Promising natural extracts for use in active food packaging

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For my grandparents

Nana (1936 – 2019), Grandad, Oma, and Opa (1926 – 2003)

Abstract

Food waste is a significant problem worldwide, with approximately one third of all food produced spoiling before consumption. One way that this food spoilage problem can be addressed is through active packaging – packaging that does more than act as a passive barrier. By introducing antioxidant and antimicrobial compounds into the packaging, bacteria and free radical scavengers can be prevented from affecting food. However, consumers are becoming more environmentally aware and the acceptability of synthetic compounds is declining. Natural extracts that still provide the antioxidant and antimicrobial benefits are one solution. Added value can be attained by using waste products to produce these extracts. Winery waste, also known as grape marc, is the by-product of the wine industry. It consists of grape skins, seeds, and stems, and has a high phenolic content, which is known to provide antioxidant and antimicrobial activity.

The aim of this thesis was to explore the properties of natural extracts and their potential application in active food packaging. To this end, grape marc was used to produce grape marc extracts (GME) rich in polyphenols. First, different extraction methods were compared, and a conventional solvent extraction was used due to its simplicity and low-cost. Biodegradable and bio-sourced films were then used as proof of concept for GME activity in films. Ethyl cellulose, fish gelatin, and soy protein isolate (SPI) films were produced at low temperature and showed good free radical scavenging with the addition of 2% GME. Ethyl cellulose films also showed activity against *Staphylococcus aureus*.

Most of the packaging used worldwide is linear low-density polyethylene (LLDPE) or high-density polyethylene (HDPE). These polymers are processed at temperatures above 150 ˚C, and so the heat tolerance of GME for use with these polymers was investigated. The

heating of GME to temperatures up to 200 ˚C did not significantly degrade the phenolic compounds, nor did it affect the free radical scavenging ability or antimicrobial activity against *S. aureus* or *Escherichia coli*. A small amount of degradation was found in GME heated to 250 ˚C.

Finally, the work with GME made way for an investigation into the potential development of antifungal active packaging. A wide range of natural extracts and essential oils (EO) were investigated for their activity against the key microorganisms in bread spoilage: fungi *Aspergillus brasiliensis* and *Penicillium citrinum*, and yeast *Pichia burtonii*. Six extracts or oils were shortlisted for further testing, chosen for their activity, New Zealand connection, and heat tolerance. Finally, three extracts were incorporated into LLDPE films. One essential oil was then discounted due to sensory issues that would be incompatible with the target application. However, the antifungal activity did not transfer to the films, although the films did show improved free radical scavenging abilities.

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Table of Contents

1 Literature Review

There are a wide variety of topics that need to be introduced in order to understand the context of the following research. The overall aims and research included in the thesis range from the valorisation of winery waste and its applications, to the potential for active packaging to decrease food spoilage, specifically in bread. The overarching theme is that of making the most of food, by reusing waste streams and decreasing waste.

First, the wine-making industry in New Zealand is described, followed by the phenolic content of grapes and the antioxidant and antibacterial properties that come from these. Antifungal natural extracts and essential oils are also of interest. Global food spoilage, especially that of bread, which can be mitigated by active packaging will also be introduced.

1.1 Wine making in New Zealand

Wine made in New Zealand is a valuable product for the country and is the country's sixth most valuable export sector. In the 2018 financial year, the value of exports of New Zealand wine was $1.7 billion, an increase of 2.5% from the previous year. The value of these exports has grown every year for 23 consecutive years.[1] Wine exports are not the only way that wine-makers contribute to New Zealand's economy. In 2017, an estimated $3.8 billion was spent by over 700,000 international 'wine tourists' around the country and over 10,000 people are employed directly by the wine industry.

