Phenolic Fraction G

As determined by mass spectroscopy analysis the thiolysed polymeric phenolic fraction G was found to consist of the monomeric subunits catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin (section 4.4.5). Catechin, epicatechin, gallic acid, and malvidin-3-glucoside were assayed for antilisterial activity. Gallic acid, though not found to be present as a sub-unit of the polymeric fraction, is a moiety of epicatechin-3-*O*-gallate and therefore included in the assay. Although the anthocyanin malvidin-3-glucoside was not detected by MS analysis of the thiolysed

fraction G, the fraction was coloured red (**Figure 4.6**) and given that malvidin-3-glucoside is the most common anthocyanin present in *Vitis vinifera* grapes (Mazza & Miniati, 1993) it was included in the antilisterial activity assay. The antilisterial activities of catechin, epicatechin, malvidin-3-glucoside, and gallic acid were assayed according to the standard protocol (section 2.3.2). A 2.0 mg sample of catechin, epicatechin, malvidin-3-glucoside, and gallic acid were weighed into separate bottles and 400 µl of 99.95% ethanol added to dissolve the compounds. The solutions were then diluted to 3.6 ml with sterile water and the pH adjusted to 3.5 with 10 mM HCl. The solutions were made up to a final volume of 3.8 ml with sterile water and sterilised by passing each through a regenerated cellulose (0.45 µm) filter. The final concentration of each solution was 0.5 mg/ml in 10% ethanol. A control of model wine solution (MWS) (0.033 M L-tartaric acid, 10% ethanol, pH 3.5) was run in parallel.

4.3.7 Fractionation of the Polymeric Phenolic Fraction Isolated from Ribier Grape Seed

The polymeric phenolic fraction of Ribier grape seed (section 3.3.5) was fractionated by molecular weight into eight polymeric fractions using the method described for the grape juice polymeric phenolic material (section 4.3.2). Eighty-eight fractions were collected. To pool the fractions 10-15 µl spots of each fraction were spotted on to TLC plates and the plates chromatographed using the solvent system, toluene:acetone:acetic acid (30:30:10) (Sun *et al*, 1998). The fractions were pooled into eight fractions based on Rf values generated by TLC. The pooled fractions were rotary evaporated at 30^{o}C to remove the solvent and then freeze-dried (Virtis). The dry weight of each fraction was measured and percentage dry weight recovery calculated. The fractions were assayed for antilisterial activity at 0.1 mg/ml and 0.05 mg/ml final concentration as described in section 4.3.3 and analysed by thiolysis (reaction time 16 hours, 0.2 M HCl, 5% benzylmercaptan) as described in section 4.3.1.3.

4.4 Results and Discussion

4.4.1 Chemical Characterisation of Polymeric Phenolic Fraction Isolated from Commercial Grape Juice

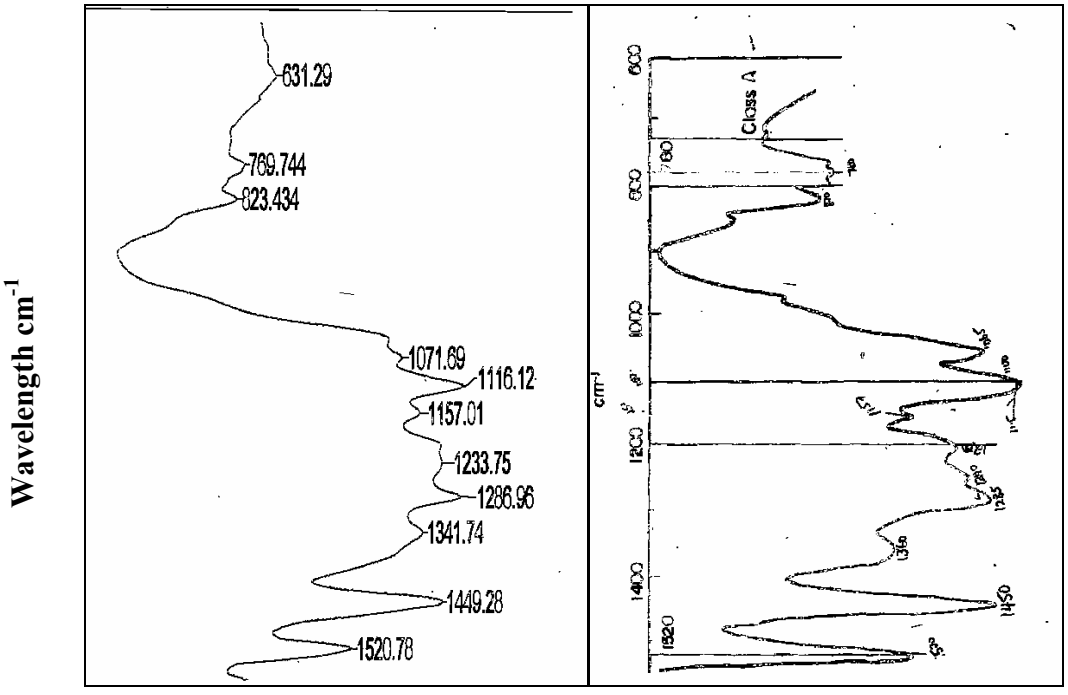
The antilisterial polymeric phenolic fraction, isolated from commercial grape juice batch 2, was analysed for elemental composition, by Fourier transform infrared (FTIR) spectroscopy and by thiolysis to characterise and identify the antilisterial factor.

4.4.1.1 Elemental Analysis of Polymeric Phenolic Fraction

The polymeric phenolic fraction was analysed for total carbon, hydrogen, oxygen, and nitrogen. The elemental analysis showed that the polymeric phenolic fraction consisted of 95.77% carbon, hydrogen, and oxygen and 0.53% nitrogen. The remaining 3.7% was not identified. On a percentage weight basis the elements were 53.43% carbon, 4.39% hydrogen, 37.96% oxygen, and 0.53% nitrogen. The low percentage of nitrogen indicated that very little protein or other nitrogenous substances were present in the polymeric phenolic fraction with the majority (>95%) found to consist of elements that make up phenol (C, H, O). Division of the percentage weight of each element by the molecular weight of the element gives the molar ratio of the element in the sample. The molar ratio of the carbon, hydrogen, and oxygen was determined to be 4.45:4.38:2.37 C:H:O. A ratio of 1:1 C:H is typical for nature-derived phenols. The results were consistent with the expected phenolic composition of the material.

4.4.1.2 Fourier Transform Infrared (FTIR) Spectroscopy Analysis of Polymeric Phenolic Fraction

The polymeric phenolic fraction was analysed for gross chemical structure by FTIR and spectra obtained were compared to proanthocyanidin spectra published by Foo (1981). A comparison of the spectra of the polymeric phenolic fraction produced by this researcher was compared to the published Infrared spectra of procyanidin polymers isolated from *Cydonia oblonga* (common quince) (Foo, 1981) (**Figure 4.1)**.



38.1 Figure 4.1 FTIR spectrum of polymeric phenolic fraction isolated from commercial grape juice batch 2 (left) and *Cydonia oblonga* procyanidin spectra (right) reported by Foo (1981).

The general spectral pattern of the polymeric phenolic fraction and the procyanidin polymers reveals a number of peaks in common (**Table 4.2**). The overall spectra of the antilisterial polymeric phenolic fraction isolated from commercial grape juice (batch 2) had a very similar pattern to that of procyanidin polymers consisting of catechin/epicatechin monomer units described by Foo (1981). Procyanidins and prodelphinidins are classes of condensed tannins (also called proanthocyanidins), which consist of chains of flavan-3-ol units (Cheynier *et al*., 1999). Procyanidins are composed of epicatechin and catechin units and prodelphinidins are composed of epigallocatechin and gallocatechin units (**Figure 4.2**).

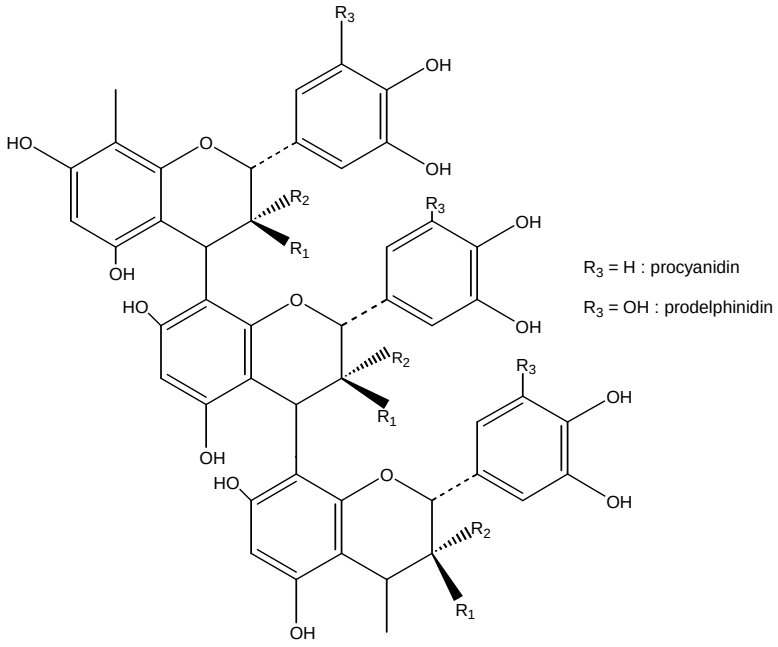
39.1

40.1 Table 4.2 Comparison of FTIR peaks of polymeric phenolic fraction isolated from commercial grape juice (batch 2) and procyanidin polymers from *Cydonia oblonga* reported by Foo (1981).

Peaks from polymeric phenolic fraction	Peaks reported for *Cydonia oblonga* procyanidins
1520	1520
1449	1450
1341	1350
1287	1285
1233	1250
1157	1200
1116	1110

1071	1065

A single peak at 1520 cm^{-1} is indicative of the presence of epicatechin or catechin (procyanidin model) (Foo, 1981). The single peak observed at 1520 cm^{-1} for the commercial grape juice polymeric phenolic sample indicated that the grape juice polymeric phenolic material contained procyanidin polymers (epicatechin/catechin). Further analyses (thiolysis and further fractionation) were performed to elucidate the chemical make-up of the antilisterial factor present in this polymeric phenolic (pigmented condensed tannin) fraction.



41.1

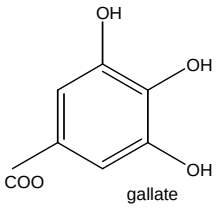
R_1 = OH, R_2 = H, R_3 = H : catechin

R_1 = H, R_2 = OH, R_3 = H : epicatechin

R_1 = H, R_2 = gallate, R_3 = H : epicatechin-3-*O*-gallate

R_1 = OH, R_2 = H, R_3 = OH : gallocatechin

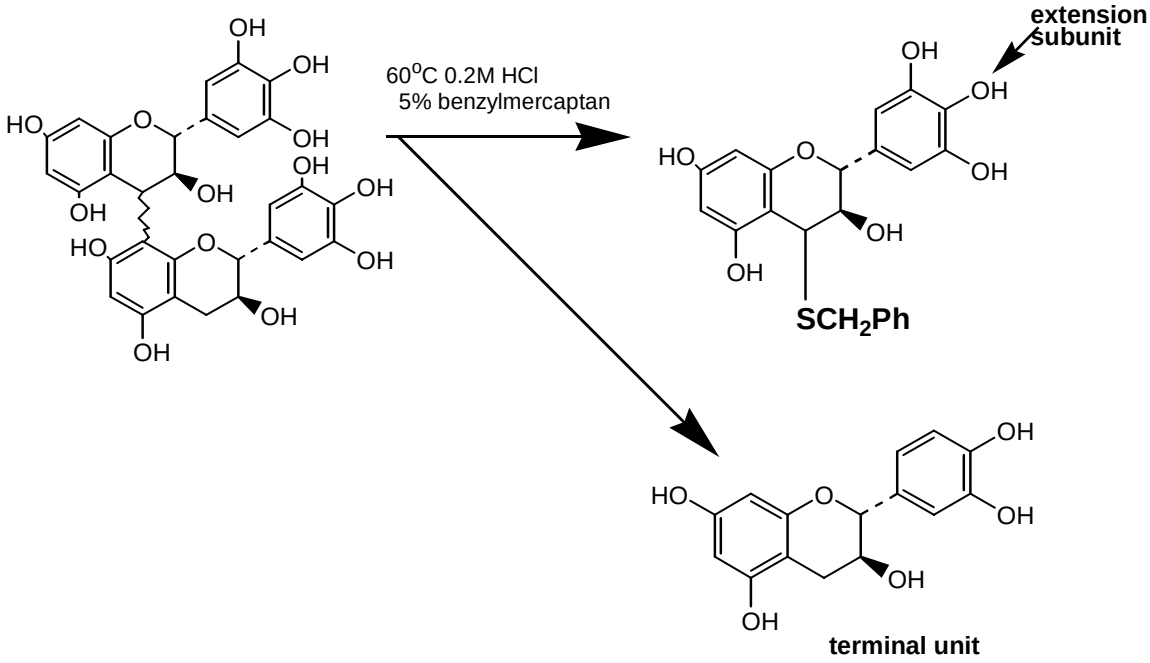
R_1 = H, R_2 = OH, R_3 = OH : epigallocatechin



42.1 Figure 4.2 Structures of typical grape proanthocyanidins (taken from Cheynier *et al*., 1999).

4.4.1.3 Optimisation of the Thiolysis Conditions of Polymeric Phenolic Fraction Isolated from Commercial Grape Juice

Thiolysis is the acid-catalysed depolymerisation of condensed tannin in the presence of a nucleophile (benzylmercaptan). The nucleophile forms a thioether with the C_4 of each polymer unit with the release of a monomer terminal unit and the formation of a thioether with the remaining extension unit (Santos-Buelga & Scalbert, 2000) (**Figure 4.3**). The thiolysis products are then separated and quantified by HPLC. The mean degree of polymerisation (mDP) is determined by calculating the ratio (mg/L) of terminal unit (catechin) plus extension unit (thioether epicatechin) to terminal units (catechin).



43.1 Figure 4.3 Example of products released by thiolysis of grape skin proanthocyanidins (modified from Kennedy & Jones, 2001). SCH_2PH = benzylmercaptan.

A typical HPLC spectrum of the thiolysis products of red wine proanthocyanidins (*Vitis vinifera* var. Merlot var. Carignan) is shown in **Figure 4.4**.

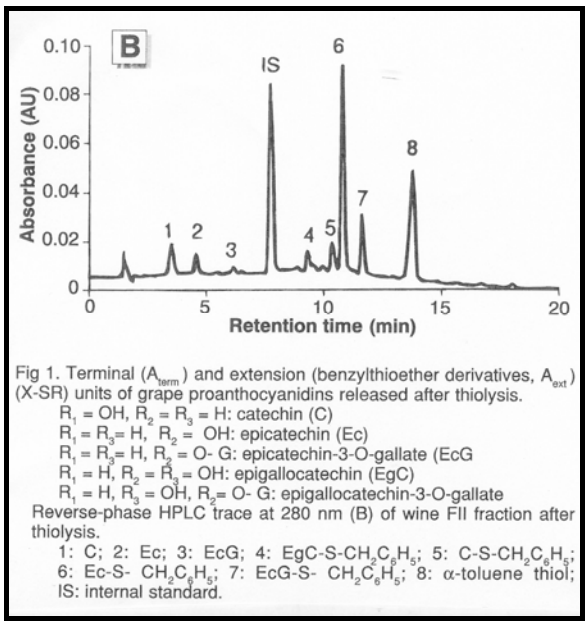
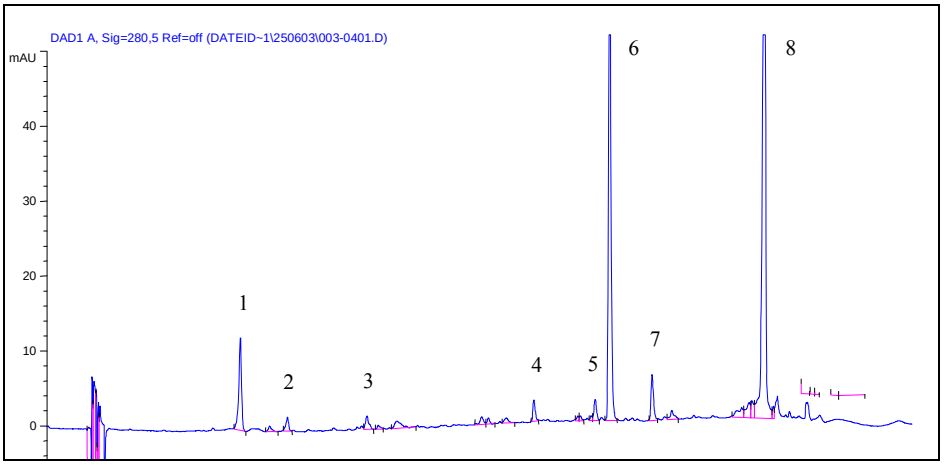


Fig 1. Terminal (A_{term}) and extension (benzylthioether derivatives, A_{ext}) (X-SR) units of grape proanthocyanidins released after thiolysis.
R_1 = OH, R_2 = R_3 = H: catechin (C)
R_1 = R_3 = H, R_2 = OH: epicatechin (Ec)
R_1 = R_3 = H, R_2 = O- G: epicatechin-3-O-gallate (EcG
R_1 = H, R_2 = R_3 = OH: epigallocatechin (EgC)
R_1 = H, R_3 = OH, R_2 = O- G: epigallocatechin-3-O-gallate
Reverse-phase HPLC trace at 280 nm (B) of wine FII fraction after thiolysis.
1: C; 2: Ec; 3: EcG; 4: EgC-S-$CH_2C_6H_5$; 5: C-S-$CH_2C_6H_5$; 6: Ec-S- $CH_2C_6H_5$; 7: EcG-S- $CH_2C_6H_5$; 8: α-toluene thiol; IS: internal standard.

44.1 **Figure 4.4 Typical HPLC spectrum of terminal and extension (benzylthioether derivatives) units of grape proanthocyanidins released after thiolysis** (taken from Sarni-Manchando *et al*., 1999).

A typical HPLC spectra (peak detection wavelength 280 nm) of the thiolysis products of Ribier grape skin polymeric phenolic fraction investigated in this thesis is shown in **Figure 4.5**.



1 = catechin; 2 = epicatechin; 3 = epicatechin-3-*O*-gallate; 4 = thioether epigallocatechin; 5 = thioether catechin; 6 = thioether epicatechin; 7 = thioether epicatechin-3-*O*-gallate; 8 = excess benzylmercaptan.

Figure 4.5 Typical HPLC spectra of terminal and extension (benzylthioether derivatives) units of Ribier grape skin polymeric phenolic material released after thiolysis (16 hours, 60°C, 0.2 M HCl, 5% benzylmercaptan).

The optimal thiolysis reaction time for the polymeric phenolic fraction was determined by calculating (HPLC peak area in mAU) the ratio of total units (terminal catechin plus extension benzylthioether epicatechin) and terminal units, measured at 280 nm. Ideally, the mean degree of polymerisation (mDP) is calculated using calibration curves generated by standards. No commercial standards of benzylthioether epicatechin or catechin were available at the time of this thesis and as such this researcher attempted to produce a standard by collection of the benzylthioether epicatechin peak from analytical HPLC. Three attempts at generation of this standard were unsuccessful. Therefore, given the lack of a benzylthioether, terminal unit (catechin) and extension unit (thioether epicatechin) peak area (milli Absorbance Units), generated by HPLC, was used to calculate the mean degree of polymerisation.

The optimal thiolysis time conditions for the polymeric phenolic fraction was found to be 16 hours (0.2 M HCl, 5% benzylmercaptan, 60°C) (**Table 4.3**).

Table 4.3 Optimisation of polymeric phenolic fraction thiolysis reaction time (terminal units (catechin) released and benzylthioether (epicatechin) produced at various reaction times (60°C with 0.2M HCl and 5% benzylmercaptan)). Peak area measured at 280 nm.

Thiolysis reaction time	Terminal Unit (Catechin)	Extension Unit (Thioether epicatechin)	Mean Degree of Polymerisation
Hours	Peak area on HPLC (mAU)		mDP
0	29.58	30.53	2.0
0.5	104.55	277.84	3.7
2	126.26	392.01	4.1
5	142.63	476.47	4.3
16	**158.33**	**585.15**	**4.7**
24	147.49	675.07	5.6
30	127.06	558.19	5.4
48	114.14	600.81	6.3

The best measure of optimal thiolysis reaction time is when the highest amount of terminal unit (catechin) is released, by acid catalysed thiolysis, together with the highest amount of

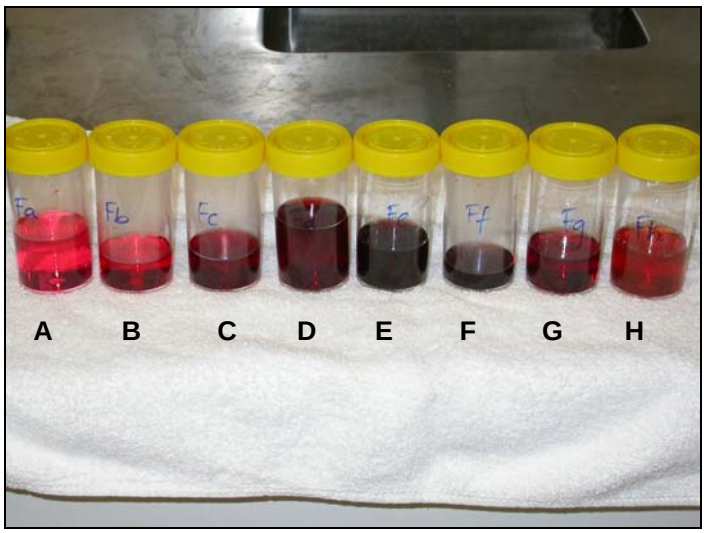
benzylthioether extension unit produced (benzylthioether epicatechin). Although this does not give the highest mDP, it is a more accurate measure of mDP as catechin begins to degrade after 16 hours, resulting in a larger ratio of catechin terminal unit to epicatechin extension unit and therefore an erroneous mDP measurement. The highest level of catechin terminal unit together with the highest amount of epicatechin extension unit was found at a thiolysis reaction time of 16 hours. Thus, 16 hours was deemed to be the optimal thiolysis reaction time of the polymeric phenolic fraction under investigation. The mDP data measured are low suggesting that the polymers may be derived from seed tannin rather than skin tannin. It is also possible that the polymeric fraction may contain a high proportion of polymers that are resistant to acid-catalysed depolymerisation resulting in low mDP measurements.

4.4.2 Fractionation of the Grape Juice Derived Polymeric Phenolic Fraction by Molecular Weight

The polymeric phenolic fraction, isolated from commercial grape juice (batch 2), was fractionated by molecular weight into 99 fractions. The fractions were chromatographed on reverse phase thin layer chromatography plates and Rf values calculated. The fractions were pooled into eight fractions based on Rf values generated by thin layer chromatography and fraction colour. The eight fractions (A, B, C, D, E, F, G, and H) generated are listed in **Table 4.4** and shown in **Figure 4.6**.

45.1 Table 4.4 Volume and fraction numbers pooled to form the eight polymeric fractions of increasing molecular weight.

Fraction Name	Fractions pooled	Total volume of fraction (ml)	Fraction Name	Fractions pooled	Total volume of fraction (ml)
A	1-12	60	**E**	40-54	70
B	13-18	30	**F**	55-67	65
C	19-24	30	**G**	68-79	55
D	25-39	75	**H**	80-99	95



46.1

47.1 **Figure 4.6 Eight aqueous fractions (A, B, C, D, E, F, G, and H) of the polymeric phenolic material isolated from commercial grape juice (batch 2).** Note the volumes of the eight fractions are not proportional to the original volumes pooled.

The 99 fractions of the polymeric phenolic fraction were eluted from Sephadex LH20 based on molecular weight using the method of Kennedy & Taylor (2003). Kennedy & Taylor (2003) found that there was not good molecular weight resolution (the polymers eluted in a continuum rather than as discrete molecular weights) of both grape seed and grape skin proanthocyanidins (*Vitis vinifera* L. cv. Pinot noir). This researcher suggests that this would also be the case for the grape juice derived polymeric phenolic material and as such the molecular weight range of the eight fractions was assumed to be a continuum rather than discrete separation of molecular weights.

The first fractions A and B were pinker in colour than fractions C, D, E, F, G, and H, which indicated the presence of monomeric anthocyanins in these two fractions (**Figure 4.6**). The presence of a monomeric anthocyanin in fractions A and B was further indicated by the observation of a pink spot with relatively high mobility (Rf 0.59) compared to the almost immobile red/brown polymeric phenolic spot (Rf 0.32) present in fractions C through H. Fractions C through H were red/brown in colour with fractions becoming browner as elution time from the matrix increased (i.e. fraction H was browner than fraction C). Additionally, when chromatographed by TLC, fractions C through H showed a single spot with a low Rf value (Rf 0.3-0.4), suggesting the presence of

unresolved high molecular weight material in these fractions. The single spot on TLC and red/brown colour of the fractions (C through H) indicated that these fractions contained pigmented polymers.

Somers (1966) studied the colour and chromatographic differences of two fractions of red wine, reporting that a fraction containing monomeric anthocyanins, as shown by relatively greater mobility of spots by paper chromatography, was bright red in colour and that a fraction that contained pigmented polymeric phenolic material, as indicated by almost complete immobility when chromatographed, was darker red in colour. He suggested that in this dark red fraction the anthocyanin pigments were incorporated into the tannin structure resulting in a colour change from bright red to dark red. Somers (1971) reported that as red wine ages it becomes more red/brown in colour concomitant with an increase in the amount of anthocyanin incorporated into the polymeric structure. Masquelier (1959) reported that the antimicrobial activity of red wine increased with an increase in wine age. This researcher suggests that it is the pigmented polymers in the grape juice that contributes the antilisterial activity to the commercial grape juice under investigation.

The 99 fractions of the grape juice polymeric phenolic material were pooled into eight fractions (A through H). The pooled fractions were freeze-dried and the dry weight of each fraction and percentage of total material calculated (**Table 4.5**).

48.1 Table 4.5 Dry weight and percentage dry weight of recovered material for the eight polymeric phenolic fractions.

Fraction	Dry weight (mg)	Percentage of total recovered dry weight
A	2.6	2.3%
B	3.3	2.9%
C	15.4	13.6%
D	43.6	38.6%
E	19.7	17.4%
F	12.3	10.9%
G	12.8	11.3%
H	3.4	3%
Total dry weight	113	
Dry weight recovery	68%	

The original dry weight of polymer phenolic material fractionated was 165 mg and the percentage dry weight recovery was 68%. The remaining material (33%) was assumed to be adsorbed onto the column matrix and not released with the most non-polar solvent used in the elution gradient (60% acetone) and/or lost through the process of pooling and freeze drying. Fractions A and B contained little phenolic material with a dry weight of 2.9 and 3.3 mg, respectively. As expected, this indicated that only a small proportion of the polymeric material in the polymeric phenolic fraction was of low molecular weight (monomeric/dimeric). The last fraction eluted from the column (fraction H) also contained relatively little material with only 3.4 mg dry weight. Fraction H contained the highest molecular weight material as it eluted from the column last and had the highest mean degree of polymerisation (section 4.4.4.1 **Table 4.7**). The small amount of material present in fraction H indicated that there may have been a relatively small proportion of high molecular weight material in the starting polymeric material and/or that a greater proportion of high molecular weight material adsorbed to the column matrix. Fraction D contained the greatest weight of material despite being only 5 ml larger in original volume than fraction E. The elution of the polymeric material from Sephadex LH20 appeared approximately normally distributed with the bulk of the material eluting in the middle of the gradient with the more non-polar solvents (i.e. 90% methanol, 10% acetone 80% methanol, and 20% acetone 65% methanol). This indicated that the molecular weight of the polymers making up the initial polymeric material was distributed over a continuum and not eluted from the column as discrete molecular weight fractions.

4.4.3 Antilisterial Activity of the Eight Polymeric Phenolic Fractions

The eight polymeric fractions were assayed for antilisterial activity at decreasing concentrations until the most active fraction was identified. At a concentration of 0.5 mg/ml, fractions F, G, and H had an extremely rapid antilisterial effect, with cell numbers reduced from 10^6 CFU/ml to 10^2 CFU/ml at time 0. As stated in section 2.4.3, this initial reduction in cell numbers reflects the rapid inhibition that occurs in the time taken (<1 minute) to perform the dilution series and spread plate the sample. Fraction E also had rapid antilisterial activity with a 2-log reduction in cell numbers at time 0. No *L. monocytogenes* colonies were detected after 10 minutes exposure to fractions E, F, G, and H (**Figure 4.7**).

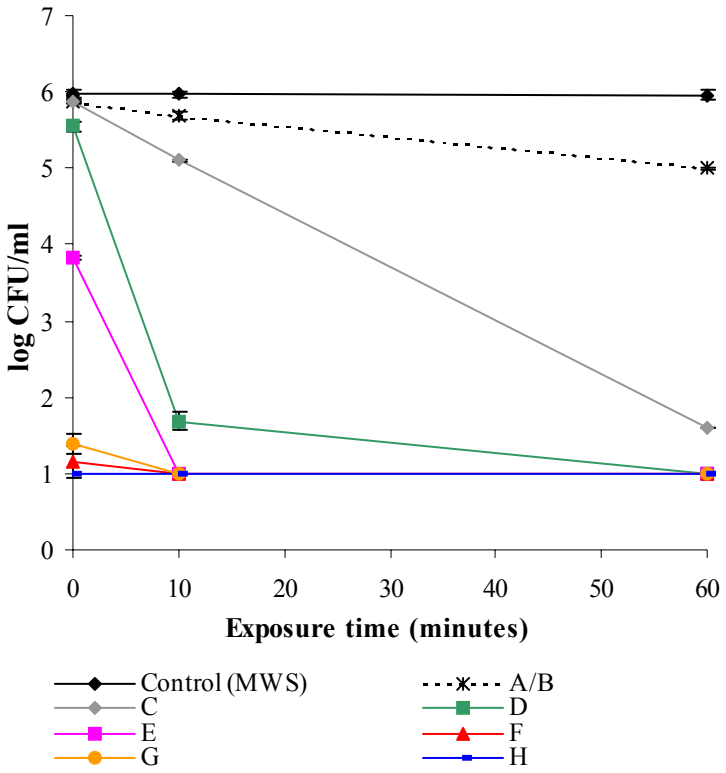
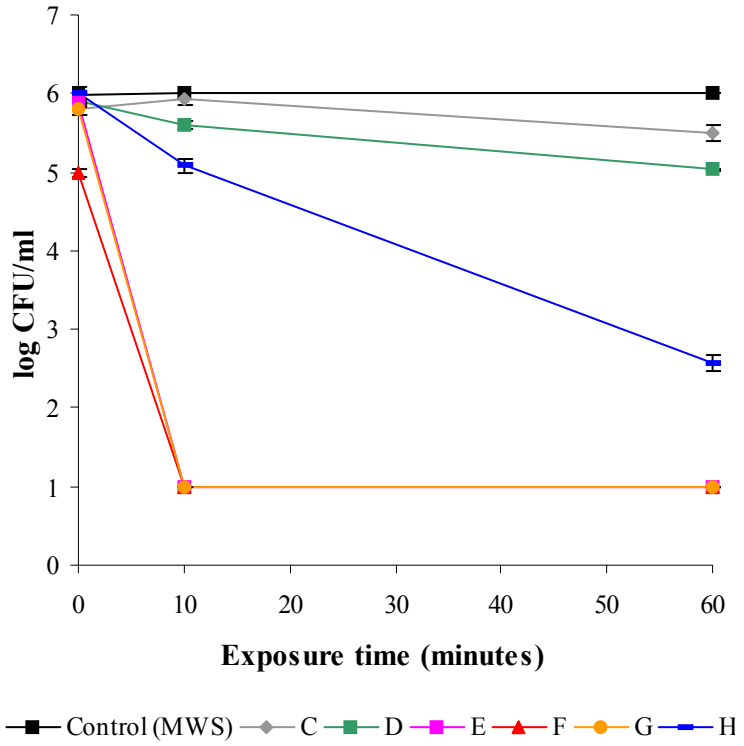


Figure 4.7 Antilisterial activities of polymeric phenolic fractions (0.5 mg/ml). Error bars are the ± standard deviation of mean log CFU/ml.