The annual grape harvest in 2018 was 419,000 tonnes, with an average of 11.1 tonnes per hectare. The major regions producing wine in New Zealand are the Hawke's Bay, Marlborough and Central Otago. Sauvignon Blanc wine is the most prevalent wine variety produced in New

Zealand and of all the wine exported from New Zealand in 2018, 86% were this variety. There were 697 wineries in 2018, with 699 individual grape growers.[1]

New Zealand's global reputation rests on its image as a 'clean and green' country. New Zealand's Ministry for the Environment estimates the value of this clean green image to be billions of dollars a year. The biggest risks to New Zealand's image include the degradation of freshwater quality from intensive agricultural land use, soil erosion and the resulting visual impact on the environment.[2] The Sustainable Winegrowing New Zealand programme was one of the first sustainability programmes established in the international wine sector. In 2016 the first Sustainability Report was published by the New Zealand Winegrowers Association, reporting that 98% of the country's vineyard producing areas were certified by the programme. The key areas of focus include biodiversity, by-products, water, energy, pest and disease management, soil, and air.[3]

1.2 Grapes

Grapes are the berry or fruit of the grapevine; whose juice is essential for wine production. It belongs to the genus *Vitis,* which is one of 14 genera in the family *Vitacae.* There are two subgenera within the genus *Vitis*: *Muscadinia* and *Euvitis* (now more commonly known as *Vitis*), with chromosomes $2n = 40$ and $2n = 38$, respectively.[4] The most important grape species for wine production is *Vitis vinifera*, one of around 60 species of the *Vitis* genus. Wine production accounts for around 80 % of the world's grape growing.[5]

The outside layer of the skin of the grape is made of wax plates and cutin, which both protect the berry from biological attack and prevent water loss from within the berry (Figure 1.1). Below this layer are the cell layers: first the true epidermis, then the hypodermis, which consists of around seven cell layers, depending on the variety, which contain most of the berry pigments. Skins can vary in thickness from 3 to 8 μm and can be between 5 and 12 percent by

weight of a mature berry, subject to the variety. The skin of the grape is used in winemaking when making red or pink wine. The strength of the colour depends on the length of the contact time and the colour of the berry.[5]

The flesh or pulp is the most important part of the grape to winemakers, along with the juice. The pulp contains the juice within the vacuoles of the pericarp (berry) cells, but no pigments.[6] The pulp has three tissues: the outer mesocarp, the inner mesocarp, and the vascular tissue separating the two. The vascular tissue itself is made up of xylem, phloem, and vascular parenchyma.[6]

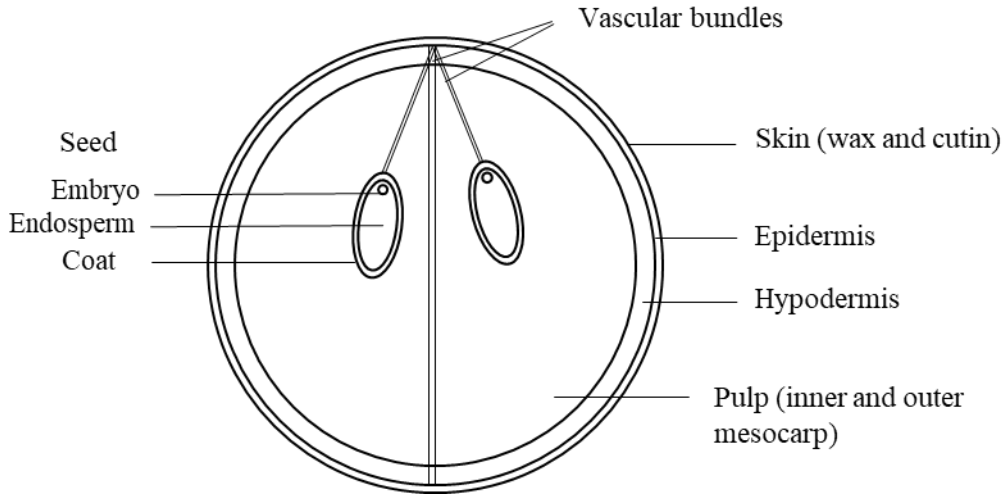

Figure 1.1. Cross-section diagram showing different structural parts of a grape.

The seeds of a grape vary in size and number depending on the variety of berry. Due to human selection, the seeds of the *Vitis vinifera* species normally make up only a small proportion (around 10 %) of the berry weight compared to other species. Seeds are of minimal use in winemaking, although they are known to be a source of tannins and of edible and industrial oil.[5]

1.3 Grape phenolics

Grapes, along with other fruits and vegetables such as cranberries and onions, are high in polyphenol content. Polyphenols are secondary metabolites, meaning they are not essential to the growth and development of the plant, though their functions are still very important. They are produced by plants in response to outside stressors, such as animal attacks, environmental conditions and to fight against fungal infections. Polyphenols are synthesised via two primary pathways: the polyketide and shikimic acid pathways.[7]

Polyphenols are a very large chemical group of which phenol (C_6H_5OH) is the basic building block. They were originally called 'vegetable tannins' due to plant matter containing the polyphenols being used in the process of turning animal skins into leather, or 'tanning'.[8] The stem, skin, and seeds of grapes are particularly rich in phenolics, although the juice and pulp also contain them.[5] In general, the skin contains pigments and tannins and the seeds contain tannins.[6] The phenolic content of grapes plays an important part for both grapes and wine, responsible for aroma, flavour, colour, astringency and bitterness.[9,10] Different phenolic compounds have different effects on these properties and are present in varying concentrations both within the grape, depending on the variety and environmental stressors experienced by the plant. In general, phenolics in grapes can be divided into two categories, flavonoids and non-flavonoids. Non-flavonoids can be further categorised as stilbenes, and phenolic acids, which include both hydroxybenzoic and hydroxycinnamic acids. The major polyphenols found in grapes include catechin, epicatechin, epicatechin gallate, procyanidin dimers, trimers, and larger oligomers, gallic acid, caftaric acid, quercetin glycosides, resveratrol, and anthocyanins (found in red grapes).[11]

1.3.1 Phenolic acids

Both hydroxybenzoic and hydroxycinnamic acids are present in grapes and wine. Hydroxybenzoic acids (Figure 1.2) have a C6-C1 skeleton and are hydroxyl derivatives of benzoic acid.

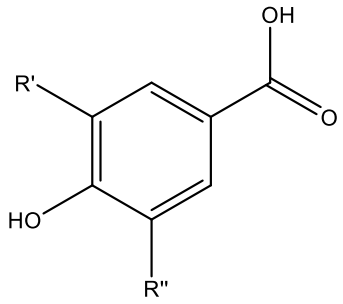

Figure 1.2. General structure of hydrobenzoic acids.

The most common hydroxybenzoic acids in grapes are vanillic, gallic, syringic, *para*-hydroxybenzoic, and protocatechuic acids. Of these, gallic acid (Figure 1.3) is the most important as it is a major component of both hydrolysable and condensed tannins, either as a precursor or as a part of their structure.

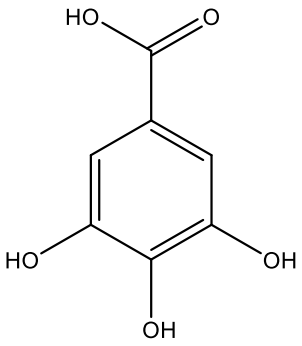

Figure 1.3. Structure of gallic acid.

Hydroxycinnamic acids (Figure 1.4), on the other hand, have a C6-C3 skeleton and are hydroxyl derivatives of cinnamic acid. They are present in grapes as tartrate esters of caffeic,

ferulic, sinapic, and *para*-coumaric acids, among others, and as free forms in wine. They are predominantly *trans* isomers, though some *cis* isomers have been reported in small amounts.[6] These are connected to the wine browning process and are precursors to volatile phenolic compounds.[10] Hydroxycinnamates are the third most abundant type of soluble polyphenols in grapes, and are found in the hypodermal skin cells and in the mesocarp and placental cells of the pulp.[6]

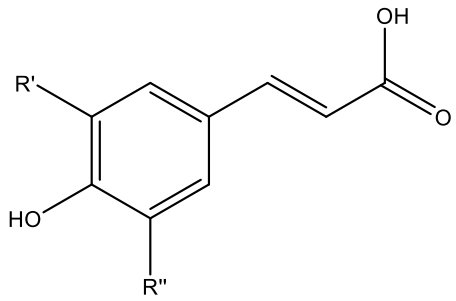

Figure 1.4. General structure of hydroxycinnamic acids.