Fraction D had slightly weaker antilisterial activity than E, F, G, and H with a 4-log reduction in cell numbers after 10 minutes. No colonies were detected after 60 minutes exposure to this fraction. The lower molecular weight fractions A+B (A and B were pooled prior to assay due to small sample weight) and C were less active than the higher molecular weight fractions D through H. Fraction C reduced *L. monocytogenes* numbers from 10^6 CFU/ml to 10^2 CFU/ml after 60 minutes. Fraction A+B had only slight antilisterial activity with a 0.5-log reduction in cell numbers after 60 minutes exposure. Based on these results, fraction A+B was deemed to have no antilisterial activity and was not assayed at a lower concentration (0.1 mg/ml). The model wine solution control (MWS) (0.033 M L-tartaric acid 10% ethanol pH 3.5) was not inhibitory to *L. monocytogenes.*

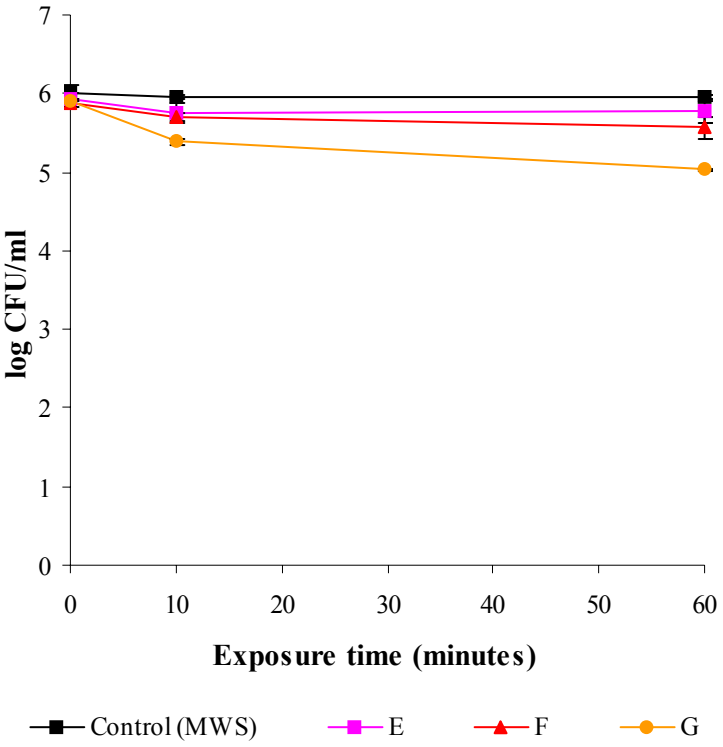
When assayed at a concentration of 0.1 mg/ml, fractions E, F, and G showed a rapid reduction in *L. monocytogenes* cell numbers with a 6-log reduction in cell numbers to no detectable colonies within 10 minutes exposure (**Figure 4.8**).



49.1 **Figure 4.8 Antilisterial activities of polymeric phenolic fractions (0.1 mg/ml).** Error bars are the ± standard deviation of mean log CFU/ml.

Fraction H was less inhibitory than fractions E, F, and G, with a 3.5-log reduction in *L. monocytogenes* numbers occurring after 60 minutes exposure to fraction H. Fractions C and D also showed a significant reduction in antilisterial activity, at 0.1 mg/ml compared to 0.5 mg/ml, with a less than 1-log reduction in *L. monocytogenes* numbers occurring after 60 minutes exposure. This result indicated that fractions E, F, and G had the greatest antilisterial activity. Fractions C, D, and H were deemed less active than E, F, and G and not assayed at a lower concentration (0.05 mg/ml).

When assayed at a concentration of 0.05 mg/ml fractions, E, F, and G had little antilisterial activity. *L. monocytogenes* numbers were reduced by less than 1-log after 60 minutes exposure to the fractions (**Figure 4.9**). Fraction G showed a slightly higher antilisterial activity than fractions E and F, though the reduction in cell numbers was less than 1-log. The results of this set of experiments indicates that fraction E, F, and G had the highest antilisterial activity of all eight fractions with fraction G showing marginally higher activity than fractions E and F.



50.1 **Figure 4.9 Antilisterial activities of polymeric phenolic fractions (0.05 mg/ml)**. Error bars are the ± standard deviation of mean log CFU/ml.

The antilisterial activity of fractions E, F, and G (0.05 mg/ml concentration) was reduced significantly with only a 50% reduction in concentration (0.1 mg/ml highly inhibitory compared to 0.05 mg/ml with little or no inhibitory activity). This may indicate that there is a threshold level of inhibitory factor required to interact with the bacteria before an inhibitory effect is observed. It seems possible that at 0.05 mg/ml the polymeric phenolic material binds to *L. monocytogenes* but that the amount of material bound to the cell is not great enough to induce a bactericidal effect.

However, at higher concretion (0.1 mg/ml) the polymeric phenolic material exceeds the assumed requisite threshold level and a bactericidal effect is observed.

It appeared that the molecular weight of the polymeric material was important in conferring the antilisterial activity of the eight polymeric fractions (A through H). Increasing elution time, an indication of changes to the molecular structure of the polymers and/or molecular weight, correlated with increasing antilisterial activity. The exception to this was fraction H, which was eluted from the matrix last, and was therefore assumed to have the highest overall molecular weight. However, fraction H had a lower antilisterial activity than fractions E, F, and G. It is feasible that polymers of a particular molecular weight range or structure have an inhibitory effect but that once the molecular weight of the polymer reaches a certain size or the molecular structure of the polymer changes the polymers become less inhibitory. Given that the fractions were assayed on a weight basis, it is also feasible that fraction H had a lower number of molecules per milligram of weight (as the polymers were larger in size) compared to fractions E, F, and G which contained more polymers per milligram of weight. This might be a reason for the lower inhibitory activity of fraction H.

Further analysis of the active fractions was performed to assess the mean degree of polymerisation (mDP) of the eight polymeric phenolic fractions (section 4.4.4).

4.4.4 Chemical Characterisation of the Eight Polymeric Phenolic Fractions

The eight polymeric phenolic fractions, isolated from the polymeric fraction of commercial grape juice batch 2, were analysed by thiolysis (refer **Figure 4.3**) and for percentage polymeric colour (section 2.3.6.3).

4.4.4.1 Thiolysis of the Eight Polymeric Phenolic Fractions

The optimal thiolysis reaction time for the polymeric phenolic was determined to be 16 hours (section 4.4.1.3) and the eight polymeric phenolic fractions were thiolysed accordingly using the set of conditions outlined in section 4.4.1.3 (0.2 M HCl, 5% benzylmercaptan). The composition of the eight polymeric phenolic fractions was determined by comparison of standard retention times (catechin, epicatechin) and by retention times of peaks identified by mass spectrometry (section 4.4.5). **Table 4.6** shows the composition of the eight fractions. To allow for the additional absorbance of the gallate moiety present in epicatechin-3-*O*-gallate and thioether epicatechin-3-*O*-gallate, a response factor was calculated by multiplying the absorbance of epicatechin-3-*O*-gallate with the ratio of the extinction coefficients of epicatechin (ε max 3580 280 nm) to epicatechin-3-*O*-

gallate (ε max 14000 279 nm) (Vuataz *et al*., 1959) i.e. a ratio of 0.256. This ratio was also assumed to be the same for thioether epicatechin and thioether epicatechin-3-*O*-gallate, however to this researchers knowledge this information was not available in the literature.

51.1 Table 4.6 Composition of the eight polymeric phenolic fractions determined by HPLC following thiolysis reaction.

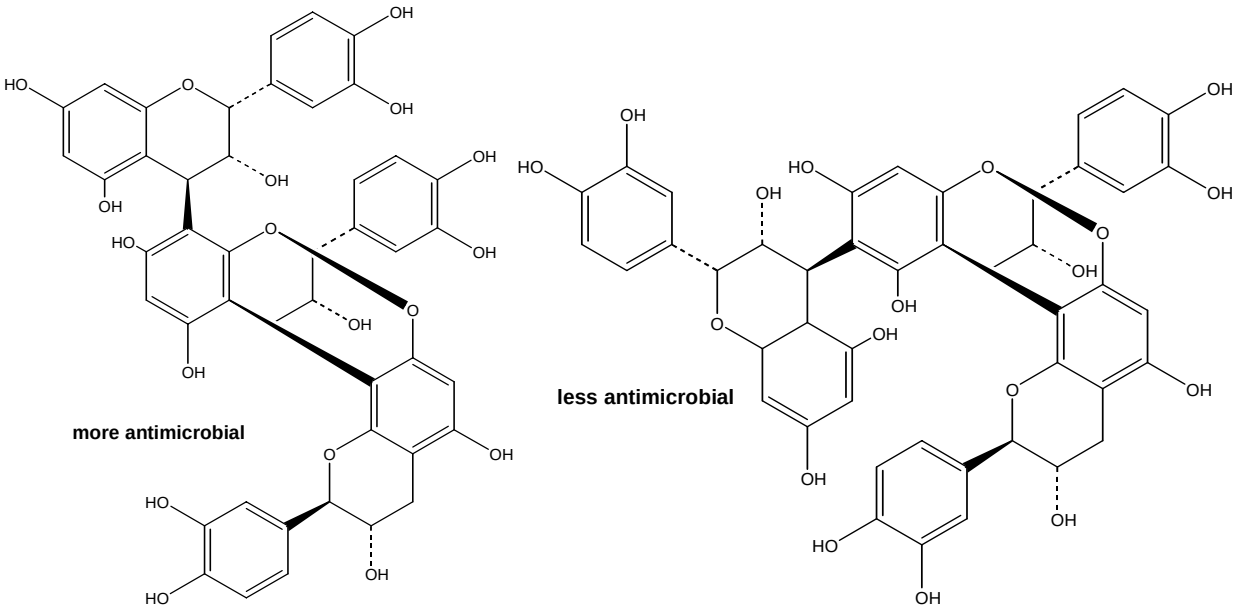
	Peak area on HPLC (mAU)*						
	Terminal Units			**Extension Units**			
Fraction	Catechin	Epicatechin	Epicatechin-3-*O*-gallate	Epigallo-catechin	Catechin	Epicatechin	Epicatechin-3-*O*-gallate
A	9.6	14.1	-	-	-	7.7	2.3
B	9.3	121.7	2.9	-	-	15.9	1.3
C	27	70.5	9.3	-	-	52	7.6
D	36.4	17.2	16.1	-	-	63.5	3.9
E	117.7	40.4	28.2	48.1	43.5	347.6	26.5
F	82	31.8	14.8	83.4	26.3	335	31.6
*G	41.4	13.1	6.4	15.5	13.6	202.2	18.5
H	76	34.5	13.6	47.4	45.5	534	45.6

*Peak detection wavelength 280 nm. All values are means of triplicate samples analysed. The proportion of constitutive units present in the fraction is important, not the actual peak areas measured by HPLC, as some fractions (example G) show lower than expected mAU due to error in weighing out small sample sizes.

Epicatechin predominated in the extension chains as seen by the presence of benzylthioether epicatechin and epicatechin-3-*O*-gallate peak areas being proportionally larger than the benzylthioether catechin peak. Catechin was more abundant as a terminal unit than epicatechin except for fractions A, B, and C. Catechin, epicatechin, and epicatechin-3-*O*-gallate were found as both terminal and extension units and epigallocatechin was found as an extension unit indicating that the grape juice polymer phenolic fractions contained both procyanidins (epicatechin/catechin) and prodelphinidins (epigallocatechin) (refer **Figure 4.2** for structures).

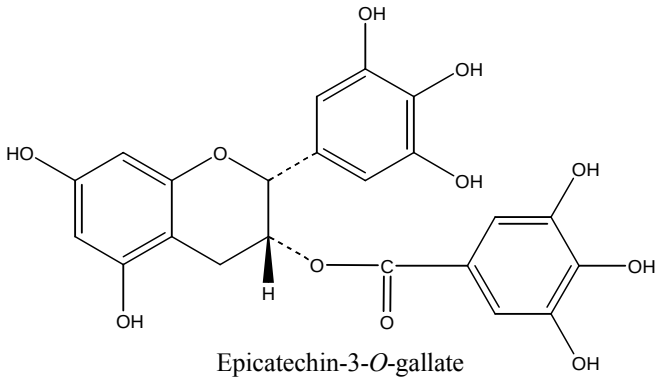
Only fractions E, F, G, and H contained epigallocatechin and catechin as extension units. The presence of epigallocatechin in the most active of the antilisterial fractions (E, F, G, and H) suggested that this compound may be important for antilisterial activity, though the antimicrobial activity of epigallocatechin has not, to this researcher's knowledge, been reported. Antimicrobial structural activity correlation studies performed by Tesaki *et al*., (1999) against *Escherichia coli, Staphylococcus aureus,* and *Salmonella* Enteritidis, revealed that gallic acid, which contains three hydroxyl groups, was a more effective antimicrobial compound than hydroxybenzoic and dihydroxybenzoic acid which contained one or two hydroxyl groups, respectively. A recent report by Friedman *et al*., (2003), on the antimicrobial activity of phenolic benzaldehydes and benzoic acids showed that *Listeria monocytogenes* was more susceptible to trihydroxylated benzadehyde than benzoic acid and that trihydroxylated benzaldehyde was more inhibitory than monohydroxybenzaldehyde or dihydroxybenzaldehyde. Epigallocatechin, which was only present in the most active fractions, (E, F, G, and H), has three hydroxyl groups on the B ring of the molecule (**Figure 4.2**) and this may contribute, in part, the higher antilisterial activity of fractions E, F, G, and H, compared to fractions A/B and C.

The antimicrobial activity of tannin components isolated from *Vaccinium vitis-idaea* L. (cowberry) was reported by Ho *et al*., (2000). The authors found that a proanthocyanidin trimer (epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-catechin) (**Figure 4.10**) had strong antimicrobial activity against periodontal disease causing bacteria (*Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis*, and *Prevotella intermedia*). Other proanthocyanidins assayed, including procyanidin B1, procyanidin B3, proanthocyanidin A1 (all dimers), and the trimers cinnamtannin B1 and epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7)-catechin were less inhibitory (**Figure 4.10**). It is apparent that structure plays an important role in the antimicrobial activity of polymeric phenolics (proanthocyanidins).



52.1 **Figure 4.10 Structures of proanthocyanidin trimers epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-catechin (more antimicrobial) and epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7) catechin (less antimicrobial)** (taken from Ho *et al*., 2000).

Mabe *et al*., (1999) reported the antimicrobial activity of six tea catechins against *Helicobacter pylori*. They found that epigallocatechin-gallate (**Figure 4.11**) had the strongest antimicrobial activity and suggested that the antimicrobial activity was due to the presence of a gallic acid moiety and the number of hydroxyl groups on the entire molecule.

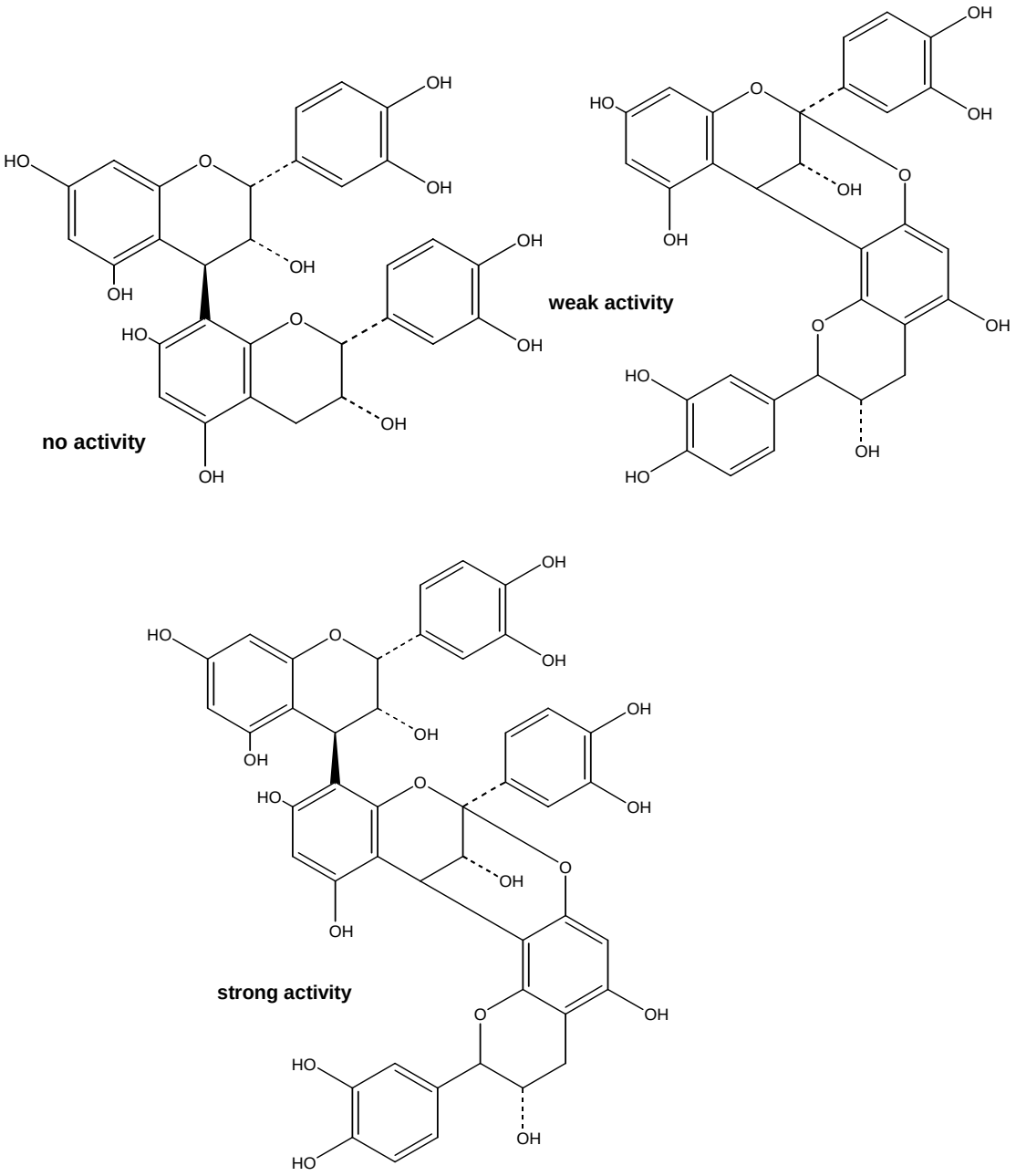


53.1 Figure 4.11 Structure of epigallocatechin-3-*O*-gallate

Mabe *et al*., (1999) found that the bactericidal action of epigallocatechin-gallate was higher at pH 7 than at pH 4 and 5, which is in contrast to this thesis finding that the commercial grape juice was

inhibitory at acidic pH and not inhibitory at pH 7.0 or above (sections 2.4.8 and 3.4.5). This suggested that either the antilisterial factor present in the commercial grape investigated in this thesis was not similar to epigallocatechin-gallate or that the presence of an anthocyanin moiety in the grape juice polymers contributed to the pH dependent activity of the grape juice. Epigallocatechin-gallate was not found in the antilisterial polymeric fraction G and therefore is unlikely to be the antilisterial factor. The antilisterial factor could be a combination of polymeric phenolic compounds, similar to epigallocatechin-gallate, which contain gallic acid moieties and hydroxyl groups, and these groups may be important in conferring antilisterial activity to the commercial grape juice.

Foo *et al*., (2000b) reported the structure of three A-type proanthocyanidin trimers isolated from cranberry that inhibited adherence of uropathogenic *E. coli* to the urinary tract. The authors reported that the monomeric polyphenol (epicatechin) and two dimeric polyphenols (epicatechin-(4β→8)-epicatechin, and epicatechin-(4β→8, 2β→*O*→7)-epicatechin, (also known as procyanidin B2 and procyanidin A2, respectively), had little or no anti-adherence activity whereas the proanthocyanidin trimers (epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7) epicatechin, epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4β→8)-epicatechin, and epicatechin-(4β→8)-epicatechin-(4β→8, 2β→*O*→7)-epicatechin) inhibited adherence of *E. coli* (**Figure 4.12**).



54.1 Figure 4.12 Structures of a proanthocyanidin dimer with no anti-adherence activity, a proanthocyanidin dimer with weak anti-adherence activity, and an A-type proanthocyanidin trimer with strong anti-adherence activity against *E. coli* (taken from Foo *et al*., 2000b)**.**

Whereas Foo *et al*., (2000b) found epicatechin-(4β→6)-epicatechin-(4β→8, 2β→*O*→7) epicatechin was active against *E. coli* adherence, Ho *et al*., (2000) found this same compound had no antimicrobial activity against *H. pylori*. It appears that the inhibitory effect of proanthocyanidins

(condensed tannins) is specific to the type of bacteria being investigated. Our finding that the commercial grape juice had inhibitory activity specific to *Listeria* species (section 2.4.2) support this theory.

The inhibitory effects of epicatechin-3-*O*-gallate and epigallocatechin-gallate, derived from the tea plant, against Human Immunodeficiency Virus enzymes (reverse transcriptase and DNA and RNA polymerase) was reported by Nakane & Ono (1990). The authors found that epigallocatechin-gallate was more inhibitory than epicatechin-3-*O*-gallate but the constitutive components, gallic acid, epicatechin, and epigallocatechin were inactive. Ikigai *et al*., (1993) studied the antimicrobial effect of green tea catechins and found that epigallocatechin-gallate had stronger antimicrobial activity than epicatechin and gram-positive bacteria were more susceptible to this action than gram-negative bacteria. They found the mode of action of epigallocatechin-gallate was bacterial membrane damage and leakage of bacterial cellular components. Ahn *et al*., (1991) reported the antimicrobial activity of the green tea polyphenols epicatechin-3-*O*-gallate and epigallocatechin-gallate but not catechin, epicatechin, gallocatechin, and epigallocatechin, against *Clostridium difficile*. They suggested that the gallate moiety was required for antimicrobial activity but the stereochemistry of the polyphenols was not important for this activity. Epigallocatechin, but not catechin and epicatechin, was found to be inhibitory to *Proteus vulgaris* and *Staphylococcus aureus* (Mori, *et al*., 1987). As suggested by Ahn *et al*., (1991) and Mabe *et al*., (1999), the presence of gallic acid moieties and the number of hydroxyl groups could be important for the antimicrobial action of polyphenols. Given that epicatechin-3-*O*-gallate contains a high number of hydroxyl groups, due to the presence of the gallate moiety, and was found to be present in seven of the eight antilisterial fractions (B, C, D, E, F, G, and H), this compound may contribute, in part, the antilisterial activity to the commercial grape juice.

The composition of the eight grape juice derived (batch 2) polymeric phenolic fractions (**Table 4.6**) was similar to that reported by a number of authors. Souquet *et al*., (1996) reported that catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin were the major constituent units of grape skin condensed tannins isolated from *Vitis vinifera* var. Merlot. Labarbe *et al*., (1999) identified the same constituent units making up *Vitis vinifera* cv. Cabernet franc grape skin proanthocyanidins but with the addition of an epigallocatechin extension unit. Sun *et al*., (1998) identified catechin, epicatechin, epicatechin-3-*O*-gallate, and their corresponding extension units with the addition of epigallocatechin in red wine made from *Vitis vinifera* cv. Tinta Miúda. Sarni-Manchando *et al*., (1999) reported the major units of red wine condensed tannins (*Vitis vinifera* var. Merlot 50% and var. Carignan 50%) as catechin, epicatechin, epicatechin-3-*O*-gallate, epigallocatechin, and

epigallocatechin-3-*O*-gallate. This thesis research did not detect epigallocatechin-3-*O*-gallate in the eight polymeric phenolic fractions (A through H). The elution order of thiolysis products of red wine proanthocyanidins reported by Sarni-Manchando *et al*., (1999), and the elution order of grape juice polymeric phenolic fraction G showed a high degree of similarity (see Sarni-Manchando result **Figure 4.4** and this thesis result in **Figures 4.5 and 4.14**). The results of thiolysis analysis in this thesis showed that the grape juice condensed tannin under investigation (derived from *Vitis vinifera* var. Ribier grapes) was very similar if not identical to the condensed tannin chemical composition reported for *Vitis vinifera* derived red wines and grape skin.

The mean degree of polymerisation (mDP) was determined for each fraction by calculating the ratio (based on HPLC mAU peak area measured at 280 nm) of terminal catechin units plus thioether epicatechin extension unit to terminal catechin units. The mDP of the eight polymeric phenolic fractions are listed in **Table 4.7**. The percentage of polymeric colour for each faction was determined using the bisulfite bleaching method outlined in section 2.3.6.3. The percentage polymeric colour is the percentage of coloured material in the fraction that is incorporated into a polymeric structure. For example 90% of the coloured material in fraction G is incorporated into a polymeric structure with the remaining 10% of the colour present as free anthocyanins.

55.1 Table 4.7 Mean degree of polymerisation and percentage polymeric colour of the eight polymeric phenolic fractions.

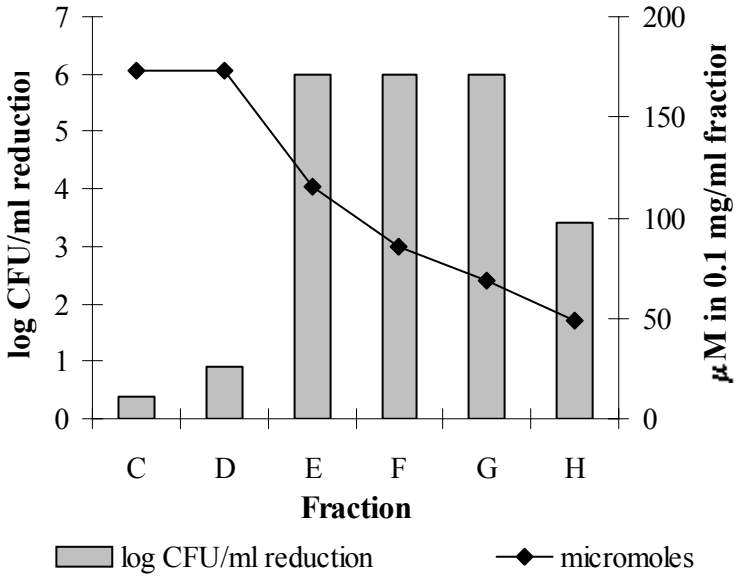
Fraction	mDP	% polymeric colour
A	1.8	ND*
B	2.9	47%
C	3.0	51%
D	2.8	64%
E	4.0	68%
F	5.0	78%
G	5.9	90%
H	8.5	74%

*Not determined due to insufficient sample weight, although fraction A was visibly red coloured.

There was a relationship between increasing mDP and the percentage polymeric colour of the fraction and increasing antilisterial activity. The most antilisterial fraction, G, contained the highest percentage (90%) polymeric colour. The least active of the fractions, A+B (assayed as A + B pooled) and C, had the lowest percentage polymeric colour with 47 and 51%, respectively. As the polymer size (mDP) increased so did the percentage of polymeric colour and the antilisterial activity of the fraction. The exception to this was fraction H, which had a higher percentage polymeric colour and mDP than fraction E but lower antilisterial activity. The most active fraction, G, had a lower mDP than fraction H but a higher percentage polymeric colour (90% compared to 74%). The combination of the percentage of polymeric colour in the fraction along with oligomer size (mDP) could confer the antilisterial activity of the fraction.

Mean degree of polymerisation increased with elution order from the Sephadex LH20 matrix. Fraction A had a mDP of 1.8 indicating that monomers and dimers were present in this fraction. Fraction A, when pooled with fraction B and assayed had very little antilisterial activity (**Figure 4.7**). Fractions B, C, and D had mDPs of 2.9, 3.0, and 2.8, respectively, indicating that dimers and trimers likely predominated in these fractions. The antilisterial activity of these fractions was not as strong as fractions with higher mDPs (i.e. fractions E, F, G, and H had mDPs of 4.0, 5.0, 5.9, and 8.5, respectively). The fraction that showed the strongest antilisterial activity (fraction G) had a mDP of 5.9. Although both fraction E and F had a similar degree of antilisterial activity they had lower mDPs of 4.0 and 5.0, respectively. Fraction H contained the largest oligomers with a mDP of 8.5. The antilisterial activity of fraction H was not as strong as the activity of fractions E, F, and G, which suggested that the average oligomer size (8.5) in this fraction may not be optimal for antilisterial activity. The fractions were assayed at equivalent weights (mg/ml). It is feasible that fraction H had a lower number of molecules per mg of weight, as the oligomers were larger in size, compared to fractions E, F, and G, thus accounting for the lower inhibitory activity of fraction H.

Figure 4.13 shows the log decrease in *L. monocytogenes* numbers, after 60 minutes exposure to each fraction, plotted against the number of μmoles of each fraction in 0.1 mg/ml solution. The number of moles was calculated by assuming that the subunit of each oligomer was catechin. For example, given that fraction C has a mDP of 3, it was assumed that the fraction consisted of mainly catechin trimers. The molecular weight of catechin is MW 290, thus a trimer has a molecular weight of MW 870. This molecular weight was used to calculate the number of μmoles of trimer in a 0.1 mg/ml solution of fraction C.



56.1 Figure 4.13 Reduction in *Listeria monocytogenes* numbers after 60 minutes exposure to 0.1 mg/ml solutions of the polymeric phenolic fractions (C through H) against the μmole concentration of each fraction.

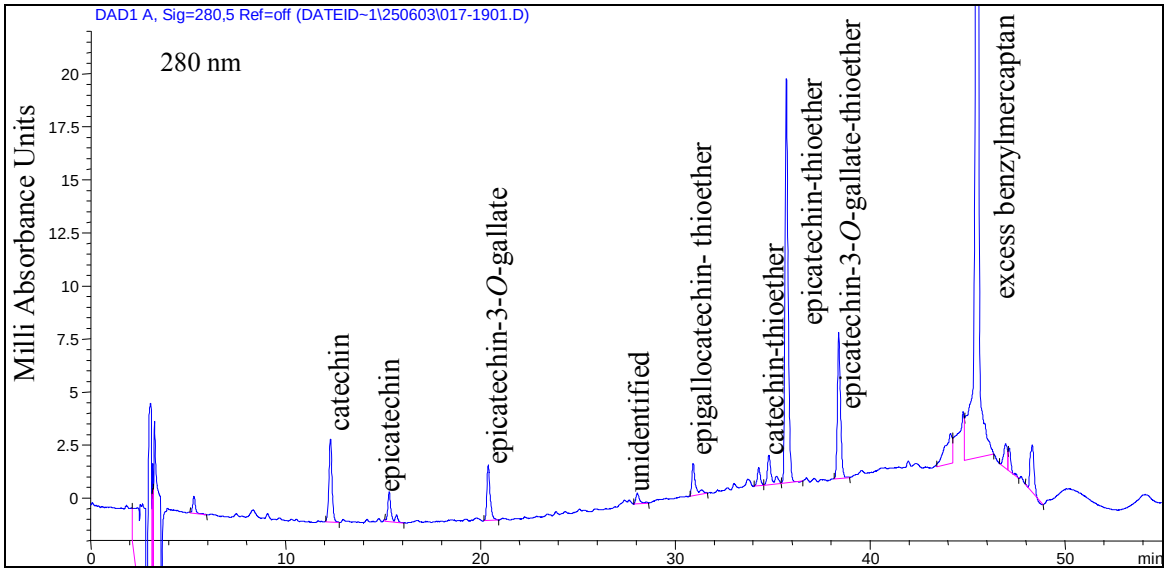
Figure 4.13 shows that the number of μmoles of oligomer in each fraction was not the sole reason for the level of antilisterial activity of the fraction. Both fractions C and D had relatively low antilisterial action despite the molar concentrations of the solutions being higher than the other antilisterial fractions assayed (E, F, G, and H). Fractions E, F, and G had a similar level of antilisterial activity when assayed at 0.1 mg/ml even though the μmole concentration of fraction E was greater than fraction F, which in turn was greater than fraction G. This could indicate that a combination of oligomer size and the number of oligomers in the assay solution contribute to the antilisterial effect. Fraction H had lower antilisterial activity than fraction E, F, and G but also had a lower μmole concentration of oligomers in solution. It is possible that if fraction H was assayed against *L. monocytogenes* at a concentration similar to that of fraction G (69 μmole) that this fraction may have had a level of inhibitory action similar to fraction G. In conclusion, this result suggests that oligomers of equal to or greater than trimers in size show the strongest antilisterial activity and that there is a required molar concentration of oligomer needed for inhibitory action.

The amount of anthocyanin containing oligomer in the fraction may have also contributed to the antilisterial activity of the polymeric phenolic fractions. As stated above, the most inhibitory

fractions (E, F, G, and H) had the highest percentage of polymeric colour. It is possible that the anthocyanins incorporated into the tannin oligomers may enhance the binding of the oligomers to the bacterial cell. At acidic pH, anthocyanins are cationic (**Figure 1.10**). It is feasible that the positive charge of the anthocyanin may interact with the negative charge of the cell wall of *L. monocytogenes*. Therefore, the higher the percentage of tannin polymers containing anthocyanin moieties the greater the ability of the tannin to bind to the bacteria and exert a bactericidal effect. This may explain in part the slightly higher antilisterial activity of fraction G.