1.3.2 Stilbenes

Stilbenes (Figure 1.5) are composed of two aromatic rings linked via an ethane bridge.[10] They have been identified in many plant species other than grapes, including cocoa (*Theobroma cacao L.*), peanut (*Arachis hypogaea L.*), strawberry (*Fragaria x ananassa Duch.*), sugar cane (*Saccharum spp.*), and cranberry (*V. macrocarpon*). Stilbenes identified in grapes and wine include resveratrol, piceid, piceatannol glucoside (or astringin), palliodol, parthenocissin, pterostilbene, and ameurensin G.[10,12] Viniferins are present in large amounts in the woody parts of the vine, where they are thought to protect against wood decay.[5]

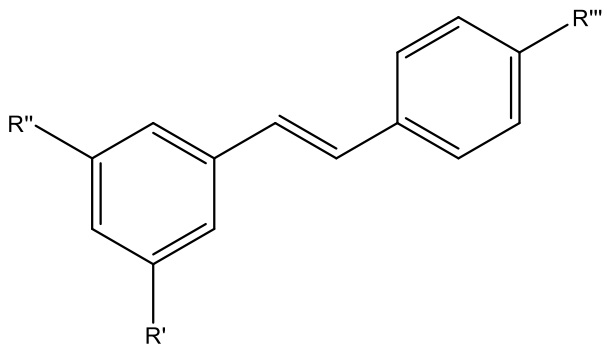

Figure 1.5. General structure of stilbenes.

The most common stilbene present in grapes is resveratrol (3,5,4'-trihydroxystilbene) (Figure 1.6). It is present in the skin of grapes and has also been found in the vine leaf. Its synthesis is affected mainly by the grape variety, but also by other factors such as climate, soil, and canopy management.[13] Wines made from Muscadinia grapes or from the variety Pinot Noir usually have high levels of resveratrol, while Cabernet Sauvignon has lower levels. In terms of climate, wines produced in cooler regions usually have more resveratrol present when compared to wines from hot, dry climates such as Australia.[5] Resveratrol may be produced by the plant in response to abiotic stress (as is common for polyphenols), or it could be a toxin produced due to fungi infection such as *Plasmopora viticola* or *Botrytis cinerea*.[10] It is known that upon grape maturation, the concentration of resveratrol decreases significantly. Red wines contain about ten times the amount of resveratrol when compared to white wines, due to the maceration process and the fact the compound is primarily found in the grape skin.[5]

The health benefits of resveratrol have been the subject of much research and public interest, including its involvement in the French Paradox, and its potential action against cancer and Alzheimer's disease.[14]

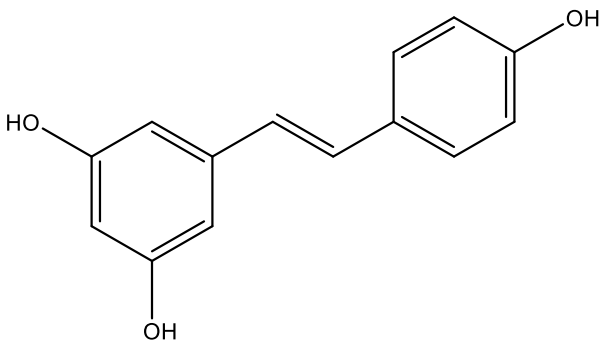

Figure 1.6. Structure of resveratrol.

1.3.3 Flavonoids

Flavonoids (Figure 1.7) are a class of compound with 15 carbons comprising two phenyl rings and one aromatic ring (C_6-C_3-C_6). The classes of flavonoids mainly differ in the degree of oxidation of the central pyran ring. They can be present in either the free or conjugated form and are often esterified through hydroxyl groups to one or two sugar molecules. The hydroxyl group used in the *O*-glycosylation process depends on the type of flavonoid. *C*-glycosides can also occur, where the sugar molecule is connected directly to the flavonoid skeleton through a C-C bond. *O*-glycosides are more likely to undergo hydrolysis than *C*-glycosides.[10]

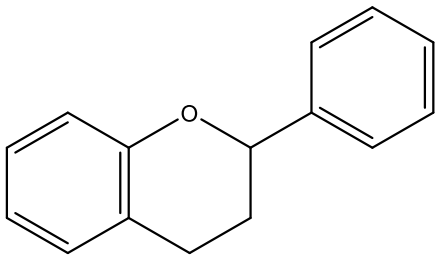

Figure 1.7. General structure of flavonoids.

1.3.3.1 Flavonols

Flavonols (Figure 1.8) have a 3-hydroxyflavone (3-hydroxy-2-phenylchromen-4-one) backbone, with different flavonols having different positions at which phenolic hydroxyl groups occur. Quercetin is one of the main flavonols found in wine, as well as kaempferol, isorhamnetin, myricetin, laricitrin, and syringetin.[10] In grape berries, the flavonols are present as the corresponding glucosides. Quercetin is found in large amounts in vine leaves, and can be used as an indicator of the exposure of the berry cluster to the sun, as increased UV exposure increases the concentration of quercetin in grape berries.[5] Flavonols are present in lower concentrations than other polyphenols, such as tannins, flavan-3-ols, anthocyanins, and phenolic acids.[6] In grapes, flavonols are found in highest concentration at flowering, and their concentrations decrease as grapes grow in size.[10]

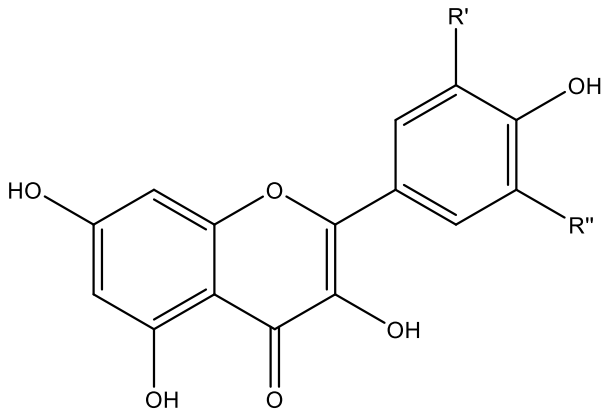

Figure 1.8. General structure of flavonols.

1.3.3.2 Anthocyanic pigments

Anthocyanins (Figure 1.9) are based on the flavylium cation (2-phenylbenzopyrylium).[10] They are responsible for the colour of grapes and of wine, which is determined by their chemical structure. This includes their degree of hydroxylation, glycosylation, and methylation.[10,15] Anthocyanidins in red grapes include delphinidin (bluish red), pelargonidin

(orange), peonidin (red), cyanidin (orange red), petunidin and especially malvidin (bluish red).[10,15,16] Anthocyanins are glycosides of anthocyanidins. The pigments are only found in the skin of the grape, in the hypodermal cells. The amounts and types of pigments present in the berry depends on the variety and whether it produces acylated pigments. Anthocyanins also have separate chemical forms based upon acid-base equilibria at vacuolar pH, notably the red-colored flavylium cation form and the blue quinonoidal anhydro base, with the ratio of these forms highly dependent on pH.[6]

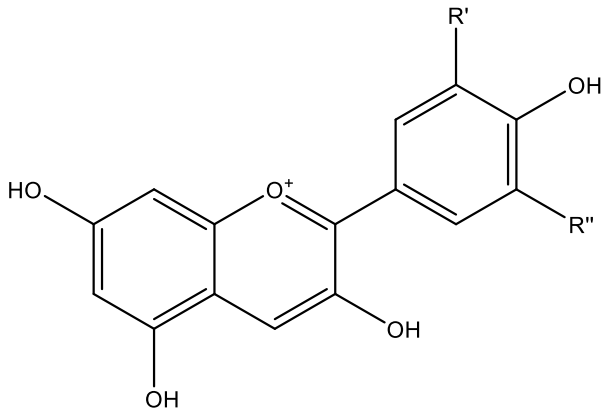

Figure 1.9. General structure of anthocyanidins.

1.3.3.3 Flavan-3-ols

In grapes, catechin and epicatechin are the two most abundant flavan-3-ols present. They are epimers, differing around the two stereocentres, with catechin having a 2,3-*trans* configuration and epicatechin having a 2,3-*cis* configuration. The general structure of flavan-3-ols is seen in Figure 1.10. This stereochemistry is established during synthesis via the chalcone isomerase reaction, which produces a flavanone, which is then used to produce a flavanonol. The flavan-3,4-diol produced from the flavanonol by dihydroflavanol reductase is then converted directly to catechin by a leucoanthocyanidin reductase (LAR). Epicatechin, in contrast, is produced from cyanidin by the enzyme anthocyanidin reductase (ANR), which

converts cyanidin and delphinidin to epicatechin and epigallocatechin, respectively. The flavan-3,4-diol is converted to the corresponding anthocyanidin by anthocyanidin synthase (ANS). As both catechin and epicatechin, along with epigallocatechin, are used as subunits in condensed tannins, this suggests that anthocyanidins are important intermediates in the synthesis of condensed tannins.[6]