4.4.5 Identification of Thiolysis Products of Polymeric Phenolic Fraction G

The products of thiolysis of fraction G were identified by comparison to standard retention times in the case of catechin and epicatechin, and by analysis of the mass of the other peaks by LC-MS. The peaks in fraction G are shown and identified in **Figure 4.14**. The following compounds were found to be produced by acid catalysed thiolysis of fraction G: catechin, epicatechin, epicatechin-3-*O*-gallate (*m/z* 443 $[M+H]^+$), the benzylthioethers of epigallocatechin (*m/z* 429 $[M+H]^+$), catechin (*m/z* 413 $[M+H]^+$), epicatechin (*m/z* 413 $[M+H]^+$), and epicatechin-3-*O*-gallate (*m/z* 565 $[M+H]^+$).



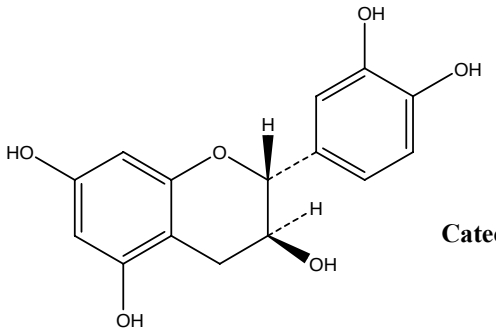
Time (minutes)

57.1 Figure 4.14 HPLC chromatograph of thiolysis products of polymeric phenolic fraction G.

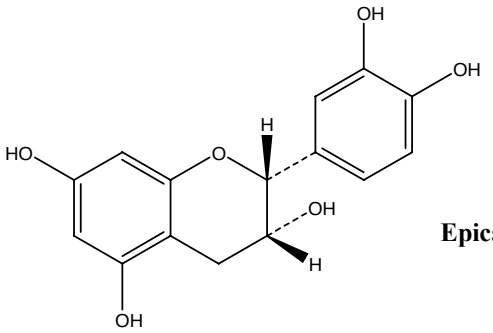
The structures of these compounds are shown in **Figure 4.15.**

58.1

59.1

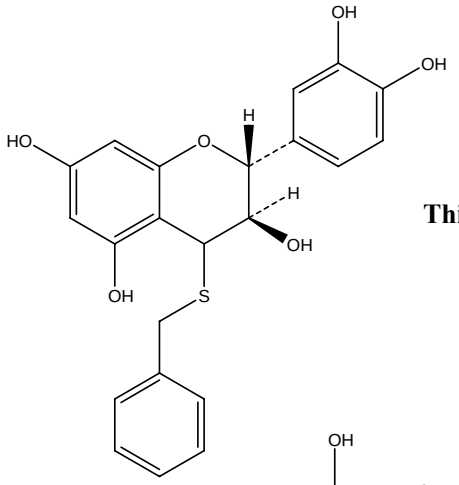


Catechin

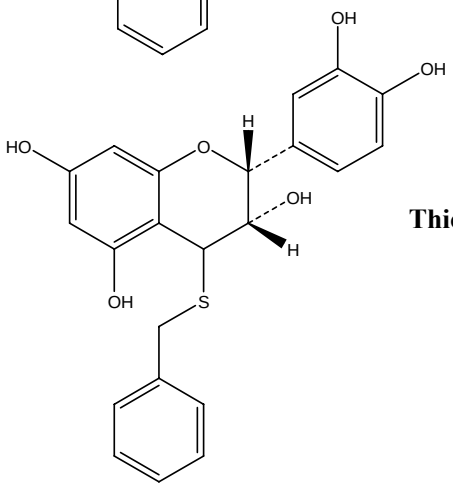


Epicatechin

60.1

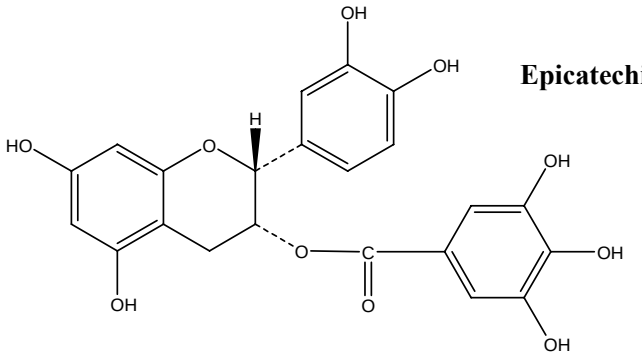


Thioether catechin

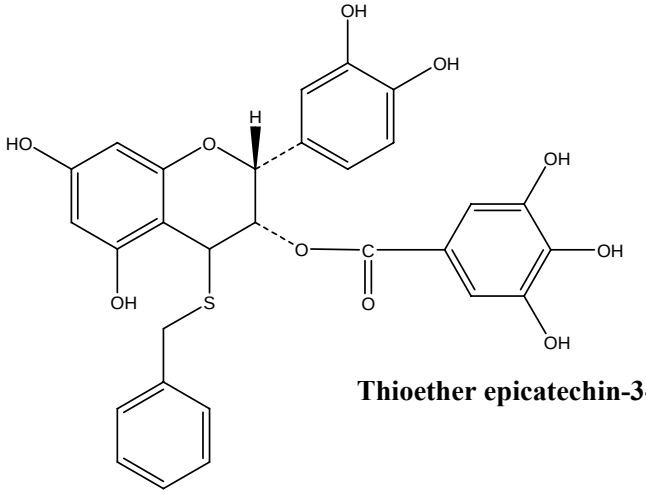


Thioether epicatechin

61.1



Epicatechin-3-*O*-gallate



Thioether epicatechin-3-*O*-gallate

62.1

63.1 Figure 4.15 Structures of the thiolysis products of polymeric phenolic fraction G.

A single peak (retention time 28 minutes) with a *m/z* 242.4 was not identified. However, this *m/z* 242 could be a fragment of the original compound due to the low molecular mass of the peak. Although the fraction under investigation was pigmented (refer **Figure 4.6**) no anthocyanin moiety was detected by this analysis method. This researcher found only one report of release of an anthocyanin moiety from the polymeric fraction of red wine or from grape skin by a thiolysis reaction. Remy *et al*., (2000) reported the detection of the anthocyanin malvidin-3-glucoside from the thiolysis of a polymeric fraction of red wine (*Vitis vinifera* var. Cabernet Sauvignon) (**Figure 4.16**).

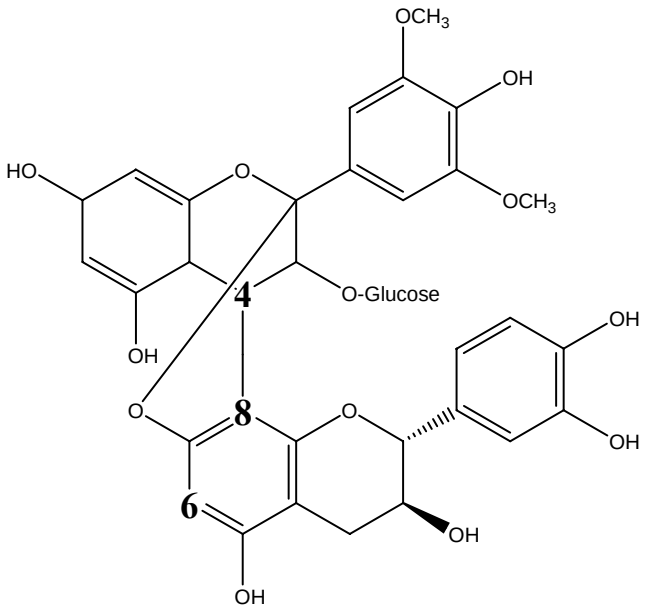


Figure 4.16 Structure of a bicyclic compound yielded by the reaction of an anthocyanin moiety (malvidin-3-glucoside) with a flavan-3-ol (taken from Remy *et al*., 2000). Note the anthocyanin is linked via the C_2 and C_4 position to the C_7 and C_8 position of the flavan-3-ol. This is an example of an A-type linkage.

A broad band of non-reacted material was observed on the HPLC chromatograph (**Figure 4.14**), beneath the excess benzylmercaptan peak (retention time 40-50 minutes), despite optimising the thiolysis reaction time for the polymeric phenolic fraction. A-type proanthocyanidins (**Figure 4.17**) are resistant to degradation by thiolysis (Lazarus *et al*., 2003) and the broad band of non-reacted material may consist of A-type proanthocyanidins. In addition, the anthocyanin moieties in polymeric phenolic fraction G may possess A-type interflavanoid linkages and this may be the reason no anthocyanin moieties were detected in the thiolysis products.

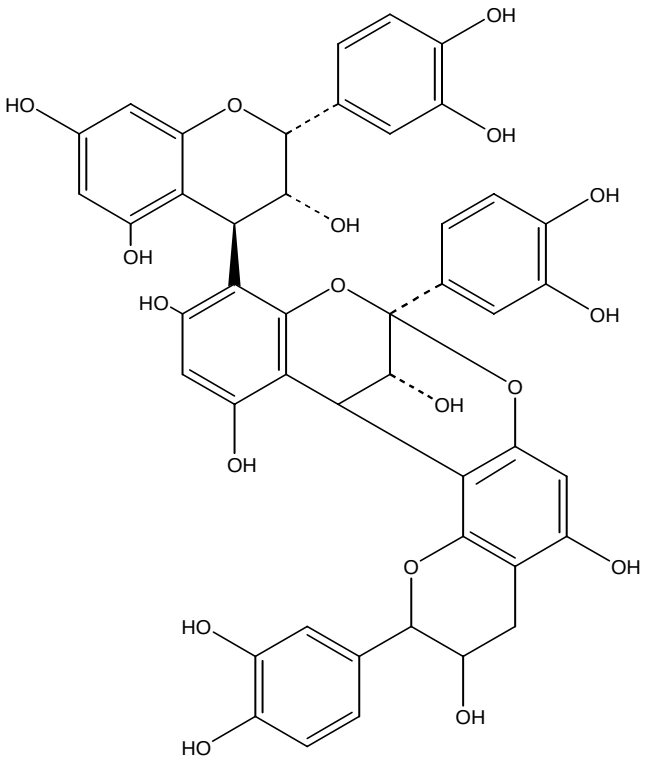


Figure 4.17 An example of an A-type proanthocyanidin trimer (taken from Foo *et al*., 2000b).

The presence of condensed tannin resistant to acid catalysed thiolysis has been reported. Kennedy *et al*., (2000) reported that the proportion of grape seed procyanidins resistant to acid catalysed hydrolysis increased with grape maturity. Kennedy & Jones (2001) also reported the presence of grape skin (*Vitis vinifera* L cv. Shiraz) proanthocyanidins resistant to acid hydrolysis. They reported a low thiolysis yield with only 58.6% of the proanthocyanidins degrading to their constitutive subunits, and suggested this was an indication of the heterogeneity of the interflavanoid bond. Larbarbe *et al*., (1999) reported the presence of grape skin (*Vitis vinifera* cv. Cabernet franc) proanthocyanidins resistant to thiolysis degradation (yield 68%) and suggested this may be due to anthocyanins included inside the polymer structure but not identified in skin tannin yet. The thiolysis degradation products of fraction G, identified by LC-MS, were in agreement with other published work (section 4.4.4.1).

4.4.5.1 Infusion Electrospray Spectroscopy-Mass Spectroscopy of Fraction G

The Australian Wine Research Institute analysed the whole unthiolysed polymeric phenolic fraction G by Infusion ES-MS in positive ion mode. A number of ions derived from some proanthocyanidins (oligomers) were recognisable. These included:

- a proanthocyanidin trimer (oligomer of three catechin units) (*m/z* 866),
- an oligomer consisting of four catechin and two gallate units (*m/z* 1459),
- a weak signal at *m/z* 2612 (oligomer of eight catechin units and two gallate units)
- a weak signal at *m/z* 2905 (oligomer of nine catechin units and two gallate units).

This analysis showed that fraction G contained a number of proanthocyanidin oligomers. Oligomers are defined as having 10 or less subunits and polymers have greater than 10 subunits. As expected, no proanthocyanidin dimers were found by this analysis as the fraction under investigation was found to have a mean degree of polymerisation of 5.9 and, as such, no dimers should have been present in this sample. The results of this analysis were in agreement with ions found by ES-MS analysis of grape seed proanthocyanidins (Peng *et al*., 2001).

The relative proportion of each size of oligomer making up fraction G was not determined by this analysis. However, the IES-MS results showed that fraction G contained oligomers that range in size from trimers to nonamers. Although fraction G had a mean degree of polymerisation of 5.9, no pentamers or hexamers were found in the analysis. However, as the mDP is a measure of average oligomer size and given that oligomers both larger and smaller than pentamers and hexamers were present within the fraction, the average size of these oligomers could result in a mDP of 5.9.

4.4.5.2 Liquid Chromatography-Mass Spectroscopy of Fraction G

The Australian Wine Research Institute analysed the polymeric phenolic fraction G by LC-MS. The pigmented polymeric material was eluted from the C18 column as a broad band and there were no resolved peaks observed. Mass spectra obtained were not informative due to poor ion signal intensity. Pigmented oligomers normally found in wine were not observed for fraction G and the AWRI suggested that the pigments in our sample are different from those found in the polymeric fraction of red wine. This result indicated that the pigmented oligomers present in the commercial

grape juice, and subsequently in the polymeric fraction G, might have been altered during the course of grape juice processing. The grape juice was concentrated prior to export to New Zealand, which does not occur in wine production, and could be a possible reason for the differences between the composition of our pigmented polymeric phenolic fraction and pigmented oligomers derived from red wines and grapes skin of *Vitis vinifera* variety. Grape varietal differences may also have contributed to the differences observed between typical red wine pigmented oligomers and our table grape derived juice. In addition, in the process of winemaking fermentation results in yeast-derived metabolites, that react with grape-derived anthocyanins and flavanols to give compounds that provide pathways for the formation of pigmented polymers that are different to those that are possible in grape juice processing (G. Jones, personal communication). Therefore this researcher suggests that the pigmented polymers found in the processed grape juice are different in structure than those found in wine.

4.4.6 Antilisterial Activity of Catechin, Epicatechin, Gallic acid, and Malvidin-3-glucoside

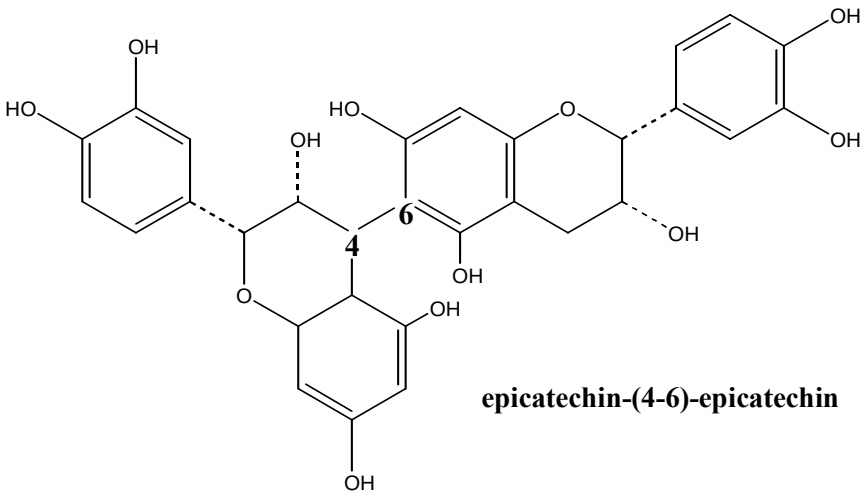
As determined by Mass Spectroscopy analysis (section 4.4.5), fraction G was found to consist of the monomeric subunits catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin. Catechin, epicatechin, gallic acid, and malvidin-3-glucoside were assayed for antilisterial activity. Gallic acid, although not present as a sub-unit of the polymeric fraction, is a moiety of epicatechin-3-*O*-gallate and therefore assayed. Although malvidin-3-glucoside was not detected by MS analysis, fraction G was coloured (**Figure 4.6**) and given that malvidin-3-glucoside is the most abundant anthocyanin in *Vitis vinifera* grapes (Mazza & Miniati, 1993), this compound was also assayed for antilisterial activity. Epicatechin-3-*O*-gallate and epigallocatechin were not assayed due to the high cost of purchasing commercial standards.

When assayed at a final concentration of 0.5 mg/ml (pH 3.5), none of the monomeric phenolic compounds that made up the antilisterial polymeric fraction G had an inhibitory effect on *L. monocytogenes* ATCC 35152 (**Table 4.8**). Epicatechin-3-*O*-gallate and epigallocatechin were not assayed and as such no comment can be made on their inhibitory activity.

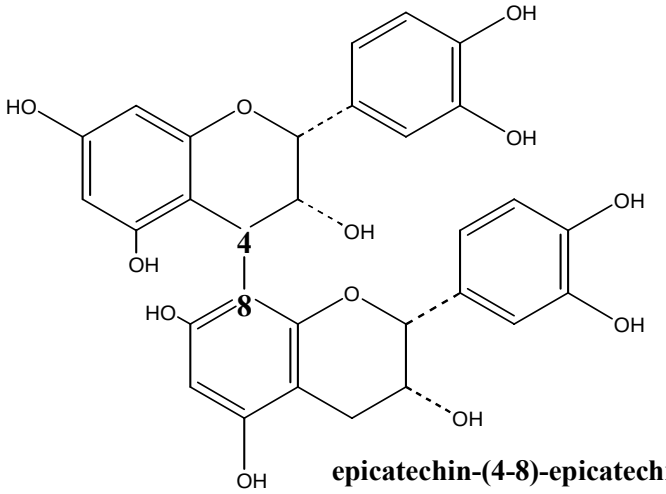
64.1 Table 4.8 Antilisterial activity of catechin, epicatechin, gallic acid, and malvidin-3-glucoside.

		Exposure time (minutes)		
		0	10	60
	Assay solution	Colony Forming Units/ ml		
Experiment 1	Model Wine Solution (MWS)	$9x10^5$	$9x10^5$	$7x10^5$
	Catechin	$8x10^5$	$7x10^5$	$7x10^5$
	Epicatechin	$9x10^5$	$6x10^5$	$6x10^5$
	Gallic acid	$8x10^5$	$9x10^5$	$8x10^5$
Experiment 2	Model Wine Solution (MWS)	$3x10^6$	$3x10^6$	$2x10^6$
	Malvidin-3-glucoside	$3x10^6$	$2x10^6$	$2x10^6$

Given that the antilisterial fraction G was inhibitory at a concentration of 0.1 mg/ml (**Figure 4.8**) and that the assay concentration of the monomer subunits (catechin, epicatechin, malvidin-3-glucoside, and gallic acid) was 0.5 mg/ml, the concentration of these compounds would not have been below the inhibitory level. Henis *et al*., (1964) reported the weaker antimicrobial activity of gallic acid (a subunit of tannic acid refer **Figure 1.11**) and catechol compared to tannic acid and carob pod condensed tannin. Payne *et al*., (1989) reported the antilisterial activity of tannic acid but the limited activity of gallic acid at concentration 0.5 mg/ml. Chung *et al*., (1993) also reported the antilisterial activity of tannic acid but not its hydrolysis products gallic acid and ellagic acid. The authors concluded that the ester linkage between glucose and gallic acid was important for antilisterial activity. This thesis research did not determine the linkages between the monomeric flavonoid subunits making up the antilisterial polymeric fraction. However, the literature reports that these linkages are not through the glucose moieties but rather direct linkages between the C_4 of the preceding monomer and C_6 or C_8 of the next flavan A-ring (**Figure 4.18**) (De Bruyne *et al*., 1999).



epicatechin-(4-6)-epicatechin



epicatechin-(4-8)-epicatechin

65.1 Figure 4.18 Common linkages between monomeric flavonoid subunits C_4-C_6 (above) and C_4-C_8 (below).

As stated previously (4.4.4.1) a number of authors (Mori *et al*., 1987; Nakane & Ono, 1990; Ahn *et al*., 1991; Ikigai *et al*., 1993; Foo *et al*., 2000a) have reported the antimicrobial and antiviral activity of polymeric phenolics such as epigallocatechin-gallate, epicatechin-3-*O*-gallate, epigallocatechin, and larger polyphenols (trimers) but not their constitutive units catechin, gallic acid, epicatechin and gallocatechin. This thesis finding agrees with these reports, as a number of the constitutive units of the antilisterial polymeric fraction G were not inhibitory to *L. monocytogenes*.

4.4.7 Fractionation of Ribier Grape Seed Tannin by Molecular Weight

The antilisterial polymeric phenolic fraction isolated from Ribier grape seed (section 3.3.5) was fractionated by molecular weight into 88 fractions according to the protocol outlined in section 4.3.2. The fractions were chromatographed on reverse phase thin layer chromatography plates using the solvent system toluene:acetone:acetic acid (30:30:10) (Sun *et al*., 1998). The fractions were pooled into eight fractions based on fraction colour and the slight differences observed in Rf values. The fractions were designated SA, SB, SC, SD, SE, SF, SG, and SH (**Table 4.9**).

66.1 Table 4.9 Volume and fraction numbers pooled to form the eight grape seed polymeric phenolic fractions of increasing molecular weight.

Fraction Name	Fractions pooled	Total volume of fraction (ml)	Fraction Name	Fractions pooled	Total volume of fraction (ml)
SA	1-17	78	**SE**	48-59	72
SB	18-24	51	**SF**	60-72	79
SC	25-31	41	**SG**	73-79	53
SD	32-47	98	**SH**	81-88	53

Once pooled the fractions were rotary evaporated at 30^{o}C, to remove the solvent, and freeze-dried (Virtis). The dry weight of each fraction and percentage of total material was calculated (**Table 4.10**).

67.1

68.1 Table 4.10 Dry weight and percentage dry weight of recovered material from the eight grape seed polymeric phenolic fractions.

Fraction	Dry weight (mg)	Percentage of total recovered dry weight
SA	0	0%
SB	1.5*	3.1%
SC	5.3	11%
SD	9.6	19.8%
SE	6	12.4%
SF	11.2	23.1%
SG	6.9	14.3%
SH	7.9	16.3%
Total dry weight	48.4	
% dry weight recovery	81%	

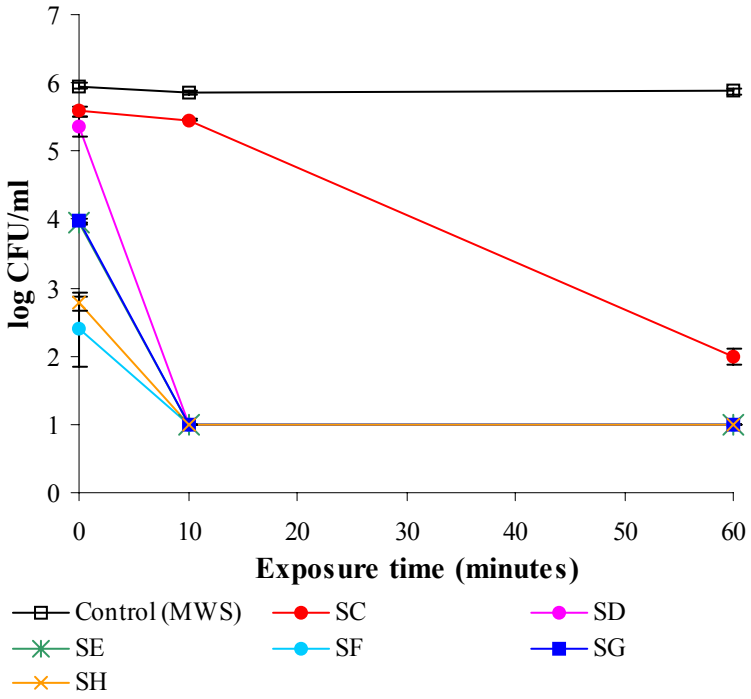
*Insufficient sample left to continue with analysis.

The percentage dry weight recovery of seed condensed tannin was 81%. This was higher than the recovery for grape juice condensed tannin (68%, section 4.4.2), and it was therefore assumed that little of the grape seed material remained adsorbed to the Sephadex LH20 matrix. Fraction SA contained no material and fraction SB only contained 3.1% of the total material recovered from the column. The lack and low level of material present in fractions SA and SB, respectively, eluted at the beginning of the gradient with 60% methanol, indicated that the seed condensed tannin contained little or no monomeric phenolic material and also precluded these fractions from further analyses. The remaining fractions contained similar levels of polymeric phenolic material (mg dry weight) with fraction SF containing the highest level (23.1%).

4.4.8 Antilisterial Activity of the Six Grape Seed Polymeric Phenolic Fractions

The six polymeric seed fractions (SC, SD, SE, SE, SF, SG, and SH) were assayed for antilisterial activity at decreasing concentrations until the most inhibitory fraction was identified.

The grape seed fractions ranged in colour from pale yellow to yellow brown. At a concentration of 0.5 mg/ml, all fractions, with the exception of fraction SC, had extremely rapid inhibitory activity with a 6-log reduction in *L. monocytogenes* numbers after 10 minutes exposure. As stated previously, the initial reduction in cell numbers at time 0 reflects the rapid inhibition that occurs in the time taken <1 minute to perform the dilution series and spread plate the sample (**Figure 4.19**).

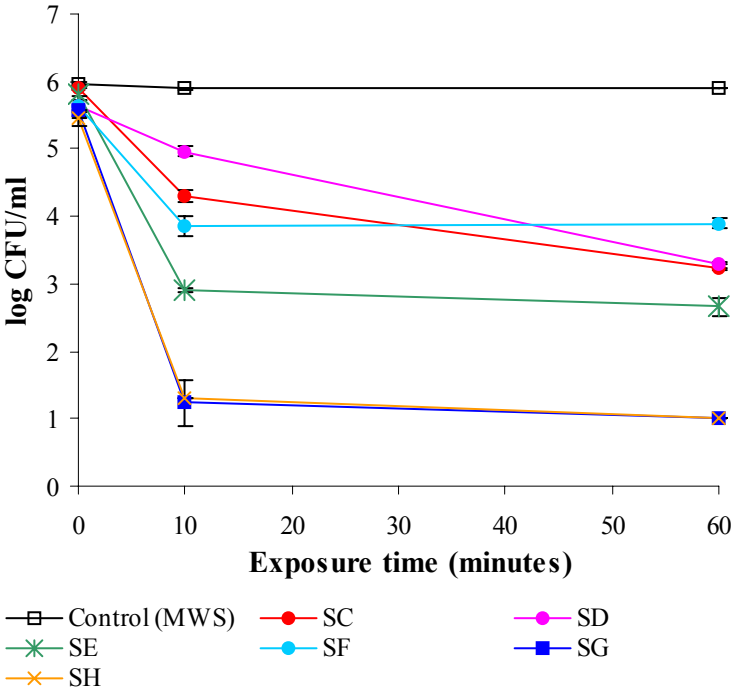


69.1 **Figure 4.19 Antilisterial activities of polymeric phenolic seed fractions (0.1 mg/ml).** Error bars are the ± standard deviation of mean log CFU/ml.

Fraction SC showed the lowest antilisterial activity of all the seed polymeric fractions with a 1.5-log reduction in cell numbers occurring within 10 minutes exposure. After 60 minutes exposure to fraction SC, cell numbers were only reduced by 4-log compared to 6-log for the other grape seed

fractions. Fraction SC was deemed inactive and not assayed at a lower concentration (0.05 mg/ml). The model wine solution control (MWS) (0.033 M L-tartaric acid 10% ethanol pH3.5) had no inhibitory effect against *L. monocytogenes*.

When assayed at a concentration of 0.05 mg/ml fractions SG and SH showed the strongest antilisterial activity against *L. monocytogenes* with cell numbers being reduced from 10^6 CFU/ml to no detectable colonies within 10 minutes exposure (**Figure 4.20**).



70.1 **Figure 4.20 Antilisterial activities of polymeric phenolic seed fractions (0.05 mg/ml).** Error bars are the ± standard deviation of mean log CFU/ml.

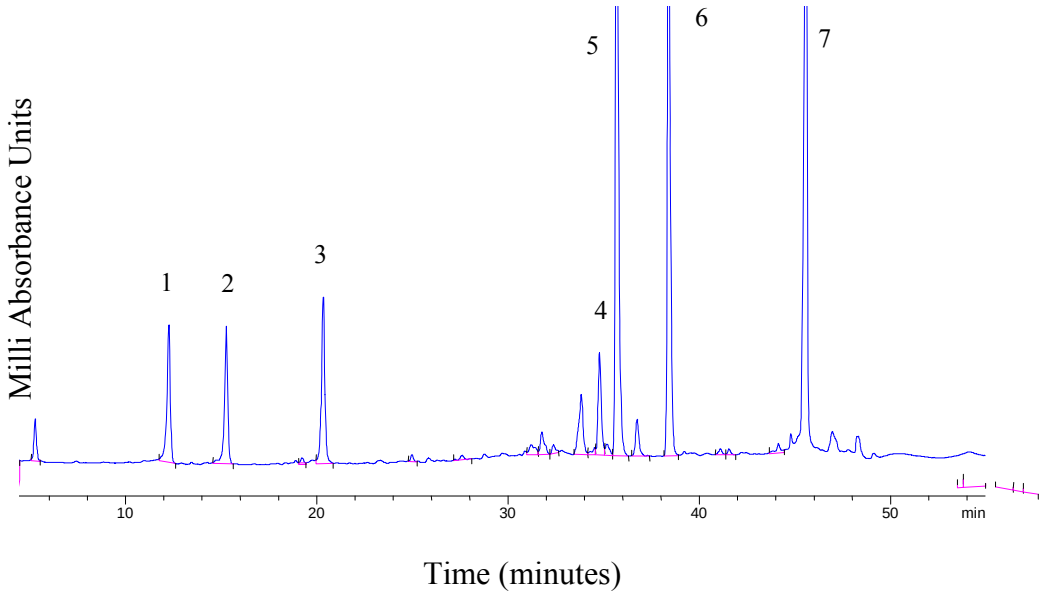
At 0.05 mg/ml, fractions SD, SE, and SF showed reduced antilisterial activity compared to the higher (0.1 mg/ml) assay concentration, whereas fractions SG and SH showed no reduction in activity at this lower concentration. Fraction SD had the weakest antilisterial activity with a 1-log reduction in cell numbers occurring within 10 minutes exposure, compared to a 2-log and 3-log reduction for fractions SF and SE, respectively. Exposure of *L. monocytogenes* to fraction SD for 60 minutes resulted in a further reduction (1.5-log) in numbers. Exposure to fractions SE and SF

for 60 minutes did not result in any further reduction in numbers (i.e. cell number were reduced after 10 minutes but no further reduction in numbers was seen at 60 minutes exposure). The lack of reduction in cell numbers, exposed to fraction SE and SF suggested that the antilisterial factor in these fractions was depleted after 10 minutes as no further reduction in cell numbers was observed at 60 minutes exposure time. A different mechanism of action may be occurring with SD as a further reduction in cell numbers was observed after 60 minutes. This may be an indication of the importance of oligomer size and/or oligomer concentration for the antilisterial effect of seed polymers. The fractions containing the largest oligomer size, SG and SH, had the highest activity, further suggesting that oligomer size could be a critical factor in the antilisterial activity of grape seed polymeric phenolics. Schofield *et al*., (2001) suggested that the antimicrobial activity of tannin was determined by the molar content and spatial configuration of the *ortho*-dihydroxyphenolic groups. The higher number of *ortho*-dihydroxyphenolic groups in the larger grape seed oligomers may confer greater antilisterial activity to fractions SG and SH. Jayaprakasha *et al*., (2003) reported the antimicrobial activity of two fractions of grape seed extract. The authors found that the fraction containing a higher proportion of larger oligomers (tetramers to nonamers) had greater antimicrobial activity compared to the fraction containing lower levels of these oligomers and higher levels of smaller oligomers (dimers and trimers). However, the authors gave no explanation for their finding.

4.4.9 Thiolysis of the Six Grape Seed Polymeric Phenolic Fractions

The optimal thiolysis reaction time for the polymeric phenolic, isolated from commercial grape juice (batch 2), was found to be 16 hours (section 4.4.1.3). The six polymeric phenolic seed fractions (SC through SH) were thiolysed using to this optimal time and the set of conditions outlined in section 4.3.1.3 (0.2 M HCl, 5% benzylmercaptan).

A chromatograph of the thiolysis products of the polymeric phenolic fraction of *Vitis vinifera* var. Ribier grape seed is shown in **Figure 4.21**.



1 = catechin; 2 = epicatechin; 3 = epicatechin-3-*O*-gallate; 4 = thioether catechin; 5 = thioether epicatechin; 6 = thioether epicatechin-3-*O*-gallate; 7 = excess benzylmercaptan.

Figure 4.21 Typical HPLC spectra of terminal and extension (benzylthioether derivatives) units of Ribier grape seed polymeric phenolic material released after thiolysis (16 hours, 60°C, 0.2 M HCl, 5% benzylmercaptan). Peak detection wavelength 280 nm.