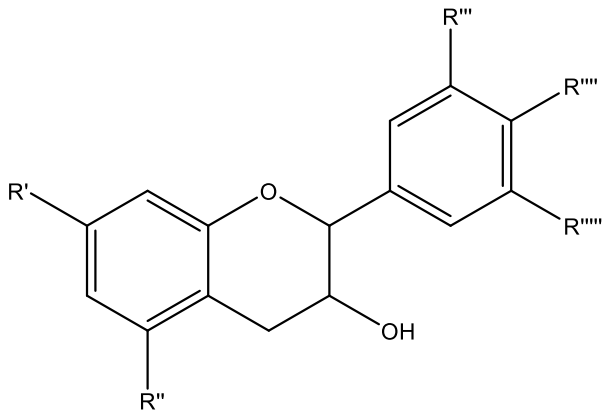

Figure 1.10. General structure of flavan-3-ols.

1.3.4 Tannins

Tannins in general have the ability to interact with proteins; the name derives from the process of tanning animal hides. Large tannins impart a sensation of astringency, via interacting with proteins in the mouth. Tannins play an important role in the ageing of wine, especially when pigmented tannins are required for red wine.[5]

1.3.4.1 Hydrolysable Tannins

Hydrolysable tannins are made up of gallic acid moieties to which are attached to a central carbohydrate, glucose or quinic acid, and typically have a molecular weight in the 500 to 2800 g/mol range. They are found at low levels in *Vitis vinifera* grapes but in higher levels in the seeds of Muscadine grapes. Hydrolysable tannins are able to be degraded to smaller fragments such as sugars and phenolic acids via change to lower pH and enzymatic and non-

enzymatic hydrolysis.[10] Grapes contain gallotannins, a class of hydrolysable tannins so called due to their ability to hydrolyse in gallic acid. Ellagitannins are introduced into wine via maturation in oak barrels.

1.3.4.2 Condensed Tannins

Condensed tannins, also known as proanthocyanidins, are much more common in grapes than hydrolysable tannins. They are oligomeric or polymeric structures consisting of monomeric compounds of flavan-3-ol molecules connected through carbon-carbon bonds. The most common of these monomers in grapes is epicatechin. Condensed tannins are found in the hypodermal layers of the skin and in the soft parenchyma of the seed. Skin and seed condensed tannins can be differentiated, whereby skin tannins are generally much larger and contain epigallocatechin as a monomer, while seed tannins contain higher proportions of epicatechin gallate monomers.[6] They can yield coloured anthocyanidins upon degradation under acidic conditions.

Condensed tannins found in *V. vinifera* grapes are largely made up of monomers of (-)-epicatechin and (+)-catechin through C4-C8 bonds, although C4-C6 bonds can also be present.[10] The exact mechanism of the synthesis of condensed tannins from these monomers is not yet fully understood. Condensed tannins range in size from dimers or trimers to molecules of more than 30 subunits.[6] The placement of the hydroxyl group is important, as the properties of the tannins can change with different placements. A hydroxyl group at position 5 of the B ring makes the tannin better able to complex with proteins.

1.4 Phenolic characterisation

1.4.1 Structural analysis

Grape phenolics, specifically tannins, can be characterised by vibrational spectroscopic techniques such as Fourier Transform Infrared (FTIR) spectroscopy. FTIR is an attractive technique that is non-destructive, fast, and cost effective. It depends on the fact that most molecules absorb light in the IR region. Infrared spectroscopy can be separated into three regions according to their wavelengths: far-infrared (400 – 10 cm^{-1}), mid-infrared or MIR (4000 – 400 cm^{-1}), and near-infrared or NIR (10000 – 4000 cm^{-1}).

Both MIR and NIR regions are commonly used in food science applications. FTIR and MIR are often used interchangeably, though it is more accurate to use FT-MIR when using the MIR region and FT-NIR when using the NIR region. NIR radiation penetrates deeper into the sample than MIR radiation, which means that MIR spectroscopy is more sensitive. NIR can therefore be more useful when analysing bulk samples with little to no sample preparation that absorb strongly. However, it can be more difficult to analyse NIR data, as the region contains broad and weak overtone and combination bands that are harder to associate with chemical groups.[17] Grape phenolics have been analysed by NIR when looking at intact samples, such as whole grape seeds and whole grapes.[18,19] MIR with attenuated total reflection (ATR) is usually used for samples such as liquid, powder, or purees, due to the strong light absorption and intense fundamental bands. In addition, MIR region contains the bands which are characteristic of chemical molecules and structures, which makes it easier to identify and quantify the individual components of a sample.[20] Therefore, when analysing grape marc extract powder, FT-MIR is used.