The grape seed polymeric fractions were analysed for the presence of catechin and epicatechin using standard retention time and for the benzylthioether extension units epicatechin and epicatechin-3-*O*-gallate by comparison to retention times determined from the grape juice polymeric fraction (section 4.4.5). To allow for the additional absorbance of the gallate moiety present in epicatechin-3-*O*-gallate and thioether epicatechin-3-*O*-gallate, a response factor was calculated by multiplying the absorbance of epicatechin-3-*O*-gallate with the ratio of the extinction coefficients of epicatechin (ε max 3580 280 nm) to epicatechin-3-*O*-gallate (ε max 14000 279 nm) (Vuataz *et al*., 1959) i.e. a ratio of 0.256. This ratio was also assumed to be the same for thioether epicatechin and thioether epicatechin-3-*O*-gallate, however to this researchers knowledge this information was not available in the literature. The mean degree of polymerisation (mDP) was

determined for each fraction by calculating the ratio (based on HPLC mAU peak area) of terminal catechin plus thioether epicatechin extension units to terminal catechin units. The mDP and basic composition of the six polymeric phenolic fractions are listed in **Table 4.11**.

The composition of the grape seed polymeric fractions was similar to that of the grape juice polymeric fractions (section 4.4.4.1). Catechin, epicatechin, and epicatechin-3-*O*-gallate were found as terminal units and catechin was more abundant as a terminal unit than epicatechin. Catechin, epicatechin and epicatechin-3-*O*-gallate were found as extension units (thioether products). These results are similar to those reported in the literature (Prieur *et al*., 1994; Saucier *et al*., 2001; Kennedy & Taylor, 2003).

71.1 Table 4.11 Composition and mDP of polymeric phenolic seed fractions determined by HPLC following thiolysis reaction.

	Peak area on HPLC (mAU)*				
	Terminal Units		**Extension Units**		
Fraction	Catechin	Epicatechin	Epicatechin	Epicatechin-3-*O*-gallate	mDP
SC	186	16	610	160	4.3
SD	117	38	258	49	3.2
SE	270	69	821	70	4.0
SF	266	41	1456	80	6.5
SG	203	30	1731	76	9.5
SH	217	28	1974	64	10.1

*Peak detection 280 nm.

Kennedy & Taylor (2003) analysed grape seed condensed tannin by thiolysis and reported the presence of the terminal units, catechin, epicatechin, and epicatechin-3-*O*-gallate and their corresponding thioether products. Kennedy & Taylor (2003) found no epigallocatechin extension unit present in grape seed. Prieur *et al*., (1994) and Saucier *et al*., (2001) also reported that grape seed condensed tannin contained only procyanidin polymers (catechin, epicatechin, and epicatechin-3-*O*-gallate) but no prodelphinidin polymers (gallocatechin and epigallocatechin). This

researcher suggests that this would also be the case for the grape seed polymeric fractions investigated in this thesis, even though the detection of epigallocatechin and gallocatechin (prodelphinidins) was not included in the analysis.

The mean degree of polymerisation of the grape seed fractions under investigation ranged from 3.2 to 10.1 (**Table 4.11**). Prieur *et al*., (1994) reported the mean degree of polymerisation of five grape seed (*Vitis vinifera*) condensed tannin fractions ranged from 2.3 to 15.1 and Labarbe *et al*., (1999) reported procyanidin fractions (*Vitis vinifera* cv. Cabernet franc) with mDPs ranging from 4.7 to 15.7. Saucier *et al*., (2001) reported mDPs of six fractions of grape seed condensed tannin, isolated from *Vitis vinifera* var. Merlot, ranging from 3.4 to 9.4 with the total unfractionated extract having a mDP of 3.4. The mean degree of polymerisation results of the Ribier grape seed fractions in this thesis was in the same size range as grape seed tannins reported by Saucier *et al*., (2001).

Overall, there was a relationship between elution order from the Sephadex LH20 matrix, increasing mDP, and increasing antilisterial activity. The exception was fraction SC which had lower antilisterial activity but a higher mDP than SD and SE. The most inhibitory of the fractions, SG and SH, had the highest mDP with 9.5 and 10.1, respectively.

When compared to the grape juice polymeric fractions the grape seed fractions were more active on a weight by weight basis. At a concentration of 0.05 mg/ml, the commercial grape juice polymeric fractions (E, F, and G) had very little antilisterial activity (**Figure 4.9**) compared to the strong antilisterial activity of seed fraction SG and SH at the same concentration (0.05 mg/ml) (**Figure 4.20**). When compared on a mean degree of polymerisation basis, the grape juice condensed tannins had a slightly lower mDP range of 2.8 to 8.5 (**Table 4.7**) relative to the grape seed fractions range of 3.2 to 10.1 (**Table 4.11**). Lazarus *et al*., (2003) reported that A-type proanthocyanidins are resistant to degradation by thiolysis and the lower mDP of the grape juice fractions compared to the seed fractions may have been due to the presence of A-type proanthocyanidins in the juice fractions. In contrast to the grape seed tannin fractions, the grape juice tannin fraction with the highest mDP (fraction H with a mDP of 8.5) did not have the highest antilisterial activity. This researcher proposes that chemical composition in combination with condensed oligomer size range may be important in conferring the antilisterial activity of both the grape juice and grape seed condensed tannin fractions. The antimicrobial activity of polyphenols may be attributed in part to the position and number of hydroxyl groups in the molecule (Ahn *et al*., 1991; Mabe *et al*., 1999; Tesaki *et al*., 1999; Ho *et al*., 2000; Friedmann *et al*., 2003). Grape seed tannins have been reported to contain 10 times as many gallate residues than grape skin tannins (the likely origin of the antilisterial factor in

the commercial grape juice) (Cheynier *et al*., 1999). This researcher proposes that the stronger inhibitory action of the grape seed tannin fractions, compared to the grape juice tannin fractions, may be due to the higher level of gallate moieties in the seed tannin. In addition, Schofield *et al*., (2001) reported that an increase in the prodelphinidin to procyanidin ratio (i.e. more procyanidin than prodelphinidin) in condensed tannin increased the ability of that tannin to complex proteins. The grape juice condensed tannin was comprised of both prodelphinidin (epigallocatechin) and procyanidin (epicatechin, epicatechin-3-*O*-gallate) oligomers whereas the seed tannin was comprised of only procyanidin oligomers. We speculate that the greater antilisterial activity of the grape seed tannin compared to the grape juice tannin may be due to the higher proportion of procyanidin oligomers, which have *ortho*-dihydroxyphenolic groups on the flavanol B ring, in seed tannin.

4.5 Summary

The antilisterial fraction isolated from commercial grape juice (batch 2) was identified as polymeric phenolic material (condensed tannin or proanthocyanidins). Elemental analysis confirmed that the fraction contained 95.9% carbon, hydrogen and oxygen with a small percentage (0.53%) of nitrogen. The ratio of carbon to hydrogen (1:1) in the fraction was typical of nature-derived phenolic compounds. Comparison of the FTIR spectra of our grape juice derived phenolic fraction with a procyanidin fraction showed a high degree of similarity between the spectra suggesting that the antilisterial polymeric phenolic fraction was condensed tannin (proanthocyanidin). FTIR analysis indicated our polymeric fraction was similar to the procyanidin (composed of catechin and epicatechin monomer units) class of condensed tannin. Fractionation of the polymeric fraction by molecular weight into eight fractions and analysis of these fractions by thiolysis revealed that the mean degree of polymerisation of the fractions ranged from 1.8 to 8.5. The fractions that had the highest antilisterial activity had a mDP range of 4.0 to 5.9, though the antilisterial activity was dispersed over six (fractions C through H) of the eight fractions. The fractions with little or no antilisterial activity (A and B) contained only monomers and dimers. The constitutive units of the most inhibitory fraction (G) were determined by mass spectroscopy and were identified as catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin. Although the fractions were red in colour no anthocyanin monomer units were detected with the thiolysis-MS analysis method. Infusion Electrospray Spectroscopy Mass Spectroscopy analysis of whole unthiolysed fraction G showed that this fraction contained a number of oligomeric proanthocyanidins with a size ranging

from trimer to nonamer. The proanthocyanidins in fraction G were composed of catechin and epicatechin subunits and gallic acid subunits. No anthocyanin was detected by thiolysis or IES-MS analysis although the fraction was red and contained 90% polymeric colour, which implies that anthocyanins were present or associated with the oligomeric structure. A search of the literature found only a single report by Remy *et al*., (2000) who detected an anthocyanin moiety (malvidin-3-glucoside) among thiolysis products of the polymeric fraction of red wine. The reason for the lack of detection of oligomers containing anthocyanins in the presence research is unclear, although this may be due to the anthocyanin moiety being linked to the oligomer via an A-type interflavanoid linkage, which is resistant to thiolysis degradation.

Determination of the percentage polymeric colour in the fractions revealed that polymeric colour ranged from 47% to 90%. When assayed for antilisterial activity, three of the eight fractions (fractions E, F, and G) showed extremely high antilisterial activity. The fraction with the highest mDP (5.9) in conjunction with the highest percentage polymeric colour (fraction G) had slightly higher antilisterial activity than the other two fractions. However, the strength of the antilisterial activity of these three fractions was found to be very similar and this suggests that antilisterial activity was likely conferred by a number of factors including percentage polymeric colour, average oligomer size (mDP) in the fraction, and concentration (μmole) of oligomers in the fraction and the composition and orientation phenolic subunits and *ortho*-hydroxyl groups within the molecule. Assay of a number of the monomer subunits (catechin, epicatechin, gallic acid, and malvidin-3-glucoside) of the polymeric fraction showed that the monomers did not have antilisterial activity. This result suggested that the polymerised state of the monomer subunits was important in conferring the antilisterial activity of the commercial grape juice and Ribier grape seed condensed tannin.

Ribier grape seed condensed tannins were also fractionated according to increasing molecular weight. The mDP of the seed fractions ranged from 3.2 to 10.1 which was slightly higher than the polymeric fractions of the commercial grape juice (batch 2). Antilisterial assay of the seed fractions revealed that antilisterial activity increased with an increase in oligomer size and when assayed at the same concentration (mg/ml) the seed fractions were more active than the grape juice fractions.

In conclusion, the antilisterial factor present in the commercial grape juice was made up, in part, of oligomeric proanthocyanidin consisting of catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin. Although anthocyanin moieties were not found by mass spectroscopy or thiolysis reaction, the fractions were coloured with the most inhibitory fractions containing high levels (up to

90%) polymeric colour, which implies that anthocyanins were probably present in the oligomer structure. The antilisterial factor from the commercial grape juice may be a combination of pigmented and non-pigmented condensed tannin (pigmented and non-pigmented proanthocyanidin oligomers) derived from the skin and seeds of Ribier grapes and is similar to if not the same as proanthocyanidins present in red wine derived from *Vitis vinifera* variety grapes and also from the skin of *Vitis vinifera* variety grapes.

5 EFFICACY OF GRAPE-DERVIED ANTILISTERIAL FACTORS AS NATURAL FOOD PRESERVATIVES

5.1 Introduction

The aim of this chapter was to assess the feasibility of the pigmented condensed tannin, isolated from commercial grape juice (batch 2), for use as a natural food preservative. *Listeria monocytogenes* is able to grow over a temperature range of -1.5^{o}C (Hudson *et al*., 1994) to 45^{o}C (Kandler & Weiss, 1986). Many food products are stored at refrigeration temperatures. However, *L. monocytogenes* is able to multiply at these temperatures. Food such as fruit and vegetables are stored at room temperature, which is also conducive to the growth of this bacterium. The inhibitory action of the grape juice tannin was assessed against *L. monocytogenes* grown at 4^{o}C, 22^{o}C, 35^{o}C, and 41^{o}C to determine whether the pigmented condensed tannin (polymeric phenolic fraction of commercial grape juice batch 2) was inhibitory over the temperature growth range of this bacterium. *L. monocytogenes* is a facultative anaerobic bacterium, able to grow under both aerobic and anaerobic conditions. Many food preservative systems use modified atmosphere packaging (MAP), which is a mixture of oxygen, carbon dioxide, and nitrogen, to inhibit the growth of pathogenic or spoilage bacteria (Szabo & Coventry, 2001). Given that *Listeria monocytogenes* is able to growth under both aerobic and anaerobic conditions these preservative systems are ineffective at controlling the growth of this bacterium. Experiments were conducted to determine whether the grape juice tannin was inhibitory to *L. monocytogenes* grown under complete anaerobic conditions or under anaerobic conditions followed by aerobic conditions. The inhibitory effect of the juice pigmented condensed tannin was also assessed against *L. monocytogenes* when in both exponential (log) and stationary growth phases. The inhibitory activity of the pigmented condensed tannin isolated from commercial grape juice (batch 2) was assessed for activity against cabbage contaminated with *L. monocytogenes*. The Ribier grape seed condensed tannin was also assessed for inhibitory action against cabbage contaminated with *L. monocytogenes*. In order to determine the physical effect of the grape juice condensed tannin on *L. monocytogenes* experiments cells exposed to increased levels of the tannin were examined by electron microscopy.

5.2 Materials

72.1 Table 5.1 List of materials used in Chapter Five.

Materials	Source
AnaeroPak Anaerobic Gas Generating System	Mitsubishi Gas Chemical Company
Copper grids	Proscitech (Australia)
Epoxy resin 812	Electron Microscopy Sciences (USA)
Fluid thioglycollate medium	Difco
Glutaraldehyde	Agar Scientific (UK)
Lead acetate	BDH
Listeria selective agar base	Oxoid
Listeria selective supplement	Oxoid
Model Grape Juice (MGJ)	(see section 2.2)
0.033 M L-tartaric acid, dextra-rotatory	BDH
Model Wine Solution (MWS)	(see section 2.2)
0.033 M L-tartaric acid, dextra-rotatory	BDH
99.95% ethanol (10% final concentration)	Merck
Osmium tetroxide	Alpha Products (USA)
pH strips Panpeha Plus pH 2.0-9.0	Schneicher & Schuell
Uranyl acetate	BDH
Whirl-Pak bags	Nasco

All other materials used in this chapter have been described previously.

5.3 Methods

5.3.1 Antimicrobial Spectrum of Polymeric Phenolic Fraction

The antimicrobial spectrum of the polymeric phenolic fraction (see section 3.3.5 for preparation details) of commercial grape juice (batch 2) was assessed for five serotypes (1/2a, 1/2c, 3a, 4a, 4b) of *Listeria monocytogenes* and for a range of food-borne pathogens including a number microorganisms phylogenetically related to *Listeria* species. Bacterial cultures were grown in brain heart infusion (BHI) broth overnight at the appropriate temperature. The cultures were then diluted 1:100, or 1:10 in the case of *Bacillus cereus*, *Brochothrix thermospacta*, and *Yersinia enterocolitica* because of low cell numbers, with 0.1% sterile peptone water. Solutions of grape juice condensed tannin (polymeric phenolic fraction isolated from commercial grape juice batch 2) (3.8 ml) were

prepared by first dissolving 2 mg of the material in 400 µl 99.95% ethanol and dilution to 3 ml with sterile water. The pH was adjusted to pH 3.5 with sterile 10 mM HCl and the solution made up to a final volume of 3.8 ml with sterile water. This gave a solution containing a final concentration of 0.5 mg/ml polymeric material in 10% ethanol. The solution was then pasteurised at 63°C for 30 minutes and assayed against the bacterial isolates in **Table 5.2** according to the standard protocol (section 2.3.2). A positive control of Model Wine Solution (MWS) (0.033M tartaric acid, 10% ethanol pH 3.5) was run in parallel for each bacterial isolate.

73.1 **Table 5.2 Bacterial isolates used to determine the antimicrobial spectrum of polymeric phenolic fraction**.

Bacterial Isolate	Growth Temperature	Gram Stain
Listeria monocytogenes serotype 1/2a ATCC 35152	37°C	+
Listeria monocytogenes serotype 1/2c ATCC 19112	37°C	+
Listeria monocytogenes serotype 3a ATCC 19113	37°C	+
Listeria monocytogenes serotype 4a ATCC 19114	37°C	+
Listeria monocytogenes serotype 4b CDC KC18	37°C	+
Listeria seeligeri ATCC 35967	37°C	+
Listeria welshimeri NCTC 11857	37°C	+
Listeria innocua ATCC 33090	37°C	+
Listeria ivanovii NZ Isolate Accession no 797	37°C	+
Listeria grayi ATCC 19120	37°C	+
Brochothrix thermospacta ATCC 11509	22°C	+
Lactobacillus rhamnosus ATCC 7469	30°C	+
Bacillus cereus NCTC 8035	30°C	+
Staphylococcus aureus ATCC 25923	37°C	+
Escherichia coli ATCC 25922	37°C	-

5.3.2 Minimum Inhibitory Concentration of Polymeric Phenolic Fraction against Listeria monocytogenes

The minimum inhibitory concentration (MIC) was determined for the polymeric phenolic fraction isolated from commercial grape juice (batch 2). A dilution series of polymeric phenolic material was prepared as described in section 5.3.1. The following concentrations of polymeric phenolic fraction were prepared: 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml, with each containing 10% ethanol as a final concentration and adjusted to pH 3.5 with sterile 10 mM HCl.

The solutions were pasteurised at 63°C for 30 minutes and assayed against *L. monocytogenes* ATCC 35152 according to the standard protocol (section 2.3.2).

5.3.3 Effect of Listeria monocytogenes Growth Conditions on the Antilisterial Activity of Polymeric Phenolic Fraction

Prior to assay, *Listeria monocytogenes* was grown at three different incubation temperatures, at mid-log growth phase, and under anaerobic conditions to determine the effect of *L. monocytogenes* growth conditions on the antilisterial activity of the polymeric phenolic fraction and to assess the feasibility of this material to be used as a natural food preservative to eliminate *L. monocytogenes* from food.

5.3.3.1 Effect of Listeria monocytogenes Growth Temperature

Listeria monocytogenes is a psychrotolerant bacterium able to grow over a range of temperatures from -1.5°C (Hudson *et al*., 1994) to 45°C (Kandler & Weiss, 1986). The optimum growth temperature for *L. monocytogenes* is 37°C and all previous experimental work was performed on *L. monocytogenes* grown at this temperature. The polymeric phenolic fraction isolated from commercial grape juice was assessed for antilisterial activity against *L. monocytogenes* grown at 4°C, 22°C, and 41°C using the standard protocol (section 2.3.2). Three broth cultures of *L. monocytogenes* were prepared by inoculating 10 ml of BHI broth with four to five colonies of *L. monocytogenes* taken from a horse blood agar plate. Broth cultures of *L. monocytogenes* were grown at 4°C for 9 days and at 22°C and 41°C for 16 hours until turbidity was observed. The broth cultures were then assayed against 1 mg/ml concentration polymeric phenolic fraction containing 10% ethanol pH 3.5 (prepared as described in section 5.3.1). Spread plates were incubated at 37°C for 24 hours. Controls consisting of Model Wine Solution (MWS) (0.033 M L-tartaric acid 10% ethanol pH 3.5) were performed in parallel.

5.3.3.2 Effect of Listeria monocytogenes Growth Phase

Bacteria have three distinct growth phases, lag phase, exponential (log) phase, and stationary phase. All previous experimental work in this thesis was performed on stationary phase *L. monocytogenes* cells. The inhibitory effect of polymeric phenolic fraction was assessed against *L. monocytogenes* grown to mid-log phase. The growth phase of *L. monocytogenes* was determined according to the protocol of Meylheuc *et al*., (2002). A 10 ml volume of BHI, equilibrated at 37°C, was inoculated with 100 µl of an overnight culture of *L. monocytogenes* grown in BHI broth as described in section 2.3.1.1. As the bacterial cells multiply the absorbance of the culture increases and this can be

measured using a spectrophotometer. The absorbance of the freshly inoculated broth culture (diluted 1:5 with reverse osmosis water) was measured at 600 nm every 30 minute on a Cary 50 Conc UV-visible spectrophotometer (Varian) until mid-log phase was reached (7 hours incubation). The culture was diluted (2 ml water to 0.5 ml culture) 1:5 prior to reading the optical density. The cells were assayed against 1 mg/ml concentration of polymeric phenolic fraction (prepared as described in section 5.3.1) according to the standard protocol (section 2.3.2). A control consisting of Model Wine Solution (MWS) (0.033 M L-tartaric acid 10% ethanol pH 3.5) was performed in parallel.

5.3.3.3 Effect of Anaerobically Grown Listeria monocytogenes

Listeria monocytogenes is able to grow both aerobically and anaerobically. All previous experimental work in this thesis was performed on aerobic *L. monocytogenes* cultures. The inhibitory effect of polymeric phenolic fraction was assessed against an anaerobic culture of *L. monocytogenes* in two parts. Firstly, a broth culture of *L. monocytogenes* was grown under anaerobic conditions prior to assay and the spread plates of the polymeric phenolic exposed cells were grown aerobically after assay. This part was designated anaerobic/aerobic. In the second part of the experiment, a broth culture of *L. monocytogenes* was grown anaerobically prior to assay and the spread plates of the polymeric phenolic exposed cells were also grown under anaerobic conditions. This was designated anaerobic/anaerobic.

A culture of *L. monocytogenes* grown under anaerobic conditions was prepared as follows. Four to five colonies of *L. monocytogenes* were inoculated in 10 ml of anaerobic fluid thioglycollate medium and incubated aerobically overnight at 37^{o}C. Growth was observed as flocculate and checked for purity by gram staining a sample of the culture. A 100 µl aliquot of the broth culture was sub-cultured into 20 ml of fluid thioglycollate media (the culture vessel contained no headspace in order to reduce oxygen diffusion) and incubated in an anaerobic chamber containing an anaeropak, thus generating an anaerobic environment (20 % CO_2, residual O_2 less than 0.1%) overnight at 37^{o}C. The anaerobically grown cells were assayed in duplicate against 1 mg/ml concentration of polymeric phenolic fraction (10% ethanol pH 3.5) (prepared as described in section 5.3.1) according to the standard protocol (section 2.3.2). After spread plating the polymeric phenolic exposed cells onto TSA plates one set of plates (including model wine solution controls) was incubated aerobically according to the standard protocol (section 2.3.2). These were designated anaerobic/aerobic. The second set of spread plates, designated as anaerobic/anaerobic, was incubated in an anaerobic chamber containing an anaeropak (including model wine solution

controls) at 37°C for 48 hours. Bacterial colonies on the agar plates were counted according to the standard protocol (section 2.3.2)

5.3.4 Antilisterial Activity of Polymeric Phenolic Fraction against Listeria monocytogenes Contaminated Cabbage

Previous experimental work showed that the antilisterial activity of the polymeric phenolic fraction of the commercial grape juice was limited to acidic pH values (refer sections 2.4.8 and 3.4.5). Also, given that the antimicrobial factor has been identified as condensed tannin which is known to precipitate with protein (section 3.4.2) (Bate-Smith 1973; Haslam, 1974), cabbage (*Brassica oleracea* var. Capitata), which is intrinsically low in protein and has a naturally acidic pH, was selected for assay. In addition, cabbage was identified as the vehicle of transmission of *L. monocytogenes* in an outbreak of listeriosis in Canada in 1981 (Schlech *et al*., 1983).

The antilisterial activity of the polymeric phenolic fraction, isolated from commercial grape juice (batch 2), was assessed on cabbage (*Brassica oleracea* var. Capitata), purchased from an Auckland, New Zealand supermarket, inoculated with *L. monocytogenes* following a modified method of Zhang and Farber (1996). As the antilisterial activity of the commercial grape juice (section 2.4.8) and the polymeric phenolic fraction was found to be pH dependent (section 3.4.5), the pH of the cabbage was determined by blending 10 g of cabbage with 20 ml of reverse osmosis water into a slurry and measuring the pH (Meterlab pH meter, Radiometer). To perform the experiment, the cabbage was cut into thin strips resembling coleslaw with a sterilised knife, and 1 gram samples were placed in sterile containers. The following cabbage samples were prepared in duplicate: control 10 minutes, control 1 hour, control 5 hours, control 24 hours, treatment 10 minutes, treatment 1 hour, treatment 5 hours, and treatment 24 hours. An overnight culture of *L monocytogenes* was diluted 1:1000 with sterile 0.1% peptone water to give a concentration of 10^7 CFU/ml. A 0.5 ml aliquot of *L. monocytogenes* was added to the 1 gram cabbage samples to give a final concentration of 10^6 CFU/g. The containers were then sealed with a screw top and shaken 30 times to evenly distribute the bacterial cells over the surface of the cabbage. The contaminated cabbage samples were stored at 4°C for 24 hours to allow attachment of the bacteria cells to the cabbage.

A 5 ml aliquot of polymeric phenolic fraction (0.5 mg/ml prepared as described in section 5.3.1) or 5 ml control Model Wine Solution (MWS) (pH 3.5) was added to duplicate cabbage samples, ensuring the cabbage was submerged in the solution. The submerged cabbage samples were then incubated at 20°C for 10 minutes, 1 hour, 5 hours, and 24 hours, respectively. After the required incubation time the polymeric phenolic material and the control model wine solution (MWS) were

decanted off and each 1 gram sample of cabbage aseptically placed into individual WHIRL bags with 10 ml 0.1% sterile peptone water. The bag containing the contaminated cabbage and peptone water was shaken 10 times. For enumeration of *L. monocytogenes*, the samples were pummelled in a stomacher (Seward) for 45 seconds. A dilution series was prepared in 0.1% peptone water and spread plated onto Listeria selective media. The plates were incubated for 48 hours and *L. monocytogenes* colonies were counted. The pH of polymeric phenolic fractions and the model wine solution controls was measured with pH strips before and after addition to the contaminated cabbage.

5.3.4.1 Antilisterial Activity of Ribier Grape Seed Polymeric Phenolic Fraction against Listeria monocytogenes Contaminated Cabbage

Previous experimental work revealed that the polymeric phenolic fraction isolated from Ribier grape seeds had antilisterial activity (section 3.4.3). Unlike the grape juice polymeric phenolic fraction, the activity of the grape seed polymers was not dependent on pH and so the pH of the seed polymer exposed cabbage was not determined for this experiment. The antilisterial activity of the grape seed polymeric phenolic fraction was assayed against cabbage (*Brassica oleracea* var. Capitata) contaminated with 10^6 CFU/g of *L. monocytogenes* cells as described in section 5.3.4. The cabbage samples were prepared as described in section 5.3.4 and the contaminated cabbage samples were then immersed in 5 ml of grape seed polymers (0.5 mg/ml concentration) and assayed as described in section 5.3.4.

5.3.5 Transmission Electron Microscopy of Listeria monocytogenes Treated with Polymeric Phenolic Fraction

The physical effect of the polymeric phenolic fraction, isolated from the commercial grape juice (batch 2), on *L. monocytogenes* was assessed by examining the treated cells by transmission electron microscopy (TEM). An overnight culture of *L. monocytogenes* was grown in BHI broth as described (section 2.3.1.1). A 1 ml aliquot of cells (10^8-10^9 CFU/ml) was harvested by centrifugation at 5 G (5000 rpm) for 10 minutes (Biofuge pico, Heraeus). Cells were washed twice with 1 ml of sterile reverse osmosis water with pelleting between washing. The washed cell pellet was suspended in 1 ml of pasteurised polymeric phenolic fraction (10% ethanol pH 3.5) at the

following polymeric phenolic fraction concentrations: 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2 mg/ml. A control of untreated cells was suspended in 1 ml of sterile reverse osmosis water. Cells were incubated at 20^{o}C for 1 hour. The inhibitory action of each concentration of polymeric phenolic fraction was determined against *L. monocytogenes* in a previous experiment (section 5.4.2). Whole and thin sections of polymeric phenolic treated and untreated cells were examined (described in sections 5.3.5.1 and 5.3.5.2) using a Transmission Electron Microscope Phillips Tecnai 12 (Eindhoven, The Netherlands).

5.3.5.1 Whole Cell TEM

Whole cells (both treated and untreated cells) were prepared for TEM. A pre-charged copper specimen grid was contacted with a 20 µl droplet of cell suspension and left to sit at 20^{o}C for 2 minutes. The grid was subsequently transferred to two sequential 20 µl droplets of sterile reverse osmosis water for 5 seconds. The grid with bacterial cells now adhered was negatively stained with uranyl acetate (2% aqueous**)** for 20 seconds. Excess uranyl acetate was blotted off with filter paper and the grid examined by TEM.

5.3.5.2 Thin Section TEM

The treated and untreated cell suspensions were pelleted as described (section 5.3.5). Each cell pellet was fixed for 3 hours using 1 ml of a 3% glutaraldehyde in 0.1 M Sörensens phosphate buffer. The pellet was then washed (3 x 10 minutes) with 1 ml of 0.1 M Sörensens phosphate buffer. The pellet was then post-fixed with a 0.5 ml solution of 1% osmium tetroxide in 0.1 M Sörensens phosphate buffer for 1 hour. The pellet was dehydrated for 10 minutes in 1 ml aliquots of the following: 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 2 aliquots of 100% ethanol, and 100% acetone. The pellet was centrifuged at 10,000 rpm for 1 minute between dehydration steps. The pellet was infiltrated with 0.5 ml of a 1:1 ratio of 812 epoxy resin: acetone for 1 hour. The resin:acetone mix was removed and replaced with 0.5 ml of 100% 812 epoxy resin and incubated at 20^{o}C for 16-24 hours. The resin was removed and replaced with fresh 0.5 ml of 100% 812 epoxy resin and the resin was cured at 60^{o}C for 48 hours. The resin embedded pellet was sectioned with a diamond knife on a Ultrotome Nova ultra-microtome (LKB, Sweden) to 70 nm thickness. The thin sections were then placed on a copper grid and stained with uranyl acetate and lead citrate and examined by TEM.

5.4 Results and Discussion

5.4.1 Antimicrobial Spectrum of Polymeric Phenolic Fraction

The antimicrobial spectrum of polymeric phenolic fraction isolated from the commercial grape juice (batch 2) showed a similar inhibitory trend to that of the commercial grape juice (batch 1) (section 2.4.2). All *Listeria* species assayed were inhibited by the polymeric phenolic fraction at a concentration of 0.5 mg/ml (**Table 5.3)**. *Listeria* isolates were reduced from 10^6 CFU/ml to no colonies detected within 60 minutes exposure. The exception was *L. welshimeri*, which was reduced from 10^6 CFU/ml to 30 CFU/ml. As stated previously the limit of detection of the assay is 10 CFU/ml or 1-log so a count of 30 was still extremely low and indicated that the polymeric phenolic fraction was inhibitory to *L. welshimeri*, though not as inhibitory as other *Listeria* species assayed. *L. welshimeri* is classified, according to the Bergey's manual (Kandler & Weiss, 1986), under the same sub-group as the other *Listeria* species assayed and as such no reason for the slight resistance of this bacterium was apparent.

Other bacterial isolates assayed were not inhibited by exposure to the polymeric phenolic fraction with the exception of *B. cereus* and *B. thermospacta*. *B. cereus* cell numbers were reduced by 1-log (10^5 reduced to 10^4) after 60 minutes exposure. This result was comparable to that observed for the commercial grape juice (section 2.4.2). *B. cereus* appeared to have some sensitivity to the polymeric phenolic fraction and the commercial grape juice (batch 1) as a small proportion of the cells was inhibited by the antilisterial factor present in these solutions. It is possible that the inhibitory factor in these solutions was not present in high enough concentration to inhibit *B. cereus* but was high enough to inhibit *Listeria* species. Ahn *et al*., (2004) found that a commercial grape seed extract (ActiVin) was more inhibitory to *L. monocytogenes* than *E. coli* and *Salmonella* Typhimurium although the authors did not suggest a reason for their finding.

74.1 Table 5.3 Antimicrobial spectrum of polymeric phenolic fraction (0.5 mg/ml) isolated from commercial grape juice (batch 2).