The infrared radiation is passed through the sample with some absorbed by the sample and some transmitted through it. The signal at the detector then gives the molecular ‘fingerprint’ of

the sample. Basically, when a sample is exposed to IR radiation at a particular frequency, the atoms of a molecule vibrate at that frequency, which results in the stretching of bond lengths and bending of angles. Vibrational excitations in molecules occur at characteristic frequencies depending on the bond involved. The detector then measures the absorption of the IR radiation by the sample, which gives a spectrum peak. Each bond type in a molecule can be excited at a different characteristic frequency. The stronger the bond, the more energy is required to vibrationally excite it, leading to a more distinct peak.

There are two regions of interest when analysing FT-MIR spectra: the functional groups region (4000 to 1800 cm^{-1}), which corresponds to IR-active stretching frequencies of molecular compounds, and the fingerprint region (1800 to 400 cm^{-1}).[21] In the fingerprint region, the frequencies correspond to combinations between the vibrational modes of parts of the molecules. This is important because it can give information about the position and number of different functional groups present.

FT-MIR can give important information about the polymerisation of tannins. An example spectrum is shown in Figure 1.11 and a selection of peaks of interest for the analysis of tannins is given in

Table 1.1. A wide distribution of degree of polymerisation of tannins in a sample gives rise to a broad band at 3700 to 3000 cm^{-1}.[21] This is due to -OH stretching bands from -OH groups in different positions of molecules that have different degrees of polymerisation. The more diverse the sample, the broader this band. Deconvolution of this –OH band can give information about the chemical interactions of the tannin molecule. The C-H stretching region for aromatic compounds is from 3100 to 2800 cm^{-1}, which overlaps the –OH band. This stretching band normally appears as a weak shoulder.

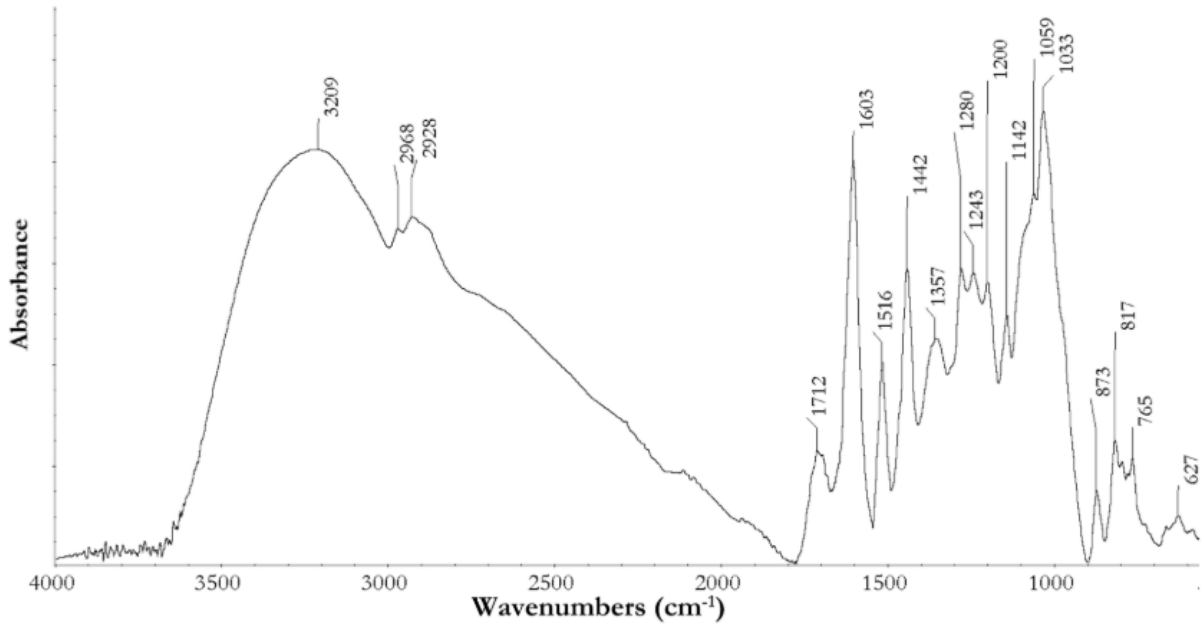

Figure 1.11. Example ATR-FTIR spectrum of grape extract labelled with identifying bands.[22]