	Exposure time (minutes)		
	0	10	60
Microorganism	Colony Forming Units/ ml		
Listeria monocytogenes serotype 1/2a	$3x10^6$	<10	<10
Listeria monocytogenes serotype 1/2c	$1x10^6$	<10	<10
Listeria monocytogenes serotype 3a	$1x10^6$	<10	<10

Listeria monocytogenes serotype 4a	$6x10^5$	<10	<10
Listeria monocytogenes serotype 4b	$3x10^6$	<10	<10
Listeria seeligeri	$7x10^5$	<10	<10
Listeria welshimeri	$1x10^6$	130	30
Listeria innocua	$2x10^6$	<10	<10
Listeria ivanovii	$4x10^6$	<10	<10
Listeria grayi	$2x10^5$	<10	<10
Brochothrix thermospacta	$3x10^5$	$3x10^4$	$7x10^2$
Lactobacillus rhamnosus	$5x10^5$	$1x10^6$	$1x10^6$
Bacillus cereus	$2x10^5$	$2x10^4$	$2x10^4$
Staphylococcus aureus	$6x10^5$	$6x10^5$	$1x10^5$
Escherichia coli	$2x10^6$	$2x10^6$	$2x10^6$
Salmonella Menston	$2x10^6$	$2x10^6$	$2x10^6$
Yersinia enterocolitica	$6x10^6$	$6x10^6$	$7x10^6$

B. thermospacta is a gram-positive food-spoilage bacterium able to grow at low temperatures (4°C). The *Brochothrix* genus and the *Listeria* genus are classified together under catalase-positive facultative anaerobes in the Bergey's manual (Kandler & Weiss, 1986) meaning these genera are more similar to each other, in terms of taxonomy (morphology, gram reaction, nutritional classification, cell-wall chemistry, nutritional requirements, temperature growth range, fermentation products generated from growth, and pH requirements) than any other bacterial species. *B. thermospacta* cell numbers were reduced by 1-log (10^5 reduced to 10^4 CFU/ml) after 60 minutes exposure to the polymeric phenolic fraction. Like *B. cereus*, *B. thermospacta* was also slightly sensitive to the inhibitory action of the grape juice polymeric phenolic fraction. *B. thermospacta* may also require a higher concentration of the inhibitory factor to completely inhibit this bacterium over the assay time period. *Lactobacillus rhamnosus* is also phylogenetically similar to *Listeria* species and is classed in the Bergey's Manual as a catalase-negative facultative anaerobe. This organism was not sensitive to the antimicrobial action of polymeric phenolic with no reduction in cell numbers observed over 60 minutes exposure. All other food-borne pathogenic bacteria assayed *(S. aureus, E. coli, Salmonella* Menston, and *Y. enterocolitica*) were not reduced in number by

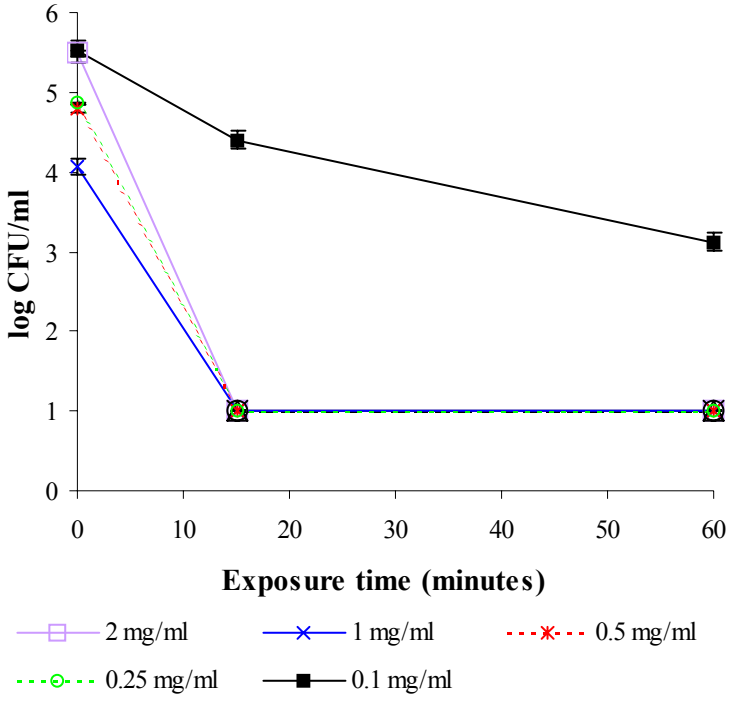
exposure to the polymeric phenolic fraction. The positive control Model Wine Solution (MWS) (0.033 M L-tartaric acid, 10% ethanol, pH 3.5) was not inhibitory to any of the bacteria assayed. The results of this experiment indicated that *Listeria* species are more susceptible to the antimicrobial effects of condensed tannin (polymeric phenolic material) than any other bacteria assayed.

The antimicrobial spectrum of the polymeric phenolic fraction is comparable to the antimicrobial spectrum of the commercial grape juice (batch 1) (section 2.4.2) with *Listeria* species being completely inhibited but other bacterial species being resistant. The exceptions are *B. cereus* and *B. thermospacta*, which were slightly inhibited. This result also indicated that no significant changes in the antilisterial factor present in the grape juice (batch 2) occurred during the grape juice fractionation process.

There are 13 serotypes of *Listeria monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) (Farber & Peterkin, 1991). Most food borne outbreaks of listeriosis are caused by serotype 4b but sporadic cases have been linked to serotypes 1/2a and 1/2b and the majority of *L. monocytogenes* isolated from foods belong to serogroup 1 (Gilot *et al*., 1996). All *L. monocytogenes* serotypes (1/2a, 1/2c, 3a, 4a, and 4b) assayed were inhibited by polymeric phenolic fraction indicating it could be applied as a natural food preservative to foods contaminated with *L. monocytogenes*, including serotypes 1/2a and 4b which have been linked to food borne listeriosis.

5.4.2 *Minimum Inhibitory Concentration of Polymeric Phenolic Fraction against Listeria monocytogenes*

The minimum inhibitory concentration (MIC) of the polymeric phenolic fraction against *L. monocytogenes* ATCC 35152 was between 0.1 and 0.25 mg/ml (**Figure 5.1**). At all concentrations above 0.2 *nocytogenes* numbers from 10^6 CFU/ml t r concentration of 0.1 mg/ml the inhibitory by 1-log within 10 minutes and by a furth entration the overall reduction in *L. monocy*



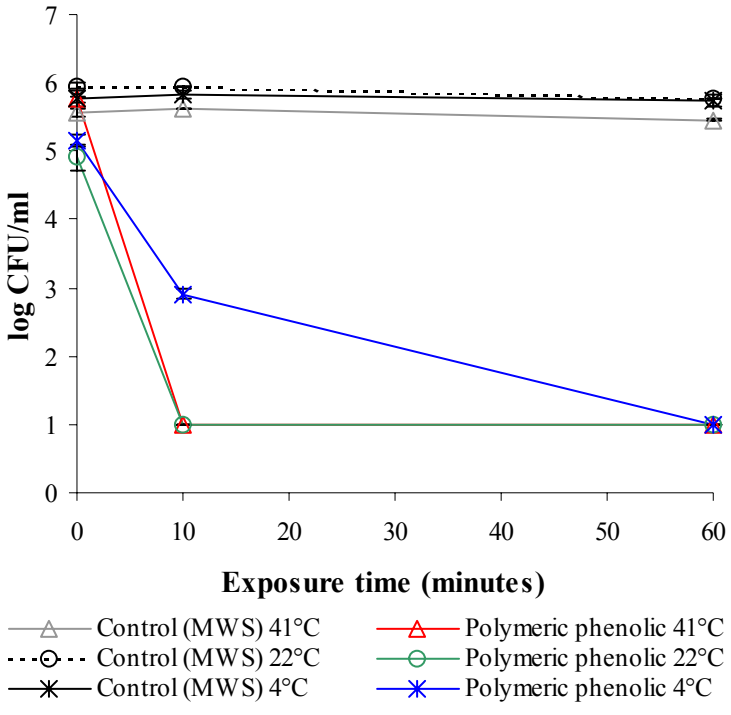
75.1 **Figure 5.1 Minimum inhibitory concentration of the polymeric phenolic fraction (isolated from commercial grape juice batch 2) against *Listeria monocytogenes* ATCC 35152.** Error bars are the ± standard deviation of mean log CFU/ml.

5.4.3 Effect of Listeria monocytogenes Growth Conditions on the Antilisterial Activity of Polymeric Phenolic Fraction Isolated from Commercial Grape Juice

5.4.3.1 Effect of Listeria monocytogenes Growth Temperature

Listeria monocytogenes is able to grow over temperatures ranging from -1.5°C (when associated with meat) (Hudson *et al*., 1994) to 45°C (Kandler & Weiss, 1986). The efficacy of the antilisterial activity of the polymeric phenolic fraction, isolated from commercial grape juice batch 2 (1 mg/ml concentration), was assessed against broth cultures of *L. monocytogenes* ATCC 35152 grown at a 4°C, 22°C, and 41°C.

L. monocytogenes growth temperature had little effect on the antilisterial activity of the polymeric phenolic fraction (**Figure 5.2**). Cells grown at 22°C and 41°C, prior to exposure to the polymeric phenolic fraction, remained sensitive to the inhibitory effect of the polymeric phenolic fraction. Cell numbers were reduced from 10^6 CFU/ml to no colonies detected within 60 minutes exposure to the polymeric phenolic fraction. This result was comparable to the reduction in cell numbers observed for *L. monocytogenes* ATCC 35152 grown at the optimal growth temperature of 37°C (serotypes 1/2a section 5.4.1). In contrast, cells grown at 4°C for 9 days, prior to exposure to the



polymeric phenolic fraction, showed a slight initial resistance to the inhibitory action with cell numbers reduced from 10^6 CFU/ml to 10^3 CFU/ml within 10 minutes; a 3-log decrease in cell number reduction compared with the other temperatures (22°C and 41°C). However, after 60 minutes exposure the 4°C cells were reduced to no detectable colonies, which is equivalent to the results observed at the higher temperatures.

76.1 **Figure 5.2 Effect of *Listeria monocytogenes* growth temperature on the antilisterial activity of polymeric phenolic fraction (1 mg/ml) (isolated from commercial grape juice batch 2).** MWS = model wine solution (0.033 M L-tartaric acid, 10% ethanol, pH 3.5). Error bars are the ± standard deviation of mean log CFU/ml.

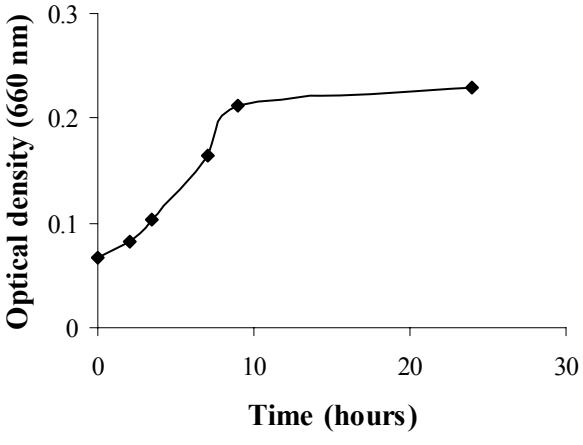
The reduction in the speed of the antilisterial activity against *L. monocytogenes* grown at 4°C (cold-adapted cells) could have been due to a change in the fatty acid composition of the cell cytoplasmic membrane. In *Listeria* species the most common fatty acids present in the cytoplasmic membrane are C_{16} and C_{18} chain length fatty acids (Püttmann *et al*., 1993). When *Listeria* cells are exposed to low temperatures (sub-optimal growth temperature), they adapt by altering the fatty acid composition of the cytoplasmic membrane. Püttmann *et al*., (1993) found that when *Listeria* was grown at 4°C the bacteria was able to change the relative composition of the fatty acids and produce more C_{16} fatty acids and less C_{18} fatty acids, with 75% of all fatty acids present being C_{16} type. The authors suggested that this adaption to low growth temperatures (cold-adaption) would increase the fluidity of the bacterial cytoplasmic membrane thereby countering the effect of sub-optimal growth temperature. Li *et al*., (2002) also reported an increase in *L. monocytogenes* membrane fluidity when grown at 10°C, compared to 30°C, which was due to increased amounts of shorter, branched-chain fatty acids. The authors reported that *L. monocytogenes* cells grown at 10°C were more sensitive to the inhibitory activity of nisin, an antimicrobial peptide, than cells grown at 30°C and suggested that the increased membrane fluidity of the cold-adapted cells made the cytoplasmic membrane more sensitive to nisin binding. In contrast to the results reported by Li *et al*., (2002), the results of this thesis research showed that the polymeric phenolic fraction had a more rapid inhibitory action against *L. monocytogenes* grown close to optimal growth temperature of 37°C (22°C and 41°C) than for sub-optimal temperatures (4°C) which suggested that bacterial membrane fluidity may not be important for antilisterial activity of the polymeric phenolic fraction. This thesis research finding suggested that cold-adaptation might confer slight resistance of *L. monocytogenes* to the inhibitory action of the polymeric phenolic fraction. However, the complete inhibition of cold-adapted cells after 60 minutes suggested that any resistance conferred may be short-lived and

not significant in terms of the antilisterial activity of this material against *L. monocytogenes* grown at low temperatures.

The control of *L. monocytogenes* is of particular concern with respect to food safety as outbreaks of listeriosis have been linked to refrigerated foods (Schlech *et al*., 1983). The enrichment of *L. monocytogenes* in foods at refrigeration temperatures (4°C) has been well documented. Walker *et al*. (1990) reported the growth of *L. monocytogenes* at -0.2 to 9.3°C in chicken broth and UHT milk. At low temperatures cell numbers were able to reach 8-log CFU/ml over a storage time of less than 30 days. Hudson *et al*., (1994) reported the increase in *L. monocytogenes* numbers to 8-log on roast beef packaged under vacuum at both -1.5°C (8-log reached 62 days) and 3°C (8-log reached 20 days). Samelis *et al*., (2002) reported an increase in *L. monocytogenes* numbers on frankfurters from 10^4 CFU/ml to 10^8 CFU/ml at 4°C over a period of 40 days. These reports suggested that modified atmosphere packaging and/or low temperature storage are not sufficient to eliminate *L. monocytogenes* from a variety of foods. Given that the infectious dose to cause listeriosis is unknown and that *L. monocytogenes* has been shown to increase in numbers at 4°C, the control or elimination of *L. monocytogenes* in food types stored at these temperatures may require the addition of antimicrobial agents. The polymeric phenolic fraction isolated in this thesis had antilisterial activity over the temperature growth range (4°C to 41°C) of *L. monocytogenes* and might be useful in eliminating or preventing the growth of this bacterial pathogen in foods stored at a range of temperatures.

5.4.3.2 Effect of Listeria monocytogenes Growth Phase

The polymeric phenolic fraction under investigation in this chapter completely inhibited stationary phase *L. monocytogenes* ATCC 35152 serotype 1/2a (section 5.4.1). The efficacy of the antilisterial activity of the polymeric phenolic fraction (1 mg/ml) was assessed against *L. monocytogenes* ATCC 35152 grown to mid-log (exponential) phase at 37°C. When grown in BHI broth at 37°C, *L. monocytogenes* reached the mid-log (exponential) growth phase after 7 hours incubation (**Figure**



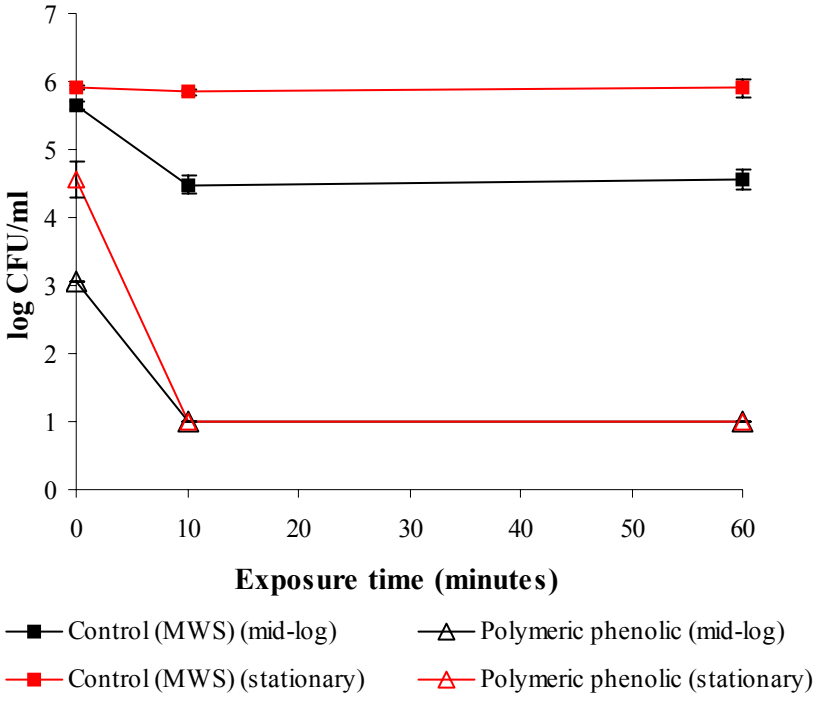
5.3), which is in agreement with the data reported by Meylheuc *et al*., (2002).

stationary phase

mid-log phase

77.1 **Figure 5.3** ***Listeria monocytogenes*** **ATCC 35152 growth curve in BHI broth at 37°C (diluted 1:5).**

Assay of mid-log phase cells against the polymeric phenolic fraction revealed that the inhibitory action was more rapid against cells in mid-log phase than cells in the stationary phase of growth (**Figure 5.4**). An initial 3-log reduction in log-phase cell numbers at time 0 was observed with a 6-log reduction occurring after 60 minutes exposure to the polymeric phenolic fraction. This is in contrast to a 0.5-log reduction in stationary phase cells at time 0. The antilisterial polymeric phenolic fraction showed a more rapid initial reduction in log phase cells compared to stationary phase cells and it is reasonable to assume that all viable log-phase cells may be eliminated within 2 to 3 minutes at this rate of reduction. In both phases of growth, the polymeric phenolic fraction reduced cell numbers from 6-log to no colonies detected within 10 minutes exposure. The control model wine solution (MWS) (0.033 M L-tartaric acid 10% ethanol pH 3.5) was also slightly inhibitory to log-phase cells with a 1-log reduction in cells occurring after 10 minutes exposure but no further reduction in cell numbers after 60 minutes. The MWS was not inhibitory to stationary



phase cells. This result indicated that similar to other bacteria *Listeria monocytogenes* is more susceptible to antimicrobial compounds, even the combination present in the MWS (0.033 M L-tartaric acid, 10% ethanol, and acidic pH 3.5), when in log phase of growth compared to stationary phase.

78.1 **Figure 5.4 Antilisterial activity of the polymeric phenolic fraction (1 mg/ml) (isolated from commercial grape juice batch 2) against mid-log and stationary phase *Listeria monocytogenes* cultures**. Error bars are the ± standard deviation of mean log CFU/ml.

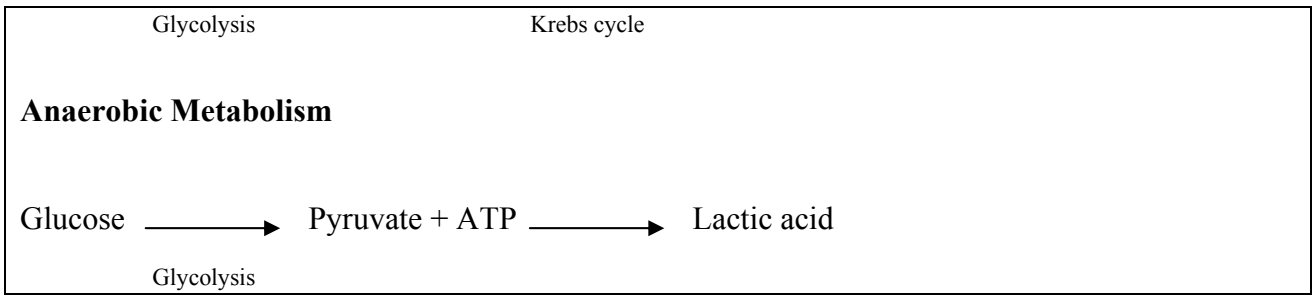
Bacterial cells in stationary phase are starved of nutrients and/or inhibited by waste accumulation. When bacteria are starved of nutrients a number of physiological changes occur that lead to an organism that is generally more resistant to a range of environmental stresses than log phase cells (Rees *et al*., 1995). This thesis result showed that the mid-log phase *L. monocytogenes* cells were more susceptible to the antilisterial effect of the polymeric phenolic fraction than stationary phase cells. Log phase cell numbers were reduced more rapidly than stationary phase cells. In conclusion, the polymeric phenolic fraction had very strong antilisterial activity against both log and stationary phase cells and could be useful in eliminating *L. monocytogenes* from both freshly contaminated foods in which *L. monocytogenes* may be rapidly multiplying, as well as foods in which *L. monocytogenes* is present but not multiplying.

5.4.3.3 Effect of Listeria monocytogenes Grown Anaerobically

Listeria monocytogenes is a facultative anaerobe and is able to grow under anaerobic conditions. Cells can grow in relatively high (e.g. 30%) CO_2 but are inhibited under 100% CO_2 conditions (Lake *et al*., 2002). Under both aerobic and anaerobic growth conditions, *L. monocytogenes* metabolises glucose to pyruvate via the Embden-Meyerhof pathway (also known as glycolysis) to pyruvate with the production of ATP. When growing aerobically pyruvate is then oxidised to CO_2 via the Krebs Cycle (Citric acid cycle) with the production of ATP. Under anaerobic growth conditions pyruvate is fermented to lactic acid (Kandler & Weiss, 1986) (**Figure 5.5**).

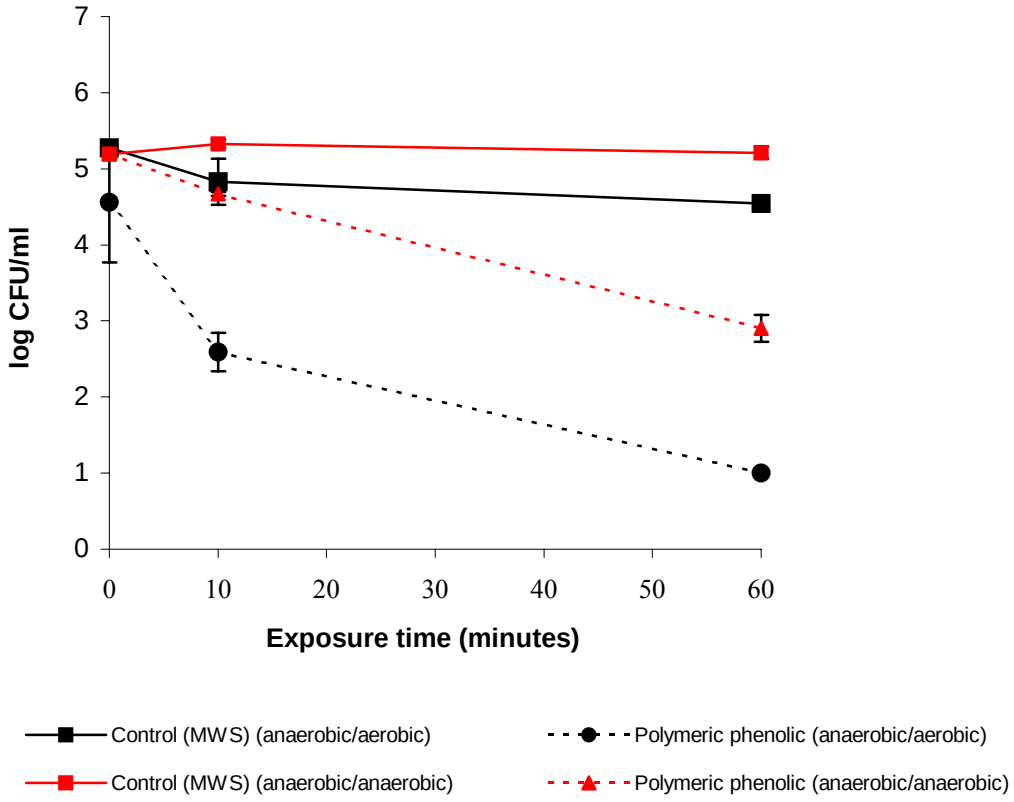
Aerobic Metabolism

Glucose ⟶ Pyruvate + ATP ⟶ CO_2 + ATP



79.1 Figure 5.5 Metabolism of glucose by *Listeria monocytogenes* under aerobic and anaerobic growth conditions.

Two sets of experiments were performed on a *L. monocytogenes* culture grown under anaerobic conditions. The experimental anaerobic conditions generated were 20% CO_2 with less than 0.1% residual O_2 which is conducive to *L. monocytogenes* growth. In the first part of the experiment, an anaerobically grown *L. monocytogenes* broth culture was exposed to the polymeric phenolic fraction and the exposed cells spread plated onto TSA and incubated aerobically according to the standard protocol (2.3.2). These were designated anaerobic/aerobic cells. The results of this part of the experiment (anaerobic/aerobic MWS and polymeric phenolic) are shown in **Figure 5.6.**



80.1 **Figure 5.6 Antilisterial activity of the polymeric phenolic fraction (1 mg/ml) (isolated from commercial grape juice batch 2) against anaerobically (20% CO_2, <0.1% O_2) grown *Listeria monocytogenes* and exposed cells grown on TSA plates aerobically and anaerobically.** Error bars are the ± standard deviation of mean log CFU/ml.

The effect of anaerobosis was a slight reduction in the speed of the antilisterial effect. *L. monocytogenes* numbers were reduced from 10^5 CFU/ml to 10^3 CFU/ml within 10 minutes exposure compared to a 6-log reduction in cell grown under completely aerobic conditions (section 5.4.1). However, after 60 minutes exposure cell numbers were reduced to no colonies detected which is in line with previous results reported for aerobically grown *L. monocytogenes* cultures. There was a slight reduction (0.5-log CFU/ml within 10 minutes but no further reduction after 60 minutes) in anaerobic/aerobic cells exposed to the control model wine solution (MWS).

The second part of the experiment consisted of a *L. monocytogenes* broth culture grown anaerobically and exposed to the polymeric phenolic fraction, with the TSA spread plates incubated

under anaerobic conditions (designated anaerobic/anaerobic cells). **Figure 5.6** (see anaerobic/anaerobic MWS and polymeric phenolic) shows that *L. monocytogenes* developed a slight resistance to the antilisterial activity of the polymeric phenolic fraction. Within 10 minutes exposure to the polymeric phenolic fraction, *L. monocytogenes* numbers were reduced by 0.5-log which was a significant reduction in antilisterial activity when compared to aerobically grown cells (6-log reduction section 5.4.3.2) and anaerobic cells grown aerobically after exposure to the polymeric phenolic fraction (2.5-log reduction, **Figure 5.6**). Even after anaerobic cells were exposed to the polymeric phenolic fraction for 60 minutes cell numbers were only slightly reduced, from 10^5 CFU/ml to 10^3 CFU/ml. After 60 minutes exposure 10^3 CFU/ml remained viable. The control model wine solution (MWS) had no inhibitory effect on *L. monocytogenes* grown anaerobically.

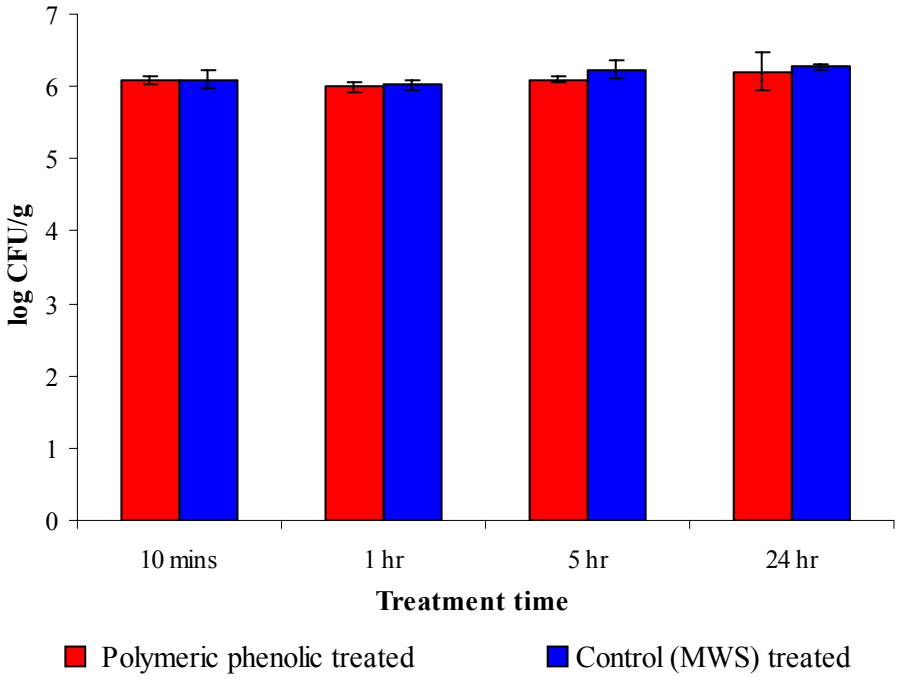
The reason for the slight decline in the inhibitory effect of the polymeric phenolic fraction against anaerobic/aerobic and the more significant decline against anaerobic/anaerobic cells was unclear. However, under anaerobic/aerobic conditions the cell numbers were reduced to zero within 60 minutes indicating that any physical or metabolic changes to *L. monocytogenes*, due to growing anaerobically, did not affect the antilisterial activity of the polymeric phenolic fraction. Completely anaerobically grown cells (both as a broth culture and then after exposure on plates incubated anaerobically) appeared to confer some resistance to *L. monocytogenes* ATCC 35152. Under anaerobic conditions or modified atmosphere conditions *L. monocytogenes* is able to slowly increase in numbers. Hudson *et al*., (1994) reported the increase in *L. monocytogenes* numbers on roast beef packaged under saturated CO_2 atmosphere. Cell numbers increased from 4-log to 8-log at 3^{o}C over a period of 62 days. Wimpfheimer *et al*., (1990) reported the inhibition of growth of *L. monocytogenes* on minced raw chicken packaged under complete anaerobic conditions (75:25 CO_2:N_2) but no inhibition of growth of *L. monocytogenes* under commercially used modified atmosphere packaging (72.5:22.5:5 CO_2:N_2:O_2). They showed that cell numbers increased under this modified atmosphere by 6-log CFU/ml over 21 days at 4^{o}C. Modified atmosphere packaging of chicken and other meats can allow *L. monocytogenes* to substantially increase in numbers, albeit over a long period of time, and therefore there may be a need for further antimicrobial hurdles to control the growth of this bacteria. Given that the polymeric phenolic fraction was able to reduce anaerobic/aerobic grown *L. monocytogenes* to no viable cells and anaerobic/anaerobic grown *L. monocytogenes* cells by 2-log within 60 minutes, it may be useful as a further hurdle in inhibiting the growth of this organism in foods stored under commercially used modified atmosphere packaging.

5.4.4 Antilisterial Activity of Polymeric Phenolic Fraction against Listeria monocytogenes Contaminated Cabbage

The polymeric phenolic fraction (isolated from commercial grape juice batch 2) was found to inhibitory to *L. monocytogenes* ATCC 35152 over a range of bacterial growth conditions (sections 5.4.3.1, 5.4.3.2, and 5.4.3.3) and therefore may be a good candidate for use as a natural food preservative. Experiments were performed on *L. monocytogenes* contaminated cabbage to assess the feasibility of polymeric phenolic fraction use as a natural preservative.

The cabbage slurry pH was found to be 6.1. However, after addition of the grape juice derived polymeric phenolic solution, or model wine solution to the *L. monocytogenes* contaminated cabbage, the pH was found to be 5.0 and 3.5, respectively, which was in the pH range for antilisterial activity by the grape juice derived polymeric phenolic fraction (sections 2.4.8 and 3.4.5).

The antilisterial activity of the polymeric phenolic fraction was suppressed when applied to *L. monocytogenes* contaminated cabbage (**Figure 5.7).** At all polymeric phenolic treatment times (10 minutes, 1 hour, 5 hours, and 24 hours) the number of viable *L. monocytogenes* cells remained at the initial cabbage inoculum level of 10^6 CFU/g. The control model wine solution (MWS) treated cabbage also showed no reduction in cell numbers over a 24 hour treatment time.



81.1 **Figure 5.7 Antilisterial effect of polymeric phenolic fraction (0.5 mg/ml) (isolated from commercial grape juice batch 2) against cabbage contaminated with *Listeria monocytogenes* ATCC 35152.** Error bars are the ± standard deviation of mean log CFU/ml.

The suppression of antilisterial activity of the polymeric phenolic material in the presence of cabbage may be explained in part by the ability of condensed tannins to bind to macromolecules. Condensed tannins have been shown to bind to proteins, carbohydrates, and polysaccharides (Bate-Smith, 1973; Haslam, 1974; Scalbert, 1991; Mueller-Harvey & McAllen, 1992). Haslam (1974) reported that the size and number of *ortho*-dihydroxyphenol groups of the tannin are important for tannin-protein complex formation. The interaction between tannin and protein is principally through hydrophobic effects, which are reinforced by the establishment of hydrogen bonds. These hydrogen bonds occur between phenolic groups, as proton donors, and the carbonyl groups of peptide bonds, as proton acceptors (Santos-Buelga & Scalbert, 2000). The suppression of the antilisterial activity of the polymeric phenolic fraction in the presence of cabbage may be due to the binding of the polymers to surface structures (possibly surface proteins or sugars) on the cabbage cells, thus preventing interaction with the bacterial cells. Other researchers have reported the inactivation of antimicrobials in the presence of food. Larson *et al*., (1996) reported that the sensitivity of *L. monocytogenes* to hop extracts was much higher in bacterial growth media than in a number of foods, including commercial coleslaw, cottage cheese, Camembert cheese, and skim and whole milk. They reported a slight reduction in *L. monocytogenes* numbers on coleslaw treated with hop extract for 11 days. However, the extended 11 day treatment period required to reduce *Listeria* numbers may not be conducive to use on fresh commercial products like vegetables as the product would not be safe for consumption until 11 days after treatment by which time the vegetable may itself be inedible. A shorter antimicrobial treatment time would be required for fresh food products such as vegetables. Ahn *et al*., (2004) reported the inhibitory activity of a commercial grape seed extract (ActiVin) against *L. monocytogenes* associated with raw ground beef. The authors reported a 1-log reduction in *L. monocytogenes* numbers in raw beef after 6 days exposure to 1% ActiVin. However, the authors reported that the antilisterial activity of ActiVin was reduced when applied to *L. monocytogenes* contaminated raw beef, compared to *L. monocytogenes* in a broth culture. Additionally, the ActiVin concentration used by Ahn *et al*., (2004) was 20 times greater than that used in this study. In this thesis experiment a 0.5 mg/ml concentration of polymeric phenolic was used as a treatment with no reduction in cell numbers seen after 24 hours. At this concentration the polymeric phenolic fraction had antilisterial activity against 'free-floating' *L. monocytogenes* (section 5.4.2). A longer treatment time or a higher

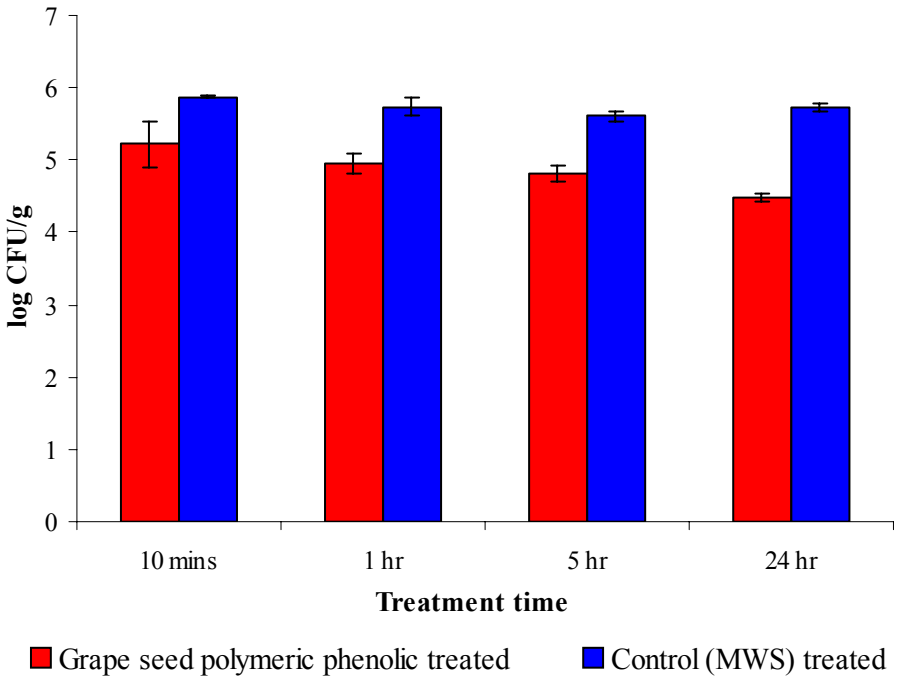
concentration of polymeric phenolic treatment may be required to give a similar reduction in *L. monocytogenes* numbers associated with cabbage.

Zaika *et al*., (1997) reported the antilisterial effect of sodium polyphosphate, a compound widely used in the food industry as an emulsifier, antioxidant, and moisture-binding agent. They reported that long chain sodium polyphosphate (chain length 13) inhibited the growth of *L. monocytogenes* in BHI broth but had little effect on the growth of *L. monocytogenes* in several mineral-rich foods such as pureed beef, green beans, and sweet potatoes. Zhang & Farber (1996) reported a decrease in *L. monocytogenes* cell numbers on shredded lettuce and cabbage treated with chlorine dioxide. They reported a 1-log reduction in cell numbers associated with vegetables compared to a 6-log reduction in *L. monocytogenes* numbers not associated with vegetables and suggested that the bacteria associated with the vegetable was somehow protected from the inhibitory effect of chlorine dioxide but did not suggest a reason for this. Adams *et al*., (1989) reported that bacteria associated with salad vegetables are protected from disinfectants due to the presence of hydrophobic pockets and folds in the leaf surface. It appears possible from this thesis result that *L. monocytogenes* might be protected from the antilisterial effect of the polymeric phenolic fraction via accumulation of the bacteria in the pockets and folds of the cabbage. It is also possible that the polymeric phenolic material interacts with the surface of the cabbage preventing it from interacting with the bacterial cells thereby reducing the inhibitory effect. The results of this thesis experiment agree with the findings of other authors (Adams *et al*., 1989; Larson *et al*., 1996; Zhang & Farber, 1996; Zaika *et al*., 1997; Ahn *et al*., 2004) that the inhibitory effect of the polymeric phenolic material was suppressed in the presence of food.

5.4.5 Antilisterial Activity of Grape Seed Polymeric Phenolic Fraction Against Listeria monocytogenes Contaminated Cabbage

Ribier grape seed polymers were found to have antilisterial activity (section 3.4.4). Unlike the grape juice polymers the activity of the seed polymers was not dependent on pH (section 3.4.5) and this may make the grape seed polymers a better candidate for use as a natural food preservative for food susceptible to *L. monocytogenes* contamination. An experiment was performed on *L. monocytogenes* contaminated cabbage to assess the feasibility of grape seed polymeric phenolic fraction for use as a natural preservative. Ribier grape seed polymeric phenolic fraction (0.5 mg/ml concentration) had antilisterial activity when assayed against *L. monocytogenes* contaminated cabbage. *L. monocytogenes* cell numbers were reduced from 5.2-log CFU/g to slightly less than 4.5-log CFU/g over a 24 hour treatment time (**Figure 5.8**). The control model wine solution (MWS) treated cabbage showed no reduction in cell numbers over the same treatment time.

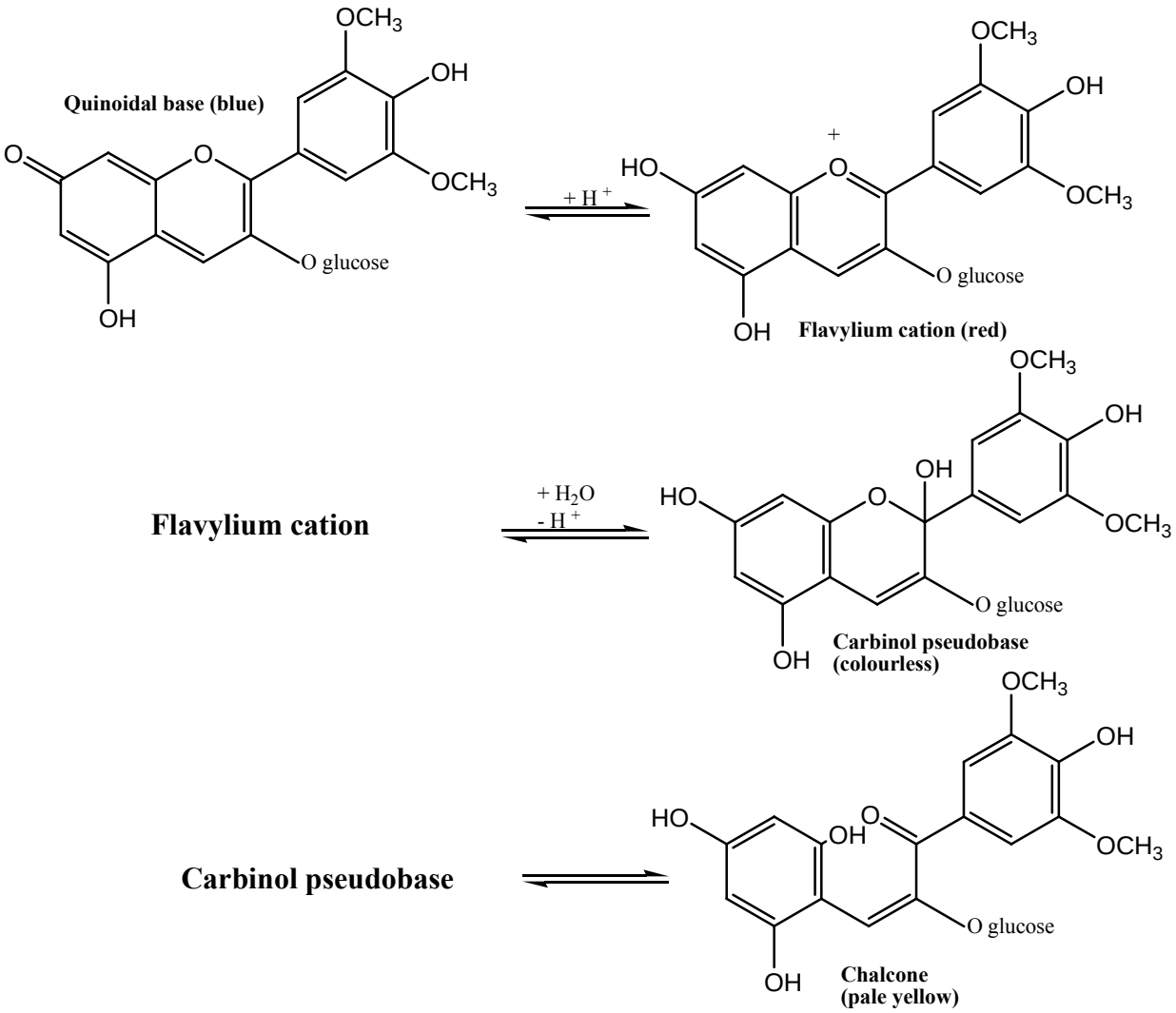
Figure 5.8 Antilisterial effect of Ribier grape seed polymeric phenolic fraction (0.5 mg/ml) against cabbage contaminated with *Listeria monocytogenes* ATCC 35152. Error bars are the ± standard deviation of mean log CFU/ml.



The grape seed polymeric phenolic fraction in this thesis showed slightly higher antilisterial activity than that reported by Ahn *et al*., (2004). Ahn *et al*., (2004) reported a 1-log reduction in *L. monocytogenes* numbers in raw beef over 6 days when treated with 1% ActiVin. This thesis research showed a 1-log reduction in *L. monocytogenes* in cabbage after 24 hours exposure to 0.5% grape seed polymeric phenolic fraction. This researcher speculates that the lower inhibitory action of the commercial grape seed extract (ActiVin) may have been due to lower concentration of tannin material in the commercial extract. The commercial grape seed extract (ActiVin) consisted of

monomeric phenolic compounds, including epicatechin, epigallocatechin, epigallocatechin gallate, ferulic acid, caffeic acid, *p*-coumaric acid, resveratrol, kaempferol, quercetin, and myricetin. The proportion of each phenolic compound in the extract was not stated. The grape seed polymeric phenolic fraction investigated in this thesis experiment consisted of condensed tannin (procyanidin polymers) rather then monomeric phenolic compounds and this may be the reason for the stronger antilisterial activity. In addition, this researcher speculates that the antilisterial activity of the Ribier grape seed tannin might be reduced when added to raw meat due to the higher concentration of compounds able to bind to tannin (i.e. protein) in meat compared to cabbage.

The grape seed polymeric phenolic material showed a greater antilisterial activity, in the presence of cabbage, than the grape juice polymeric phenolic material. A possible reason for this could have been the effect of pH. The antilisterial activity of the grape seed polymers was pH independent (section 3.4.5) whereas the grape juice polymers are only active at pH 5.0 and below (sections 2.4.8 and 3.4.5). The pH of the contaminated cabbage after the addition of the grape juice polymeric phenolic material was pH 5.0, which is the highest pH at which the grape juice polymers have antilisterial activity. The grape juice condensed tannin is pigmented suggesting the polymers contain anthocyanin moieties (**Figure 4.6**). Anthocyanins undergo reversible structural transformations with change in pH (**Figure 5.9**). At pH 5.0, it is fesible that a greater proportion of the anthocyanin will not be in the flavylium cationic form but rather in the anion quinonoidal base and anionic chalcone forms compared to pH 3.5 (Asenstorfer *et al*., 2003). It is possible that the antilisterial activity of the grape juice condensed tannin occurs when the cationic form of the anthocyanin interacts with the negatively charged bacterial cell membrane and, if there is less cationic form present, there is therefore less interaction of the pigmented polymeric material with the bacterial cell which in turn decreases the antilisterial effect. The grape seed condensed tannins do not contain anthocyanin moieties and are also not dependent on acidic pH for antilisterial activity. Therefore, a different mode of antimicrobial action may exist for these types of non-pigmented condensed tannin.



82.1

83.1 Figure 5.9 pH equilibrium of malvidin-3-glucoside (Brouillard & Delaporte, 1977).

A second theory is proposed for the higher antilisterial activity of the grape seed compared to the grape juice polymers. Haslam (1974) reported that the number of *ortho*-dihydroxyphenol groups of condensed tannin are important for protein binding. Cheynier *et al*., (1999) found that grape seed condensed tannins contained about 20% esterified gallate, whereas grape skin condensed tannins had only about 2%. Given that it is possible that the grape seed polymers contain higher numbers of gallate moieties and therefore a higher numbers of *ortho*-dihydroxyphenol groups, this may increase their ability to bind to the bacterial cell compared to the juice polymers.

It is suggested that

1. Due to the possibity of grape seed oligomers containing a higher proportion of *ortho*-dihydroxyphenol groups compared to the juice oligomers more grape seed oligomers than grape juice oligomers may be able to bind to *L. monocytogenes* thereby increasing the inhibitory effect of the seed oligomers. Possibly, a certain proportion of the *ortho*-dihydroxyphenol groups bind to the protein in the cabbage but with the grape seed oligomers there is sufficient unbound *ortho*-dihydroxyphenol groups available to bind to the bacterial cell membrane. In contrast, the lower levels of *ortho*-dihydroxyphenol groups in the juice oligomers may result in less binding to the bacterial cell.

2. The antilisterial activity of the grape juice tannin is pH dependent. When assayed the cabbage-bacterial mixture was at pH 5.0. This is the upper pH limit for antilisterial by the juice tannin and as such the reduction of antilisterial activity in the presence of cabbage may have been due to the anthocyanin moieties in the tannin being in the quinonoidal and chalcone forms rather than a proportion being in the cationic flavylium form as seem at more acidic pH's. This may affect the ability of these pigmented oligomers to bind to the bacterial cell. This is not significant for the antilisterial activity of grape seed oligomers, which are not pH dependent.

One or a combination of these factors may be responsible for the greater antilisterial activity of the grape seed tannin compared to the grape juice tannin.

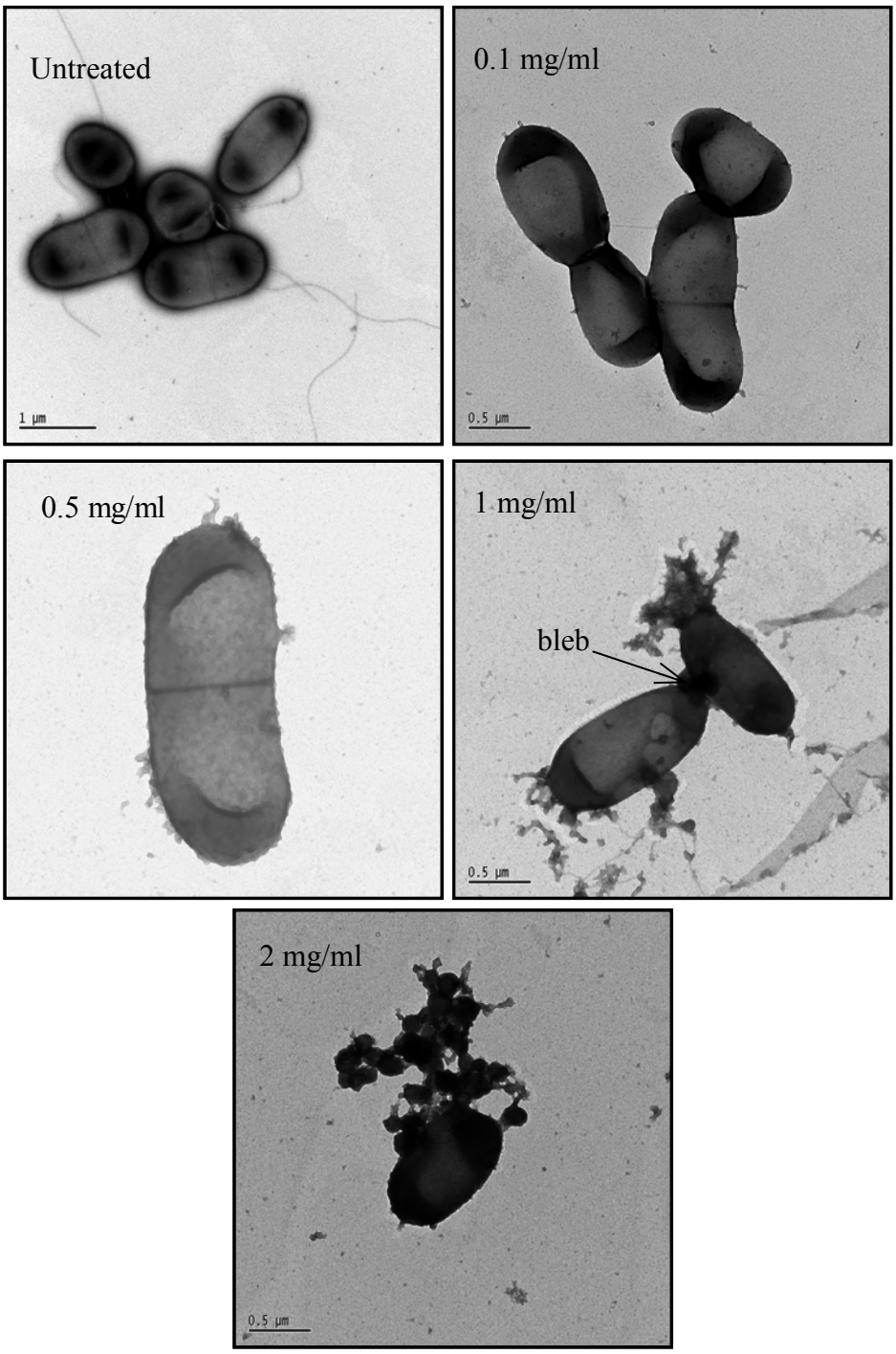
5.4.6 Transmission Electron Microscopy of Listeria monocytogenes Treated with Polymeric Phenolic Fraction Isolated from Commercial Grape Juice

Whole cells of *Listeria monocytogenes* treated with increasing concentrations of the polymeric phenolic fraction (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 m/ml), isolated from commercial grape juice batch 2, and thin sections of *L. monocytogenes* treated with polymeric phenolic fraction (2 mg/ml) were analysed for morphological changes by transmission electron microscopy (TEM).

5.4.6.1 Whole Cell TEM of Listeria monocytogenes Treated with Polymeric Phenolic Fraction

L. monocytogenes was treated for 60 minutes at 20^{o}C with increasing concentration of polymeric phenolic fraction in order to mimic the experimental conditions under which this fraction is inhibitory (section 5.4.2). Previous results show that at a concentration of 0.1 mg/ml the fraction is only slightly inhibitory to *Listeria* whereas higher concentrations (0.5, 1, 2 mg/ml) were very inhibitory.

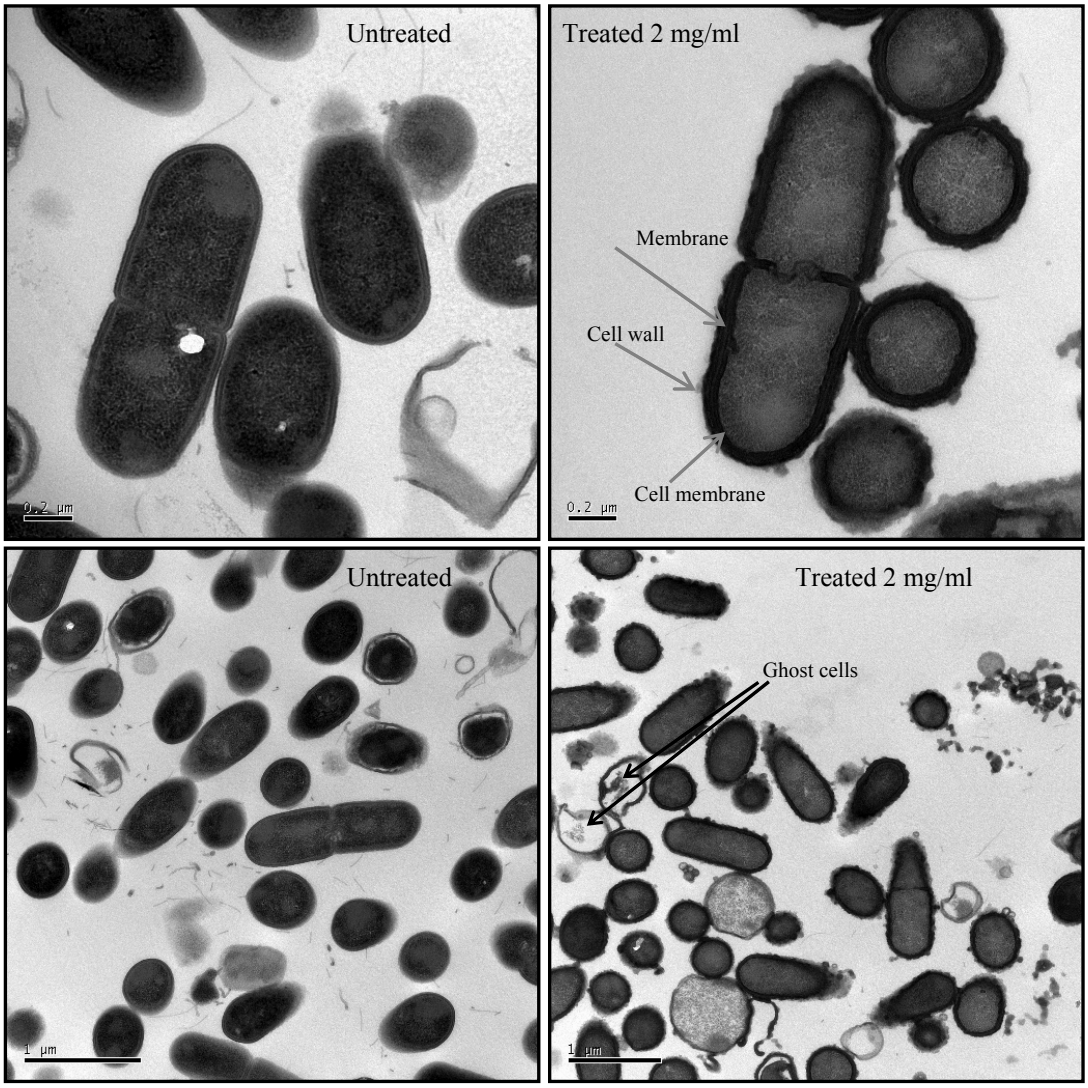
The untreated *L. monocytogenes* cells were intact and no obvious breakage or protrusions from the cell wall or cell membrane were observed (**Figure 5.10**). The cells had peritrichous flagella, as expected for *L. monocytogenes* cells grown at 20-28^{o}C. When exposed to 0.1 mg/ml polymeric phenolic fraction the cells appeared intact with a few slight protrusions from the surface of the cell visible. At this concentration, a 2-log reduction in cell numbers was observed (see MIC results section 5.4.2). It is unclear whether the cells shown in the microscopy image were viable but they appeared more similar to the untreated control cells than to the cells exposed to higher concentrations of the polymeric phenolic fraction (1 and 2 mg/ml). The cells exposed to the polymeric phenolic material showed no flagella present. This may be an artefact of the cell preparation process as some control cells also showed lack of flagella. At higher concentrations (bactericidal) of polymeric phenolic the cells appear to have breakages or perturbation of the cell membrane and increased numbers of blebs are observed as the concentration of the polymeric phenolic treated increased (**Figure 5.10**). Kiura *et al*., (2002) reported an increase in cell membrane breakages and bleb numbers occurring on gram-negative *Pseudomonas aeruginosa* cells treated with increasing concentrations of free chlorine and suggest that breakage of the bacterial cell wall and cell membrane may have caused the bactericidal effect. Beveridge *et al*., (1991) reported the blebbing of the outer membrane of *E. coli* (gram-negative) cells treated with a sub-biocidal (killing) level of Betaine. Bacterial cells treated with sub-inhibitory concentrations of tannic acid and a condensed tannin rich carob pod extract resulted in a change in cell morphology of *Pseudomonas fluorescens, E coli,* and *Cellvibrio fulvus* which formed chains or filaments, whereas the morphology of other bacterial species including *V. cholerae, S. aureus, B. subtilis*, and *A. aerogenes* were not affected when subjected to tannin inhibition (Henis, *et al*., 1964). The authors suggested that the site of action of tannins was the cell envelope (cell wall and cell membrane). This thesis result showed that no major change in cell morphology, aside from the blebbing and some evidence of cell lysis, resulted from the exposure of *L. monocytogenes* cells to the polymeric phenolic fraction.



84.1 Figure 5.10 Transmission electron microscopic images of *Listeria monocytogenes* untreated and treated with increasing concentrations of polymeric phenolic fraction isolated from commercial grape juice batch 2.

5.4.6.2 Thin Sections of Listeria monocytogenes Treated with Polymeric Phenolic Fraction

Thin sections of *L. monocytogenes* cells treated with a bactericidal concentration (2 mg/ml) of polymeric phenolic fraction show marked changes in cell wall and/or cell membrane structure (**Figure 5.11**). When compared to the untreated cells, the treated cells appear to have thickening of the cell surface and breakages or disorganisation of the cell membrane. The thickening of the cell wall could have been the result of adherence of the polymeric phenolic material to the cell wall or inner cytoplasmic membrane. However, this is unclear. The bottom treatment image in **Figure 5.11** shows the appearance of cells that have lost their cell contents (ghost cells) due to treatment with polymeric phenolic fraction. There is no evidence of blebbing in the thin section images, which could have been due to the inability of blebs to survive the thin section preparation process.



85.1 Figure 5.11 Thin section transmission electron microscopic images of *Listeria monocytogenes* untreated and treated with 2 mg/ml polymeric phenolic fraction isolated from commercial grape juice batch 2.

5.5 Summary

The polymeric phenolic fraction isolated from the commercial grape juice (batch 2) used in this chapter had a narrow spectrum of antimicrobial activity analogous to that of the commercial grape juice (batch 1). The polymeric phenolic fraction was active against all *Listeria* species assayed including *L. monocytogenes* serotypes 1/2a and 4b, which have been associated with food-borne listeriosis. The minimum inhibitory concentration of the fraction was 0.25 mg/ml and the antilisterial activity was comparable to unmodified whole grape juice (batch 1) with a 6-log reduction in cell numbers occurring within 10 minutes exposure.

The polymeric phenolic fraction was inhibitory to *L. monocytogenes* over a range of bacterial growth parameters. It was active against *L. monocytogenes* over a growth temperature range of 4^{o}C to 41^{o}C. It was also found to be more inhibitory to *L. monocytogenes* in log phase of growth than in stationary phase growth, which is in general agreement with the literature (Rees *et al*., 1995). The polymeric phenolic fraction was active against *L. monocytogenes* grown under anaerobic/aerobic conditions but was less active against this bacterium when grown under totally anaerobic conditions (i.e. anaerobic/anaerobic). The results of growth parameter experiments suggested that the polymeric phenolic fraction could be a good candidate for use as a natural food preservative to eliminate or prevent the growth of *L. monocytogenes* in a range of foods stored under a number of different commercial storage conditions such as chilled storage and/or modified atmosphere packaging.

When assessed for efficacy as a natural food preservative against *L. monocytogenes* contaminated cabbage, the inhibitory activity of the polymeric phenolic material isolated from commercial grape juice batch 2 was suppressed. The polymeric phenolic fraction did not reduce *L. monocytogenes* numbers when associated with cabbage. Comparison with the grape seed polymeric phenolic fraction showed that the seed polymeric phenolics were able to reduce *L. monocytogenes* numbers on cabbage to a greater extent than the grape juice polymeric phenolic fraction. This researcher proposes that the pH independent activity of the seed condensed tannin together with the higher numbers of *ortho*-dihydroxyphenol groups, compared to grape juice condensed tannin, could contribute to the greater inhibitory effect of the grape seed polymeric fraction.

When *L. monocytogenes* cells were assessed for gross morphological changes by electron microscopy, changes in the outer cell wall or cytoplasmic membrane were observed as were the formation of blebs and thickening of the bacterial cell wall. Breakages in the bacterial cytoplasmic

membrane were also observed as were the presence of cells depleted of their cell contents (ghost cells). The mode of action of the antilisterial polymeric phenolic fraction was not determined but it appeared that total cell lysis does not occur for all *L. monocytogenes* cells treated with the polymeric phenolic fraction as the majority of cells appear intact.

6 DISCUSSION AND CONCLUSIONS

The aims of this thesis were to

- Characterise the antimicrobial efficacy of a commercial grape juice.
- Compared and contrast the antilisterial action of extracts from different parts of the grape berry.
- Isolate and characterise the antimicrobial factor(s) present on the commercial grape juice.
- Assess the efficacy of grape-derived antimicrobial factor(s) as additives to food to eliminate *L. monocytogenes* contamination.

All of these aims were achieved.

6.1 General Discussion

A commercial red grape juice and the skin and seed extracts of *Vitis vinifera* var. Ribier grapes have strong inhibitory action against the food-borne bacterial pathogen *Listeria monocytogenes*. The antimicrobial activity of the grape juice, grape skin, and seed extracts was specific to *Listeria* species. To this researcher's knowledge, this is the first report of the inhibitory effect of grape juice and grape skin extract against *Listeria monocytogenes*. The rapid lethality of the commercial grape juice, skin, and seed extracts against *L. monocytogenes* (6-log reduction in cell numbers within 10 minutes exposure) was comparable to the lethality of red wines against other microorganisms (Masquelier 1959; Sheth *et al*., 1988; Weisse *et al*., 1995; Marimón & Bujanda, 1998; Marimón *et al*., 1998; Jayaprakasha *et al*., 2003; Just & Daeschel, 2003). The antimicrobial spectrum of grape seed extracts reported by Jayaprakasha *et al*., (2003) and Ahn *et al*., (2004) showed a wider spectrum of antimicrobial activity than the grape seed extract in this thesis. Jayaprakasha *et al*., (2003) reported that grape seed extract was more inhibitory to gram-positive bacteria including *Bacillus cereus, Bacillus subtilis, Bacillus coagulans,* and *Staphylococcus aureus* (grape seed extract concentration 0.85-1 mg/ml) than the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (grape seed extract concentration 1.25-1.5 mg/ml). Ahn *et al*., (2004)

reported that the commercial grape seed extract (ActiVin) was inhibitory to *L. monocytogenes, E. coli* and *Salmonella* Typhimurium, with *L. monocytogenes* being more sensitive than the other bacterial species. Ahn *et al*., (2004) reported a 6-log reduction in *L. monocytogenes* numbers exposed to 6 mg/ml grape seed extract (ActiVin), whereas this thesis research found that exposure of *L. monocytogenes* to Ribier grape seed extract at a concentration of 0.25 mg/ml reduced cell numbers by 6-log CFU/ml within 10 minutes. This thesis researcher suggests that enhanced antilisterial activity of Ribier grape seed extract was due to the high proportion of polymeric phenolic material (condensed tannin) in the extract compared to the high proportion of monomeric polyphenols in ActiVin. Further, this thesis result indicated that *L. monocytogenes* was more sensitive than other bacteria to the inhibitory action the commercial grape juice, grape skin, and seed extracts derived from *Vitis vinifera* var. Ribier grapes. The sensitivity of *L. monocytogenes* to grape derived polymeric phenolics was an interesting finding and reason for this finding warrants further investigation in the future.

The antilisterial factor in the grape juice, skin, and seed extracts has been identified as condensed tannin (polymeric phenolic material). While the antilisterial factor in the commercial grape juice and the Ribier grape skin was identified as pigmented and non-pigmented condensed tannin, the antilisterial factor from Ribier grape seed was shown to be non-pigmented condensed tannin. A number of experiments on the commercial grape juice (batch 1) and the condensed tannin fraction of the juice (batch 2) initially identified a likely antilisterial factor. The behaviour of the grape juice in the presence of metal ions (magnesium and iron), protein (BSA), and PVPP indicated that the antilisterial factor was tannin. Tannin is known to complex with metal ions and the antimicrobial action of condensed tannin and anthocyanins has been suggested to be due to the ability of these compounds to chelate metal ions necessary for microbial growth (Scalbert, 1991). The present research found that the addition of ions (Fe^{3+} and Mg^{2+}) to the grape juice resulted in suppression of antilisterial activity suggesting that the antilisterial factor was the condensed tannin present in the grape juice. Hseih *et al*., (2001) found that the addition of metal ions, including Ca^{2+}, Zn^{2+}, Fe^{3+}, and Mg^{2+}, reduced the antilisterial activity of an extract consisting of *Corni fructus* (Dogwood fruit):cinnamon:Chinese chive (8:1:1). Powers (1964) found that the addition of Mg^{2+}reversed the inhibitory effect of malvidin-3-glucoside against *S. aureus*. The antimicrobial activity of tannins is probably determined by the molar content and spatial configuration of the *ortho*-dihydroxyphenolic groups (Schofield *et al*., 2001). Khokher & Owusu Apenten (2003) found that the presence of a 3,4 dihydroxy group on the flavonoid B ring of epicatechin was associated with high iron binding efficiency, whereas the presence of a 3,4,5 trihydroxy group of the B ring of epigallocatechin or the 3,4,5 trihydroxy group of the gallate moiety of epicatechin-3-*O*-gallate was associated with reduced

iron binding. The authors concluded that the presence of *ortho*-dihydroxyphenol groups were important for iron binding. This researcher suggests that the factor that confers the antilisterial activity to the commercial grape juice may be the *ortho*-dihydroxyphenol groups, as seen in epicatechin, of the condensed tannin present in the juice. In the presence of metal ions ($Fe^{3+}$and Mg^{2+}) the *ortho*-dihydroxyphenol groups of the grape juice tannin may be bound to metal ions which would prevent the *ortho*-dihydroxyphenol groups interacting with the bacterial cell, resulting in the loss of antilisterial activity.

Tannin is able to bind to proteins, carbohydrates, and polysaccharides (Bate-Smith, 1973; Haslam, 1974; Scalbert, 1991; Mueller-Harvey & McAllen, 1992). In the present research we found that the addition of protein (BSA) to the grape juice derived pigmented condensed tannin, and removal of the assumed protein-tannin complexes from the assay system, resulted in the suppression of the antilisterial activity of the juice. This result indicated that the inhibitory factor was condensed tannin. Previous researchers have also found that the addition of protein to antimicrobial plant extracts and grape juice resulted in the suppression of the antimicrobial action. Konowalchuk & Spiers, (1976) showed that the addition of 1% gelatine to a virus-grape juice complex resulted in the restoration of poliovirus infectivity. Chung *et al*., (1990) found that the antilisterial activity of a Chinese medicinal plant, Siu Mao Heung, was suppressed by the addition of 1% Bacto-peptone. Nguyen-the & Lund, (1992) found that the antilisterial activity of carrot tissue was suppressed by 0.05% bovine serum albumin (BSA). PVPP is known to bind to phenolic compounds (Amerine & Ough, 1980; Zoecklein *et al*., 1995a). PVPP treatment of the grape juice, and removal of the assumed PVPP-polyphenol complexes from the assay system, resulted in the loss of inhibitory action of the resultant decolourised grape juice. We identified the antilisterial factor present in the commercial grape juice as pigmented polymeric phenolic material (pigmented condensed tannin). This is in agreement with the findings of Masquelier (1959), who found that decolourisation of red wine, with charcoal, resulted in the loss of antimicrobial activity of the red wine against *E. coli*. Masquelier (1959) postulated that the inhibitory factor was the colouring material and not ethanol or organic acids present in the red wine.

The dependence on acidic pH for antilisterial activity of the commercial grape juice was an interesting finding. The present research found that at pH $\leq$5.0 the commercial grape juice had antilisterial activity but increasing the pH of the grape juice to pH 6.4 and above resulted in the complete suppression of the antilisterial activity concomitant with a change in grape juice colour from red to opaque black. When the grape juice samples were adjusted back to pH 3.5 the colour of the juice turned from black back to red concomitant with the recovery of antilisterial activity.

Monomeric anthocyanin pigments undergo reversible structural transformations with change in pH (**Figure 5.9**). Somers (1971) proposed the presence of the flavylium form of anthocyanins in the polymer structures of red wine. He reported that the equilibrium of red wine polymeric pigments was more complex than that of monomeric anthocyanins and reported the colour stability of the wine polymeric pigments, which retained more than 50% of the maximum colour in the pH range of 0.75 to 4.76. Based on Somers (1971) findings and this thesis finding that at pH ≤5.0 the grape juice was coloured red and was inhibitory to *L. monocytogenes*, we theorise that the pH dependent inhibitory action may have been due to a proportion of the anthocyanin moiety being in a stabilised flavylium cation form which may interact with the negatively charged bacterial cell. When the commercial grape juice was at pH ≥6.4 the antilisterial activity was lost and this may be because the majority of the anthocyanin moiety in the polymeric structure may be in a quinonoidal base form.

The structure of the anthocyanin moieties incorporated into polymers is not clearly elucidated and because anthocyanins undergo structural transformations with change in pH a number of species of anthocyanin may exist in the polymer structure. The structure proposed by Francia-Aricha *et al*., (1997) showed that the anthocyanin moiety of the pigmented condensed tannin was in the flavylium form (**Figure 6.1**), however this does not imply that the flavylium form is the only form of the anthocyanin present.

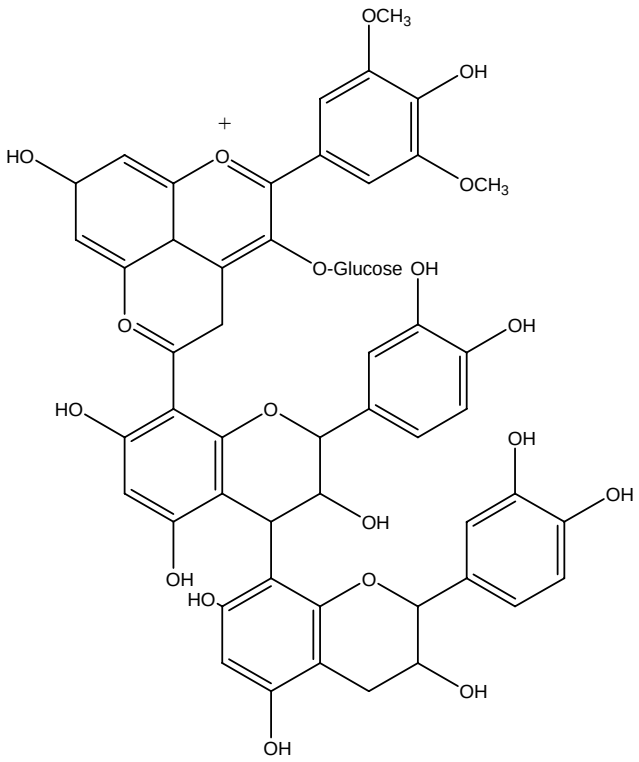


Figure 6.1 Proposed linkage of an anthocyanin (malvidin-3-glucoside) and procyanidin dimer (taken from Francia-Aricha *et al*., 1997). The anthocyanin is linked via the C_4 position to a vinyl residue at the C_8 position of the procyanidin polymer.

Given that the antilisterial factor in the commercial grape juice was identified as anthocyanin containing polymeric phenolic material (pigmented condensed tannin), it appears that the anthocyanin moiety may be involved to some extent in the inhibitory activity of the pigmented condensed tannin. Bacterial cells have a net negative charge on the cell wall, although the magnitude of this charge varies from strain to strain. *Listeria monocytogenes* has a high net negative charge and a lower cell surface hydrophobicity compared to many other bacteria (Dykes *et al*., 2001). This researcher speculates that the positively charged anthocyanin moiety of the condensed tannin may be the initial interaction point of the tannin with the negatively charged surface of *L. monocytogenes* cell. The mechanism of the inhibitory action of the pigmented condensed tannin was not determined in this thesis but electron microscopy images of *L. monocytogenes* showed that cell lysis, bleb formation, cell membrane breakage, and thickening of the bacterial cell wall occurred after exposed to inhibitory levels of the polymeric phenolic material (pigmented condensed tannin).

The grape juice under investigation is derived from *Vitis vinifera* variety Ribier black table grapes. The condensed tannin fraction of Ribier grape skin and seed was found to be inhibitory to *L. monocytogenes*. In order to identify the likely source of the antilisterial factor in the commercial grape juice, whether skin and/or seed, a number of comparative experiments were performed. The condensed tannin derived from the commercial grape juice (batch 2) and the skin of Ribier grapes was found to be dependent on acidic pH for activity whereas the seed condensed tannin was inhibitory at both acidic and neutral pH. The effect of dialysis on the antilisterial activity of the commercial grape juice, skin, and seed condensed tannins also revealed a difference in the antilisterial factor present in these fractions. When dialysed, the seed tannin lost more weight (90%) compared to the grape juice (73%) and skin (72%) tannin. Concomitant with a loss of grape seed tannin material, resulting from dialysis, was a significant loss in antilisterial activity. In contrast, the grape juice and skin tannin, although greatly reduced in weight by dialysis, did not show a loss of antilisterial activity. Together, the pH and dialysis effect results showed that the

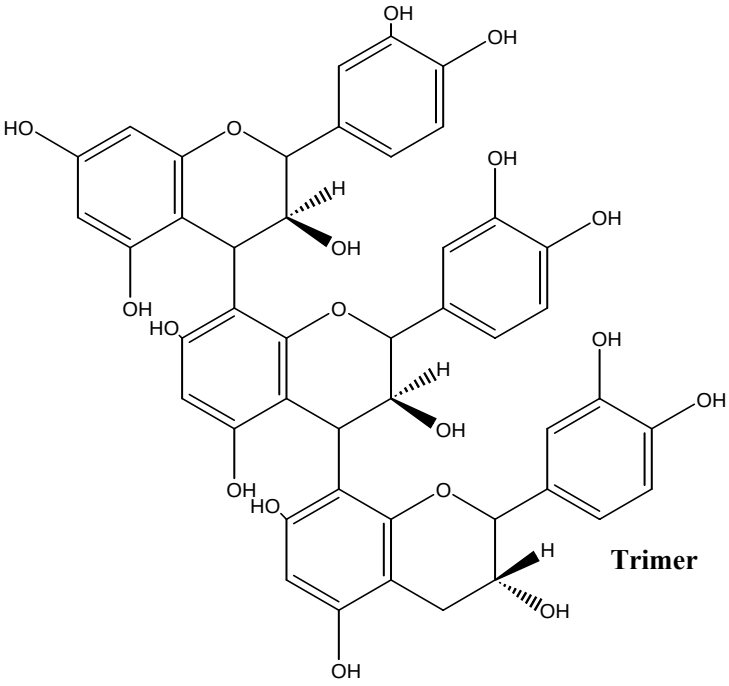
grape seed tannin differed in nature to both the grape juice polymeric phenolics (condensed tannin) and skin tannin. The grape juice and the grape skin polymeric phenolic material had pH dependent antilisterial activity and the antilisterial activity was not lost by dialysis. These findings indicated that the origin of the antilisterial factor in the commercial grape juice was likely derived from the skin of Ribier grapes.

The antimicrobial spectrum of the grape juice, skin, and seed polymeric phenolic material was found to be narrow with only *L. monocytogenes* being significantly inhibited by all three polymeric samples. Jayaprakasha *et al*., (2003) found that grape seed tannin was inhibitory to both gram-positive and gram-negative bacteria, but the authors did not include *Listeria monocytogenes* in their study. Jayaprakasha *et al*., (2003) found that the grape seed fraction containing a higher percentage of procyanidin tetramers through to nonamers was more inhibitory than the fraction containing a higher percentage of procyanidin dimers and trimers. Ahn *et al*., (2004) found that a commercial grape seed extract (ActiVin) was more inhibitory to *L. monocytogenes* than *E. coli* and *Salmonella* Typhimurium, albeit at a high concentration (6 mg/ml). The commercial grape seed extract studied by Ahn *et al*., (2004) contained mainly monomeric polyphenols including epicatechin, epigallocatechin, and epigallocatechin-gallate. This present research found that the Ribier grape seed condensed tannin was comprised of procyanidins ranging from trimers to nonamers (mDP 3.2-10.1) and antilisterial activity of grape seed tannin increased with increasing mDP. This investigator suggests that the number and possibly the spatial configuration of *ortho*-hydroxyphenol groups in the tannin may be important for antilisterial activity and that the may be the reason for the stronger activity of the Ribier grape seed tannin compared to ActiVin (Ahn *et al*., 2004) and the procyanidin dimers and trimers (Jayaprakasha *et al*., 2003). In addition, this thesis research used a concentration of grape seed tannin (0.25 mg/ml) for antimicrobial assay which was lower than that used by Jayaprakasha *et al*., (2003) (0.85 mg/ml) and Ahn *et al*., (2004) (6 mg/ml). If the concentration of both grape seed and grape juice and skin tannin was increased in the assay system used in this thesis, a wider antimicrobial spectrum might have been discovered. However, the use of a low concentration of tannin resulted in the finding that *Listeria* species are particularly susceptible to the inhibitory effect of condensed tannin and provides further avenues for research into this phenomenon.

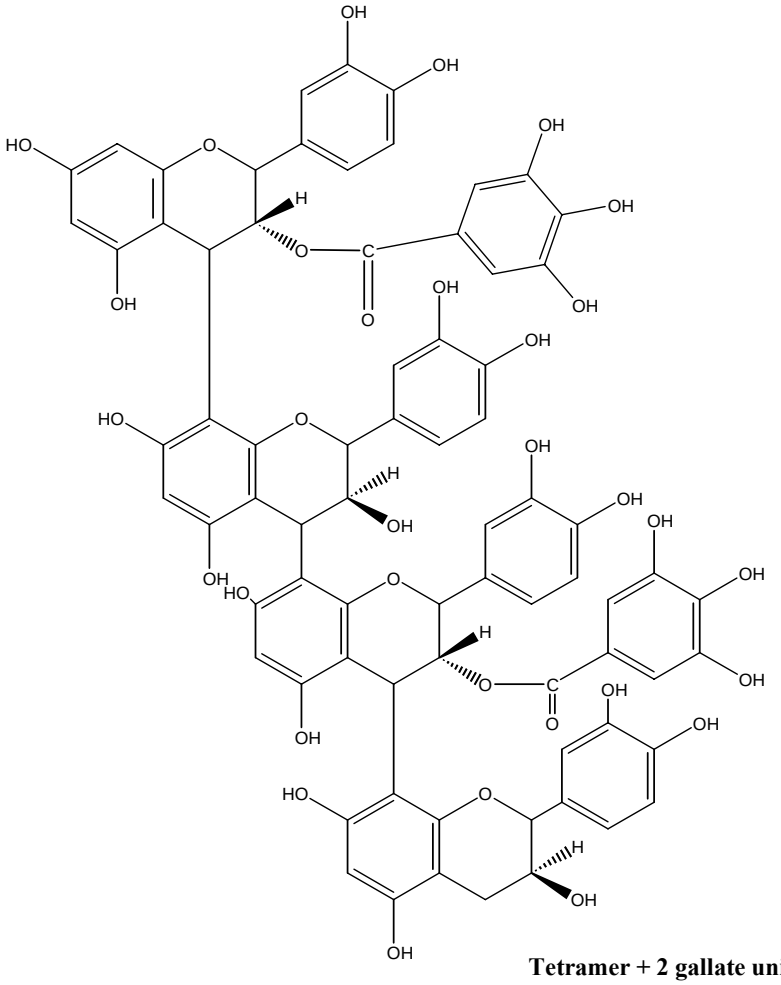
The antilisterial factor in the commercial grape juice was identified as pigmented condensed tannin (anthocyanin containing polymeric phenolic material) using a number of analytical chemistry techniques. Elemental analysis of the grape juice tannin and comparison of the FTIR spectra of the grape juice tannin with published procyanidin (a class of condensed tannin) spectra (Foo, 1981),

indicated that the antilisterial factor in the commercial grape juice was phenolic and was comprised of a mixture of procyanidin (consisting of catechin, epicatechin, and epicatechin-3-*O*-gallate) and prodelphinidin polymers (consisting of epigallocatechin). To further identify the antilisterial factor in the commercial grape juice the pigmented polymeric phenolic material was fractionated by molecular weight and the fractions analysed by thiolysis and for antilisterial activity. The eight fractions, with the exception of fraction A+B (pooled), had antilisterial activity. The mean degree of polymerisation of the active fractions (C, D, E, F, G, and H) ranged from 2.8 to 8.5, with the most active fractions E, F, and G having a mDP of 4.0, 5.0, and 5.9, respectively. Fraction G, with a mDP of 5.9, showed slightly stronger antilisterial activity than the other fractions and was analysed by IES-MS. A number of oligomers in fraction G were identified as follows: a proanthocyanidin trimer (an oligomer of three catechin units *m/z* 866), an oligomer consisting of four catechin and two gallate units (*m/z* 1459), an oligomer of eight catechin units and two gallate units (*m/z* 2612), and an oligomer of nine catechin units and two gallate units (*m/z* 2905).

The possible structures of the aforementioned oligomers are shown in **Figure 6.2**. The flavan-3-ol subunits can be linked C_4-C_8 and C_4-C_6. C_4-C_8 linkages are shown in the proposed structures.

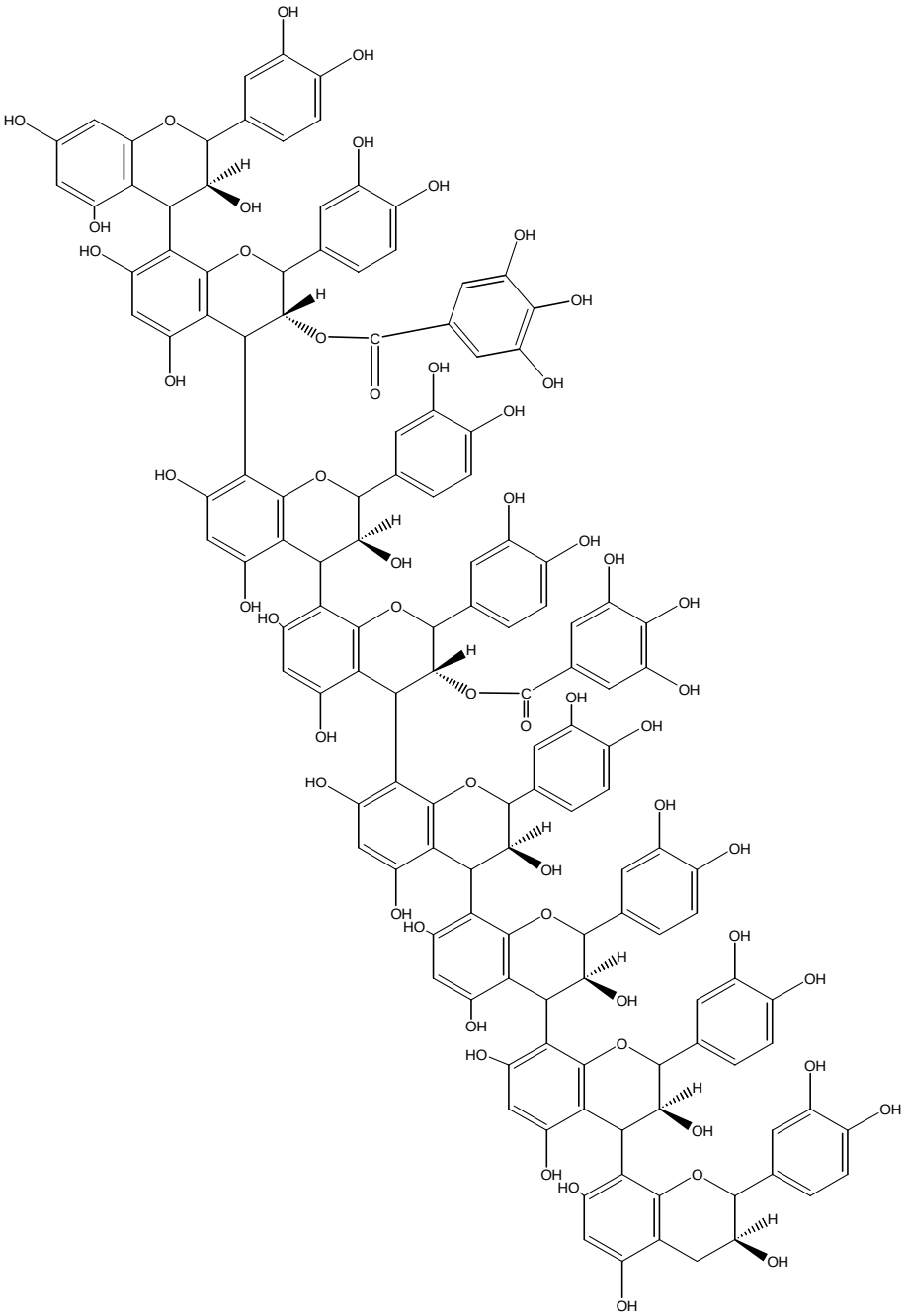


Trimer

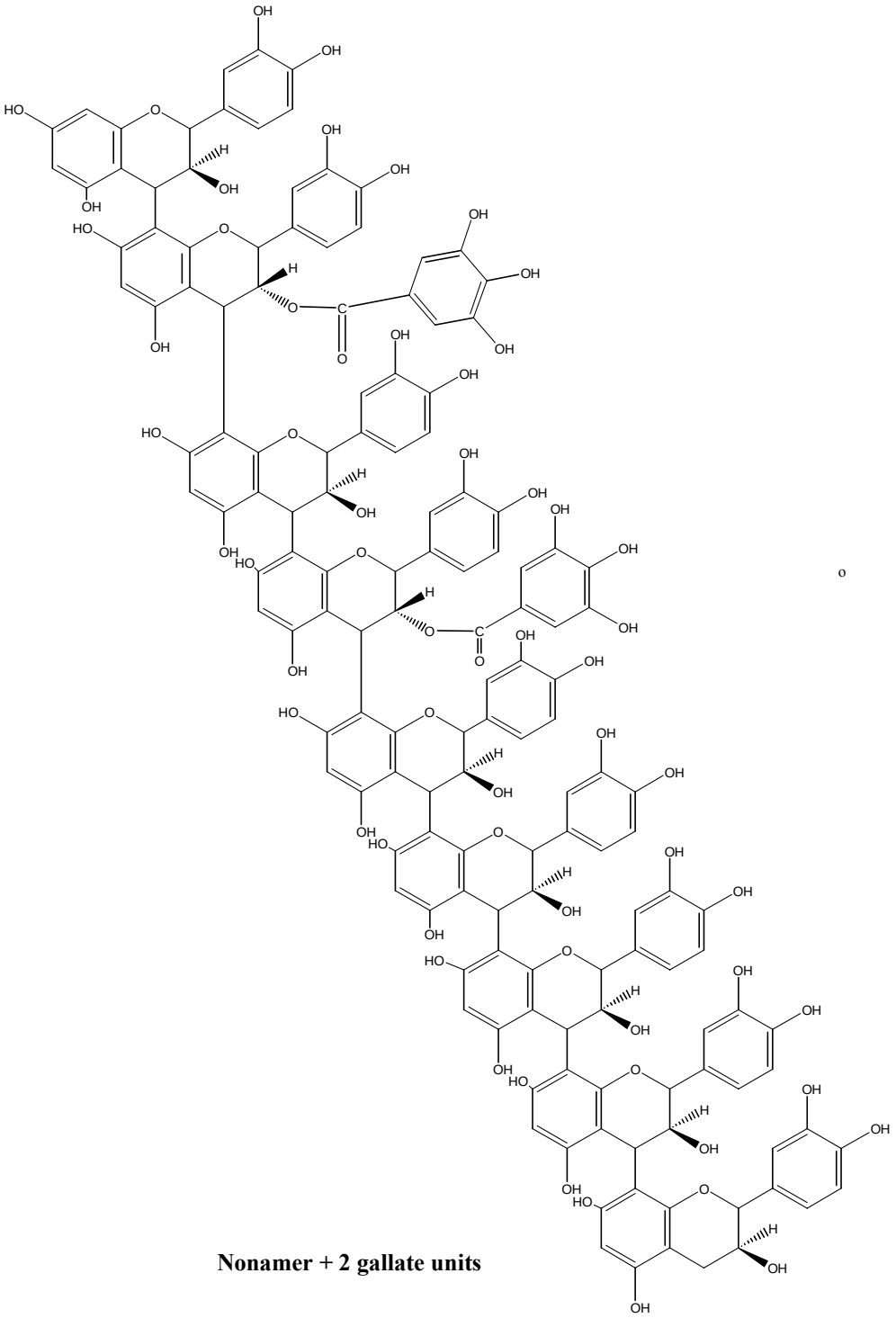


Tetramer + 2 gallate units

o



Octomer+ 2 gallate units



Nonamer + 2 gallate units

Figure 6.2 Proposed structures of some oligomers identified in antilisterial fraction G.

Although fraction G had a mDP of 5.9, no pentamers or hexamers were found by mass spectroscopy analysis and no anthocyanin moieties were identified in fraction G. However, it is apparent that this fraction contains oligomers with anthocyanins incorporated into the structure, as the fraction was pigmented (red). An example of a proposed structure of an anthocyanin containing proanthocyanidin pentamer is shown in **Figures 6.3**. The structural subunits are taken from the composition of fraction G as determined by HPLC following thiolysis (refer **Table 4.6**). The structure assumes only one anthocyanin moiety (malvidin-3-glucoside) was present in each pentamer, though this was not proved in this research. The anthocyanin was proposed to be at the top of the oligomer based on findings and proposed structures of other researchers (Somers, 1971; Francia-Aricha *et al*., 1997; Remy *et al*., 2000). The anthocyanin was assumed to be malvidin-3-glucoside as this is the most abundant anthocyanin in *Vitis vinifera* variety grapes (Mazza & Miniati, 1993) and was found to be present as a monomer in the polymeric phenolic fraction of the commercial grape juice (refer **Figure 3.2**). We propose that malvidin-3-glucoside was in the flavylium cation (red) form as Fraction G was red in colour (refer **Figure 4.6**). Further, we propose that the anthocyanin is linked to the oligomer via an A-type linkage because no anthocyanin moieties were detected by thiolysis, which may be due to the anthocyanin being linked via an A-type linkage resistant to thiolysis degradation. The terminal units of the pentamer can be catechin, epicatechin, and epicatechin-3-*O*-gallate with a likely ratio, based on thiolysis product peak area, of 3:1:2, respectively. The extension units of the pentamer can be epigallocatechin, catechin, epicatechin, and epicatechin-3-*O*-gallate with a likely ratio of 1:1:15:5, respectively. The linkage between the subunits can be C_4-C_8 and C_4-C_6. C_4-C_8 linkages are shown in the proposed structure. Note that this proposed structure is only one of approximately 5000 possible structures with the same subunits.

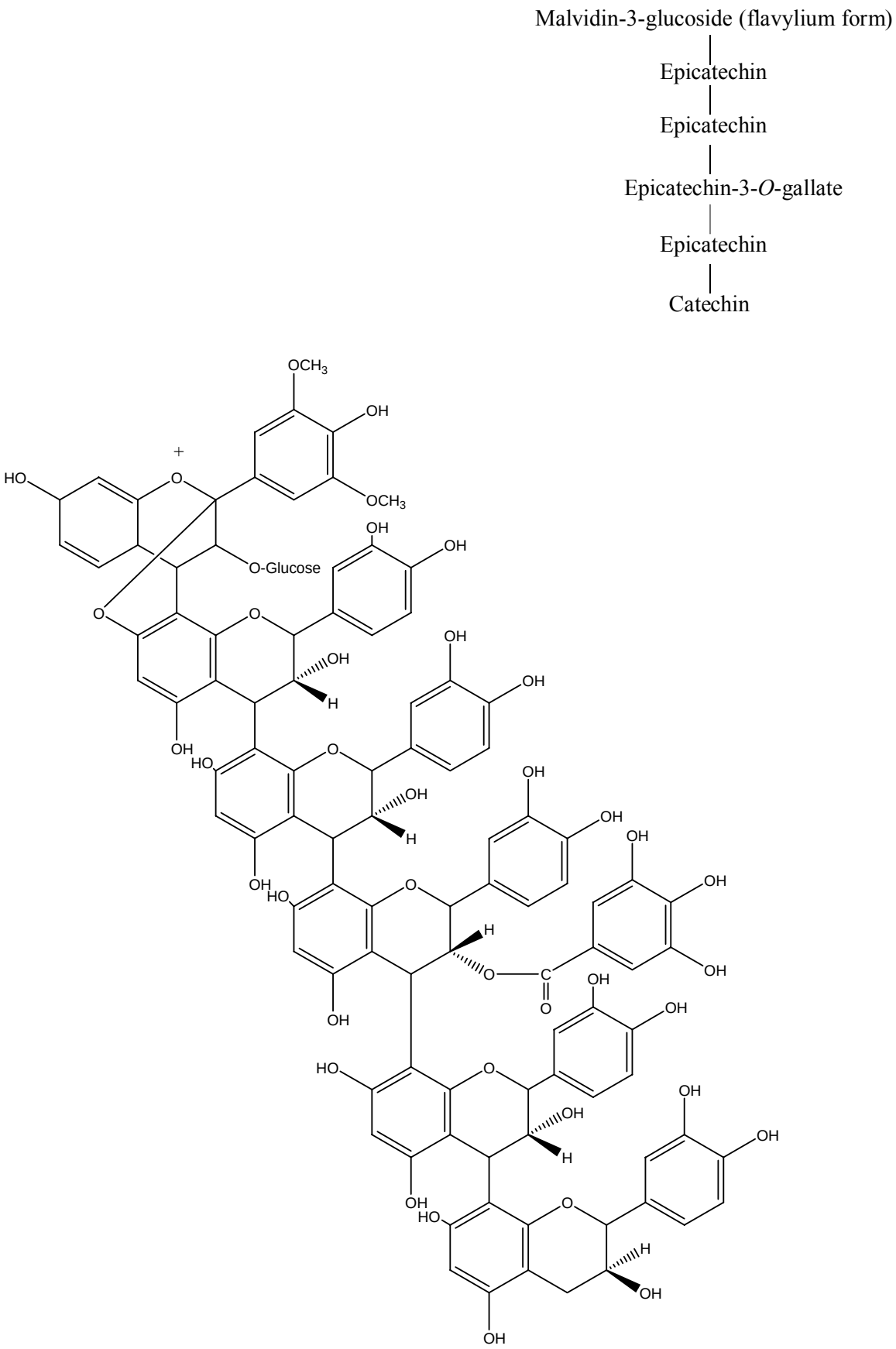


Figure 6.3 Proposed structure of an antilisterial anthocyanin (malvidin-3-glucoside) containing proanthocyanidin pentamer in commercial grape juice fraction G.

Fraction G contained the highest percentage polymeric colour. As stated previously, this researcher proposes that the positively charged anthocyanin moiety might interact with the negatively charged bacterial cell causing cell death. The higher the percentage of oligomers containing anthocyanin moieties the higher the proportion of oligomers able to interact with the bacterial cell and therefore, the higher the proportion of cells inhibited. This could be the reason for fraction G showing slightly stronger antilisterial activity compared to the other polymeric fractions. In addition, the size range of the proanthocyanidin oligomers in fraction G along with the strong antilisterial activity of other fractions (E and F) suggested that the antilisterial activity is due to either a single oligomer of a certain size and composition that is present in all the active fractions, but more abundant in the more inhibitory fractions, or that oligomers of a certain size range (i.e. mDP 4.0-5.9) are optimal for antilisterial activity and the more active fractions (i.e. E, F, and G) contain more oligomers within this size range.

The monomeric subunits comprising fraction G were identified as catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin. These monomer subunits are identical to subunits of proanthocyanidins (containing both procyanidin and prodelphinidin oligomers) present in *Vitis vinifera* derived red wine and *Vitis vinifera* grape skin (Souquet *et al*., 1996; Sun *et al*., 1998, Labarbe *et al*., 1999; Sarni-Manchando *et al*., 1999). Note that the presence of these proanthocyanidins (condensed tannin) in red wine and *V. vinifera* grape skin reported by these authors may also explain the high antilisterial activity of New Zealand red wine and the Ribier grape skin polymeric phenolic materials found in this thesis research.

Mass spectroscopy analysis of fraction G showed the fraction contained oligomers consisting of catechin, epicatechin, epigallocatechin, and epicatechin-3-*O*-gallate. Previous researchers have suggested that the number of hydroxyl groups and the presence of gallate moieties are important for the antimicrobial effect of a range of polymeric phenolic compounds (Ahn *et al*., 1991; Mabe *et al*., 1999; Tesaki *et al*., 1999). However, this thesis research along with the findings of a number of other authors (Mori *et al*., 1987; Nakane & Ono, 1990; Ahn *et al*., 1991; Ikigai *et al*., 1993; Foo *et al*., 2000a) found that a number of monomeric subunits of fraction G (i.e. catechin, gallic acid, epicatechin) did not have antimicrobial activity. It is highly likely that rather than the number of hydroxyl groups and *ortho*-dihydroxyphenolic groups present in an oligomer it might be the position or orientation of these groups that confers the inhibitory activity.

The Ribier grape seed condensed tannin (polymeric phenolic material) had antilisterial activity and was also fractionated by molecular weight in order to further identify the antilisterial factor. The

fractions were assessed for antilisterial activity and analysed by thiolysis. The mean degree of polymerisation of the fractions ranged from 3.2 to 10.1. Concomitant with an increase in mDP was an increase in antilisterial activity. The seed oligomers were found to consist of the same phenolic compounds as the juice polymers, though no epigallocatechin was present in the seed tannin. The grape seed condensed tannin consists of procyanidin oligomers only which is in agreement with previous findings by other authors (Prieur *et al*., 1994; Saucier *et al*., 2001; Kennedy & Taylor, 2003). The structures of typical grape seed procyanidins are shown in **Figure 6.4 and 6.5**.

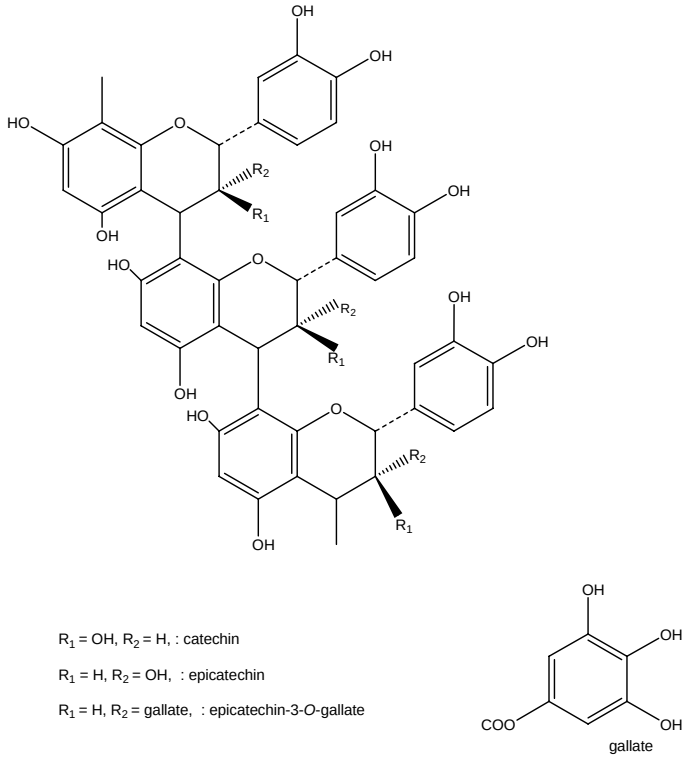


Figure 6.4 Structure of a procyanidin trimer (modified from Cheynier *et al*., 1999). Note the procyanidin subunits are linked via the C_4 position of the top subunit to the C_8 position of the neighbouring subunit. The subunits can be linked C_4-C_8 and C_4-C_6.

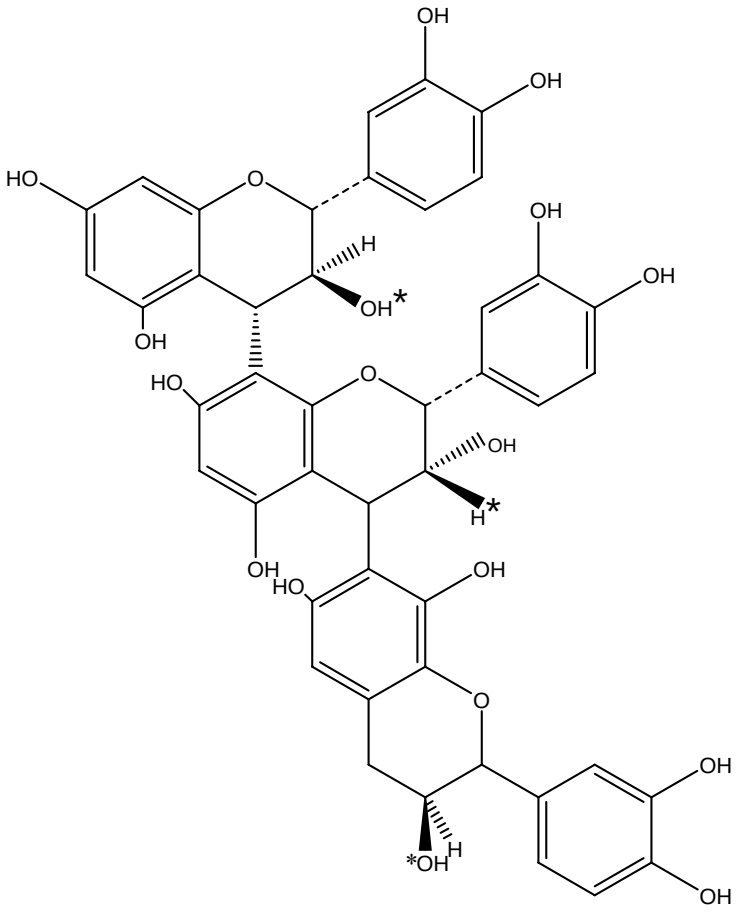


Figure 6.5 Structure of a grape seed procyanidin trimer (taken from de Freitas *et al*., 1998). Note the top subunit is linked via through the C_4 position to the C_8 position next subunit, which is in turn linked via the C_4 position to the C_6 position of the following subunit. *The gallate residues are linked to the C_3 position of the flavonoid B ring.

The seed tannins were more inhibitory, contained no anthocyanin moieties, were independent of pH for activity, and showed the relationship between increasing mDP and increasing antilisterial activity. Therefore, a different theory was proposed for the inhibitory action of the Ribier grape seed condensed tannin. Grape seed tannins have been reported to contain 10 times as many gallate residues compared to grape skin tannins (Cheynier *et al*., 1999). This researcher proposes that the stronger inhibitory action of the grape seed tannin fractions, compared to the grape juice tannin fractions, may be due to the higher level of gallate moieties in the seed tannin. In addition, Schofield *et al*., (2001) reported that an increase in the procyanidin to prodelphinidin ratio (i.e.

more procyanidin than prodelphinidin) in condensed tannin increased the ability of that tannin to complex proteins. The grape juice condensed tannin was comprised of both prodelphinidin (epigallocatechin) and procyanidin (epicatechin, epicatechin-3-*O*-gallate) oligomers whereas the seed tannin was comprised of only procyanidin oligomers. We speculate that the greater antilisterial activity of the grape seed tannin compared to the grape juice tannin may be due to the higher proportion of procyanidin oligomers, which have *ortho*-dihydroxyphenolic groups on the flavanol B ring, in seed tannin. Further, Kennedy & Taylor (2003) suggested that grape seed polymers might have a more extended conformation than grape skin condensed tannin (analogous to grape juice tannin batch 2). We propose that the higher level of gallate residues (and therefore hydroxyl groups) and *ortho*-dihydroxyphenolic groups together with an extended conformation, compared to the grape juice tannin, may allow greater interaction of the hydroxyl groups with the bacterial cell, resulting in the stronger inhibitory action of the seed tannin. In addition, the larger the oligomer size, the more hydroxyl groups likely to be in the oligomer and the stronger the inhibitory effect of the oligomer as seen in the relationship of increasing mean degree of polymerisation and increasing antilisterial activity. The lack of pH dependence for the antilisterial activity of the grape seed tannin and the absence of anthocyanins and pH dependence for the grape juice tannin may be attributed to the presence of anthocyanins in these pigmented tannins, indicating that anthocyanins and pH dependent inhibitory activity are linked.

The slightly larger size range of the grape seed oligomeric fractions (mDP 3.2-10.1) compared to the grape juice oligomers (mDP 1.8-8.5), suggested that the seed oligomers might contain more hydroxyl groups and *ortho*-dihydroxphenolic groups and this could be a reason for the stronger inhibitory action of the grape seed tannin. This thesis research concluded that the antilisterial factor in the commercial grape juice was pigmented condensed tannin (procyanidin and prodelphinidin oligomers), the antilisterial factor in the Ribier grape seed was condensed tannin (procyanidin oligomers), and that oligomer size greater than dimers was required for the inhibitory activity.

The usefulness of the grape juice polymeric phenolic material (pigmented condensed tannin) as a natural food preservative for food susceptible to *Listeria monocytogenes* contamination appears limited. Although the polymeric phenolic material was able to inhibit *L. monocytogenes* over a range of bacterial growth temperatures (4^{o}C to 41^{o}C), under aerobic and anaerobic conditions, and log and stationary phase cultures, the inhibitory activity was suppressed when *L. monocytogenes* cells were associated with cabbage. This result was not unexpected as the antilisterial factor was identified as condensed tannin and tannin is known to bind to protein, carbohydrates, and polysaccharides. Therefore the antilisterial factor is as likely to bind to these macromolecules

present on the food as it is to bind to *L. monocytogenes* cells. Other researchers have found that the inhibitory effect of antimicrobial compounds is suppressed in the presence of food (Adams *et al*., 1989; Larson *et al*., 1996; Zhang & Farber 1996; Zaika *et al*., 1997). The pigmented condensed tannin would not be useful in eliminating *L. monocytogenes* from food already contaminated as the disease-causing dose of this bacterium is unknown and only the complete elimination of this bacterium ensures the safety of the food. It is possible that using higher levels of polymeric phenolic to treat the contaminated cabbage may result in the complete inhibition of *L. monocytogenes*. However, these higher levels could impart undesirable flavour and colour characteristics to the food.

The Ribier grape seed tannin had inhibitory activity against *L. monocytogenes* associated with cabbage. However, as found with the juice tannin, this activity was not as strong compared to the inhibitory effect against "free floating" *L. monocytogenes* cells. Grape seed condensed tannin has the potential to be developed as a natural preservative for food susceptible to contamination by *Listeria monocytogenes*.

The difference in the inhibitory activities of the grape juice tannin and the grape seed tannin may be due in part to the pH of the contaminated cabbage assay system. As stated previously, the grape juice tannin is inhibitory at acidic pH only whereas the activity of the seed tannin is not affected by pH. The pH of the grape juice tannin treated cabbage was found to be pH 5.0 and therefore within the acidic inhibitory range. However, given that the proportion of positively charged anthocyanin moieties increases with increasingly acidic pH, this researcher suggests that at pH 5.0, compared to pH 3.5, less of the anthocyanin present in the grape juice tannin would be positively charged and therefore less of the pigmented polymeric phenolic material would interact with the negatively charged bacterial cell. In addition, in the presence of food, which is able to bind tannin, even less of the pigmented polymer would be available to interact with the bacterial cells. The grape seed tannin does not contain anthocyanin moieties and therefore the pH of the cabbage does not affect the inhibitory action of these tannins. In addition, on a weight for weight basis, the grape seed tannin was found to be more inhibitory against free floating *L. monocytogenes* cells compared with the grape juice tannin. This stronger inhibitory action of the seed tannin against free *L. monocytogenes* cells was also observed for *L. monocytogenes* associated with food.

6.2 Conclusions

- A commercial grape juice was found that has inhibitory activity against all species of *Listeria*, including all serotypes of the food-borne pathogen *Listeria monocytogenes*.

- Polymeric phenolic (condensed tannin) extracts of the skin and seed of *Vitis vinifera* var. Ribier grapes had antilisterial activity.

- The possible origin of the antilisterial factor in the commercial grape juice may be the skin of Ribier grapes.

- The antilisterial factor present in the commercial grape juice was identified as pigmented condensed tannin consisting, in part, of oligomers that contain procyanidin and prodelphinidin subunits.

- The antilisterial factor in Ribier grape seed was identified as condensed tannin consisting, in part, of procyanidin oligomers.

- Condensed tannins larger than dimers exhibited strong antilisterial activity. Future investigation is required to determine the optimal oligomer size and/or size range that confer antilisterial activity.

- Pigmented condensed tannin was inhibitory to free floating *Listeria monocytogenes* over a range of the bacterium's growth parameters.

- The inhibitory activity of the pigmented condensed tannin was suppressed when *Listeria monocytogenes* was associated with cabbage held at pH 5.0.

- The grape seed condensed tannin showed inhibitory activity against *Listeria monocytogenes* associated with cabbage.

- The pigmented condensed tannin caused irreversible damage to *Listeria monocytogenes* with cell lysis, bleb formation, cell membrane damage, and cell wall thickening observed.

6.3 Future Research

- The antilisterial factor in the commercial grape juice was found to be pigmented condensed tannin of a particular average size range. The optimal antilisterial factor, whether it is of a certain size or has a required conformation or number and orientation of hydroxyl groups is still unknown. A method of separating larger amounts (mg) of proanthocyanidin oligomers needs to be developed so each oligomer can be defined and assessed for antilisterial activity. A comparison of the activities of these oligomers can then be made and structure-activity relationships determined in order to elucidate the critical factor within these tannins that imparts the inhibitory activity.

- The *ortho*-dihydroxyphenol groups may be responsible for the antilisterial activity of the grape juice and grape skin and seed condensed tannin. Acetylation or methylation of the hydroxyl groups and assay of these against *Listeria monocytogenes* could indicate the importance of these groups in inhibitory action. An assay system in which the non-polar derivatized tannin are soluble and that is also conducive to the growth of *Listeria monocytogenes* needs to be developed.

- The commercial grape juice investigated in this thesis was derived from *Vitis vinifera* var. Ribier black table grapes. It would be of interest to assess the antimicrobial efficacy of condensed tannins isolated from different grape varieties (i.e. varieties of *Vitis vinifera* used for wine-making) in order to discover condensed tannin with stronger antimicrobial activity than *Vitis vinifera* var. Ribier grapes.

- The application of the tannins and commercial grape juice to provide protection against ingested *Listeria monocytogenes* contaminated food needs to be investigated. Previous research has shown that red wine is inhibitory to *Salmonella* species in a model stomach system (Just & Daeschel, 2003). Initially, a simple model stomach system consisting of food and synthetic gastric fluid could be developed to assess the antilisterial activity of the commercial grape juice and the Ribier grape derived pigmented and non-pigmented condensed tannins in a model stomach environment. A more elaborate dynamic model of the stomach and gastrointestinal tract, described by Gänzle *et al*., (1999), could also be used assess the antilisterial activity of the tannins in the human stomach environment.

- An important finding in this thesis was the susceptibility of *Listeria monocytogenes* to condensed tannin. This bacterium is of increasing concern to public health and an understanding of possible target sights to inhibit this bacterium would be helpful in developing biocides to control *Listeria monocytogenes*. The mechanism of action of the antilisterial activity of the grape tannins should be investigated and the site of interaction of the tannin with the bacterial cell identified. This information could enable the development of new effective antilisterial biocides for use in the food and pharmaceutical industries.

- In this thesis, the Ribier grape seed condensed tannin was shown to have potential as a natural food preservative. This potential needs to be investigated further by conducting experiments on a range of foods. It would be useful to assess the antilisterial activity of the seed tannin in conjunction with other traditional food preservative methods, such as modified atmosphere packaging, in order to determine the antimicrobial efficacy of this tannin and the minimum inhibitory concentration needed to give an antimicrobial effect. Ahn *et al*., (2004) and Jayaprakasha *et al*., (2003) found that grape seed tannin was inhibitory to a wide range of bacteria. It would also be interesting to determine the antimicrobial spectrum of the grape seed (and juice) tannins at increasing concentration to assess whether these tannins had the potential to inhibit a wider range of pathogenic bacteria.

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Appendix A Tables of Data for Figures

Table A1 Minimum Inhibitory Concentration of Grape Juice (batch 1) against *Listeria monocytogenes* ATCC 35152

% Grape Juice	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
100%	0	274300	5.438226	167000	5.222716	5.330471	0.1523
	10	<10	1	<10	1	1	0

	60	<10	1	<10	1	1	0
50%	0	250000	5.39794	380000	5.579784	5.488862	0.1285
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
33.3%	0	250000	5.39794	210100	5.322426	5.360183	0.0533
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
25%	0	289300	5.461348	201100	5.303412	5.38238	0.1116
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
20%	0	259200	5.413635	236900	5.374565	5.3941	0.0276
	10	110	2.041393	100	2	2.020696	0.0292
	60	<10	1	<10	1	1	0
12.5%	0	184000	5.264818	143000	5.155366	5.210077	0.0774
	10	17750	4.249198	6150	3.788875	4.019037	0.3254
	60	150	2.176091	240	2.380211	2.278151	0.1443
10%	0	120000	5.079181	193000	5.285557	5.182369	0.1459
	10	41870	4.621903	27300	4.436163	4.529033	0.1313
	60	15640	4.194237	19740	4.295347	4.244792	0.0714

Table A2 Survival of *Listeria monocytogenes* in three different batches of the same commercial grape juice

Grape juice batch number	Exposure time (mins)	CFU/ml Rep 1	Log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MGJ	0	640000	5.80618	620000	5.792392	5.799286	0.00975
	10	390000	5.59106	380000	5.57978	5.585424	0.00797

	60	460000	5.66275	320000	5.50515	5.583954	0.11144
Batch 1	0	260000	5.41497	190000	5.278754	5.346863	0.09632
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
Batch 2	0	70000	5.84509	830000	5.919078	5.882088	0.05231
	10	2000	3.3010	1000	3	3.150515	0.21286
	60	40	1.60206	70	1.845098	1.723579	0.17185
Batch 3	0	680000	5.83250	920000	5.963788	5.66111	0.0.9282
	10	1000	3	1000	3	3	0
	60	1000	3	4000	3.60206	3.30103	0.42572

Table A3 Effect of PVPP treatment on the antilisterial activity of grape juice (batch 1).

Assay solution	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MGJ	0	2020000	6.30535	2020000	6.30535	6.30535	0
	10	1400000	6.14612	1900000	6.278754	6.212441	0.09378

	60	1950000	6.29003	2130000	6.32838	6.309207	0.02711
Untreated grape juice	0	120000	5.07918	132000	6.120574	5.599878	0.73637
	10	<10	1	<10	1	1	0
	6	<10	1	<10	1	1	0
PVPP treated	0	2320000	6.36548	2200000	6.342423	6.353955	0.01631
	10	2060000	6.31386	1570000	6.1959	6.254883	0.08341
	60	626000	5.79657	644000	5.808886	5.80273	0.00870

Table A4 Effect of the addition of magnesium and iron cations on the antilisterial activity of grape juice (batch 1).

Assay solution	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MGJ Fe^{3+}	0	2000000	6.30103	1200000	6.079181	6.190106	0.15687

	10	2300000	6.36172	1600000	6.20412	6.282924	0.11144
	60	1500000	6.176091	1100000	6.041393	6.108742	0.09524
Grape juice Fe^{3+}	0	1100000	6.04139	1400000	6.146128	6.09376	0.07405
	10	400000	5.60206	1200000	6.079181	5.840621	0.33737
	60	200000	5.30103	100000	5	5.150515	0.21286
MGJ Mg^{2+}	0	1350000	6.13033	4300000	6.633468	6.381901	0.35577
	10	2200000	6.34242	1350000	6.130334	6.236378	0.14997
	60	1150000	6.06069	2250000	6.352183	6.20644	0.20611
Grape juice Mg^{2+}	0	1850000	6.26717	2250000	6.352183	6.309677	0.06011
	10	1150000	6.06069	1100000	6.041393	6.051045	0.01365
	60	900000	5.95424	300000	5.477121	5.715682	0.33737

Table A5 Antilisterial activity of monomeric and polymeric grape juice phenolics.

Assay solution	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MGJ	0	1600000	6.20412	800000	5.90309	6.053605	0.21286
	10	1600000	6.20412	1400000	6.146128	6.175124	0.04100

	60	1000000	6	900000	5.954243	5.977121	0.03235
Monomers	0	1400000	6.14612	1300000	6.113943	6.130036	0.02275
	10	11000	4.04139	5000	3.69897	3.870181	0.24212
	60	620	2.79239	730	2.863323	2.827857	0.05015
Polymers	0	70000	4.84509	10000	4	4.422549	0.59757
	10	10	1	10	1	1	0
	60	10	1	10	1	1	0
Acetone Control	0	700000	5.84509	3500000	6.544068	6.194583	0.49424
	10	97000	4.98677	154000	5.187521	5.087146	0.14195
	60	15000	4.17609	10000	4	4.088046	0.12451

Table A6 Effect of protein (BSA) addition on the antilisterial activity of the polymeric phenolic fraction of commercial grape juice (batch 2).

mg/ml BSA added	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev

0 mg/ml	0	970000	5.98677	114000	6.0569	6.02184	0.04959
	10	1000000	6	890000	5.94939	5.9747	0.03579
	60	1090000	6.03743	990000	5.99564	6.01653	0.02955
0.5 mg/ml	0	440000	5.64345	540000	5.73239	5.68792	0.06289
	10	73000	4.86332	74000	4.86923	4.86628	0.00418
	60	55000	4.74036	24000	4.38021	4.56029	0.25467
0.25 mg/ml	0	94000	4.97313	94000	4.97313	4.97313	0
	10	7200	3.85733	6000	3.77815	3.81774	0.05599
	60	390	2.59106	320	2.50515	2.54811	0.06075
0.1 mg/ml	0	39000	4.59106	30000	4.47712	4.53409	0.08057
	10	40	1.60206	90	1.95424	1.77815	0.24903
	60	<10	1	<10	1	1	0
0.01 mg/ml	0	44000	4.64345	54000	4.73239	4.68792	0.06289
	10	<10	1	60	1.77815	1.38908	0.55024
	60	<10	1	<10	1	1	0
0.001 mg/ml	0	2100	3.32222	2300	3.36173	3.34197	0.02794
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0

Table A7 Antilisterial activity of skin, seed, and juice/pulp extracts of Ribier grapes.

Grape extract assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev

MGJ	0	5400000	6.73239	5000000	6.69897	6.715682	0.02363
	10	5800000	6.76342	4600000	6.662758	6.713093	0.07118
	60	5700000	6.75587	4300000	6.633468	6.694672	0.08655
Grape skin	0	360000	5.55630	490000	5.690196	5.623249	0.09467
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
Grape seed	0	59000	4.77085	54000	4.732394	4.751623	0.02719
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
Grape juice/pulp	0	4530000	6.65609	4770000	6.678518	6.667308	0.01585
	10	4430000	6.64640	4350000	6.638489	6.642446	0.00559
	60	4370000	6.64048	4250000	6.628389	6.634435	0.00855

Table A8 Antilisterial activity of dialysis retentates of phenolic fractions of commercial grape juice, Ribier grape skin, and seed.

Grape extract	Exposure time	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev

assayed	(mins)						
MWS	0	830000	5.919078	840000	5.924279	5.921679	0.003678
	10	980000	5.991226	950000	5.977724	5.984475	0.009548
	60	730000	5.863323	1010000	6.004321	5.933822	0.099701
Juice	0	720000	5.857332	710000	5.851258	5.854295	0.004295
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
Skin	0	150000	5.176091	182000	5.260071	5.218081	0.059383
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
Seed	0	930000	5.968483	750000	5.875061	5.921772	0.066059
	10	102000	5.0086	120000	5.079181	5.043891	0.049908
	60	12500	4.09691	13000	4.113943	4.105427	0.012044

Table A 9 Antilisterial activity of grape juice polymeric phenolic fractions at a concentration of 0.5 mg/ml.

Fraction Assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	1030000	6.012837	860000	5.934498	5.973668	0.055394
	10	880000	5.944483	1010000	6.004321	5.974402	0.042312
	60	820000	5.913814	1010000	6.004321	5.959068	0.063998
A/B	0	760000	5.88081	740000	5.869232	5.875023	0.00819
	10	540000	5.73239	450000	5.653213	5.692803	0.05599
	60	102000	5.0086	99000	4.995635	5.002118	0.00916
C	0	720000	5.85733	780000	5.892095	5.874714	0.02458
	10	131000	5.11727	121000	5.082785	5.100028	0.02438
	60	40	1.60206	40	1.60206	1.60206	0
D	0	390000	5.59106	320000	5.50515	5.548107	0.06075
	10	60	1.77815	40	1.60206	1.690106	0.12451
	60	<10	1	<10	1	1	0
E	0	7000	3.84509	6400	3.80618	3.825639	0.02751
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
F	0	20	1.30103	<10	1	1.150515	0.21286
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
G	0	30	1.47712	20	1.30103	1.389076	0.12451
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
H	0	<10	1	<10	1	1	0
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0

Table A10 Antilisterial activity of grape juice polymeric phenolic fractions at a concentration of 0.1 mg/ml

Fraction Assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	980000	5.991226	960000	5.982271	5.986749	0.006332
	10	1020000	6.0086	1020000	6.0086	6.0086	0
	60	1010000	6.004321	1020000	6.0086	6.006461	0.003026
C	0	570000	5.75587	750000	5.87506	5.81547	0.08428
	10	970000	5.98677	770000	5.88649	5.93663	0.07091
	60	270000	5.43136	370000	5.5682	5.49978	0.09676
D	0	950000	5.97772	720000	5.85733	5.91753	0.08513
	10	360000	5.5563	440000	5.64345	5.59988	0.06162
	60	105000	5.02119	109000	5.03743	5.02931	0.01148
E	0	770000	5.88649	760000	5.88081	5.88365	0.00401
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
F	0	88000	4.94448	107000	5.02938	4.98693	0.06003
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
G	0	600000	5.77815	660000	5.81954	5.79885	0.02927
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
H	0	1150000	6.0607	890000	5.94939	6.00504	0.07871
	10	141000	5.14922	103000	5.01284	5.08103	0.09644
	60	440	2.64345	310	2.49136	2.56741	0.10754

Table A11 Antilisterial activity of grape juice polymeric phenolic fractions at a concentration of 0.05 mg/ml.

Fraction Assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	900000	5.954243	1210000	6.082785	6.018514	0.090894
	10	940000	5.973128	880000	5.944483	5.958805	0.020255
	60	840000	5.924279	960000	5.982271	5.953275	0.041006
E	0	980000	5.99122	720000	5.857332	5.924279	0.09467
	10	700000	5.84509	460000	5.662758	5.753928	0.12893
	60	470000	5.67209	760000	5.880814	5.776456	0.14758
F	0	810000	5.90848	680000	5.832509	5.870497	0.05372
	10	460000	5.66275	550000	5.740363	5.70156	0.05487
	60	460000	5.66275	290000	5.462398	5.562578	0.14167
G	0	810000	5.90848	790000	5.897627	5.903056	0.00767
	10	230000	5.36172	260000	5.414973	5.388351	0.03765
	60	108000	5.03342	106000	5.025306	5.029365	0.00574

Table A12 Antilisterial activity of polymeric phenolic seed fractions at a concentration of 0.1 mg/ml.

Fraction Assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	970000	5.986772	840000	5.924279	5.955526	0.044189
	10	700000	5.845098	760000	5.880814	5.862956	0.025255
	60	700000	5.845098	810000	5.908485	5.876792	0.044821
SC	0	340000	5.447158	430000	5.462398	5.454778	0.010776
	10	280000	5.531479	290000	5.633468	5.582474	0.072117
	60	80	1.90309	120	2.079181	1.991136	0.124515
SD	0	299000	5.475671	182000	5.260071	5.367871	0.152452
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
SE	0	9000	3.954243	8500	3.929419	3.941831	0.017553
	10	<10	1	<10	1	1	0
	60	1	10	1	1	0	0
SF	0	100	2	600	2.778151	2.389076	0.550236
	10	10	1	10	1	1	0
	60	10	1	10	1	1	0
SG	0	9000	3.954243	10000	4	3.977121	0.032355
	10	10	1	10	1	1	0
	60	10	1	10	1	1	0
SH	0	700	2.845098	500	2.69897	2.772034	0.103328
	10	10	1	10	1	1	0
	60	10	1	10	1	1	0

Table A13 Antilisterial activity of polymeric phenolic seed fractions at a concentration of 0.05 mg/ml.

Fraction Assayed	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	880000	5.944483	960000	5.982271	5.963377	0.026721
	10	800000	5.90309	760000	5.880814	5.891952	0.015752
	60	800000	5.90309	790000	5.897627	5.900359	0.003863
SD	0	560000	5.748188	350000	5.544068	5.646128	0.144335
	10	104000	5.017033	82000	4.913814	4.965424	0.072987
	60	2090	3.320146	1760	3.245513	3.282829	0.052774
SE	0	670000	5.826075			5.826075	
	10	760	2.880814	860	2.934498	2.907656	0.037961
	60	560	2.748188	370	2.568202	2.658195	0.12727
SF	0	370000	5.568202	500000	5.69897	5.633586	0.092467
	10	9000	3.954243	5600	3.748188	3.851215	0.145703
	60	8800	3.944483	7100	3.851258	3.897871	0.06592
SG	0	490000	5.690196	310000	5.491362	5.590779	0.140597
	10	<10	1	30	1.477121	1.238561	0.337376
	60	<10	1	<10	1	1	0
SH	0	239000	5.378398	343000	5.535294	5.456846	0.110942
	10	20	1.30103	20	1.30103	1.30103	0
	60	<10	1	<10	1	1	0

Table A14 Minimum inhibitory concentration of the polymeric phenolic fraction (isolated from commercial grape juice batch 2) against *L. monocytogenes* ATCC 35152.

mg/ml	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
0.1 mg/ml	0	410000	5.612784	260000	5.414973	5.513879	0.139873
	10	21000	4.322219	30000	4.477121	4.39967	0.109532
	60	1100	3.041393	1600	3.20412	3.122756	0.115066
0.25 mg/ml	0	75000	4.875061	72000	4.857332	4.866197	0.012536
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
0.5 mg/ml	0	67000	4.826075	58000	4.763428	4.794751	0.044298
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
1 mg/ml	0	14000	4.146128	10000	4	4.073064	0.103328
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
2 mg/ml	0	290000	5.462398	330000	5.518514	5.490456	0.03968
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0

Table A15 Effect of *Listeria monocytogenes* growth temperature on the antilisterial activity of polymeric phenolic fraction.

Bacterial growth temerature	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS 4°C	0	610000	5.78533	540000	5.732394	5.758862	0.03743
	10	750000	5.87506	600000	5.778151	5.826606	0.06852
	60	490000	5.69019	650000	5.812913	5.751555	0.08677
Phenolic 4°C	0	122000	5.08636	169000	5.227887	5.157123	0.10007
	10	920	2.96378	720	2.857332	2.91056	0.07527
	60	<10	1	<10	1	1	0
MWS 22°C	0	1000000	6	830000	5.919078	5.959539	0.05722
	10	890000	5.94939	900000	5.954243	5.951816	0.00343
	60	580000	5.76342	590000	5.770852	5.76714	0.00525
Phenolic 22°C	0	112000	5.04921	59000	4.770852	4.910035	0.19683
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0
MWS 41°C	0	330000	5.51851	410000	5.612784	5.565649	0.06665
	10	420000	5.62324	430000	5.633468	5.628359	0.00722
	60	300000	5.47712	280000	5.447158	5.46214	0.02118
Phenolic 41°C	0	500000	5.69897	730000	5.863323	5.781146	0.11621
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0

Table A16 Effect of mid-log phase *L. monocytogenes* culture on the antilisterial activity of polymeric phenolic fraction.

Assay solutions	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS	0	401000	5.60314	479000	5.68034	5.64174	0.05458
	10	38000	4.57978	24000	4.38021	4.48	0.14112
	60	45000	4.65321	29000	4.4624	4.55781	0.13493
mid-log	0	1120	3.04922	1140	3.0569	3.05306	0.00544
	10	<10	1	<10	1	1	0
	60	<10	1	<10	1	1	0

Table A17 Antilisterial activity of the polymeric phenolic fraction against anaerobically grown *Listeria monocytogenes* and exposed cells grown on TSA plates aerobically and anaerobically.

Assay solutions	Exposure time (mins)	CFU/ml Rep 1	log CFU/ml Rep 1	CFU/ml Rep 2	log CFU/ml Rep 2	Mean log CFU/ml	Std dev
MWS anaerobic/ anaerobic	0	143000	5.155336	170000	5.230449	5.192892	0.053113
	10	194000	5.287802	233000	5.367356	5.327579	0.056253
	60	186000	5.269513	142000	5.152288	5.210901	0.08289
Polymer anaerobic/ anaerobic	0	155000	5.190332	160000	5.20412	5.197226	0.00975
	10	49000	4.690196	45000	4.653213	4.671704	0.026151
	60	1060	3.025306	600	2.778151	2.901729	0.174765
MWS anaerobic/ anerobic	0	178000	5.25042	196000	5.292256	5.271338	0.029583
	10	110000	5.041393	41000	4.612784	4.827088	0.303072
	60	31000	4.491362	40000	4.60206	4.546711	0.078276
Polymer anaerobic/ aerobic	0	133000	5.123852	10000	4	4.561926	0.794683
	10	260	2.414973	590	2.770852	2.592913	0.251644
	60	<10	1	<10	1	1	0

Appendix B IES-MS Spectra generated from Polymeric Phenolic Fraction G