Table 1.1. Vibrational spectral frequencies and their assignment to common features of tannins in the solid state.[21]

Peak (cm^{-1})	Assignment
615	C-O out-of-plane bending
670 – 900	Aromatic CH out-of-plane bending (multiple peaks)
675	C-OH bending
750	H-bonded OH stretching in carboxylic groups
770	CC, CH, C-O, and ring vibrations
835	Deformation aromatic hydroxyl groups/CH deformation aromatic ring
875	Ring deformation/breathing
930 – 780	CH_2OH bending
999	Stretching CC and bending CC
1015	C-O stretching aliphatic secondary alcohol, stretching C-C and bending C-H
1040 – 1037	Symmetric stretching C-O
1064	OH bending vibration
1082	CH out-of-plane deformation and OH out-of-plane deformation
1150 – 1350	Combination C-O stretching and OH bending vibrations
1175	C-O stretching and C-OH deformation
1200	C-OH bending
1283	Stretching C=C aromatic compounds
1390 – 1310	C-OH deformation of phenols
1450	Stretching OH, stretching C-O-C, bending OH
1510, 1470	Stretching C=C aromatic compounds
1650 – 1430	Aromatic C=C bondings
1745	C=O stretching, catechinic acid rearrangement during extraction process
3100 – 2800	Aromatic CH stretching
3600 – 3000	OH stretching

1.4.2 Phenolic antioxidant capacity

Antioxidants scavenge free radicals, removing potentially dangerous oxidising agents from the surrounding environment. This is significant, as the accumulation of reactive oxygen species (ROS) may cause the modifications of cellular molecules such as lipids, proteins and nucleic acids. Damage by ROS to DNA causes mutagenesis and aging. Phenolic compounds

have good antioxidant activity, which can be measured through various antioxidant assays. In general, the higher the total phenolic content, the higher antioxidant activity. The degree of antioxidant activity of classes of polyphenols depends on the redox properties of the phenolic hydroxy groups and the potential for electron delocalisation.[23]

The antioxidant and chemo-preventive properties of resveratrol have been documented by the scientific community.[24,25] It also has shown some beneficial effects against Alzheimer's disease. Three antioxidant mechanisms have been suggested for resveratrol: a) scavenging oxygen radicals formed in the mitochondria, b) inhibition of lipid peroxidation, and c) competition with the site of ROS generation.[26] Resveratrol can scavenge both oxygen and hydroxyl radicals.[27–29]

Flavonoids show antioxidant, antimicrobial, anti-viral, anti-carcinogen, and neuro-protective properties.[30] They scavenge free radicals by electron transfer processes. The antioxidant properties of flavonoids are due to the aromatic -OH groups in the structure.[31] Flavan-3-ols such as catechin have shown protective effects against lipid peroxidation and low-density lipoprotein oxidation.[32]

The antioxidant effect of tannins is due to the ability of the hydroxyl groups to form hydrogen bonds with free radicals. As such, tannins are an important source of radical oxygen scavengers. As reactive oxygen species have been found in many disease states, tannins may be useful as bioactive radical scavengers. One issue is the bioavailability of tannins, as their size prevents them from effectively crossing through the intestinal mucosa. However, tannins must be hydrolysed by enzymes, or metabolised by gut microflora, in order to obtain molecules that can be absorbed.

Gallic acid is a good antioxidant in emulsion or lipid systems.[33,34] It is used in the food industry as an active packaging additive and within food to prevent spoilage or rancidity caused

by lipid peroxidation.[35] It can be used as a standard in antioxidant electron transfer assays, where the tested compound is expressed in terms of 'gallic acid equivalents' (GAE).

The gallic acid groups found in hydrolysable tannins provide active hydroxyl sites which may be used for antioxidant activity. Hydrolysable tannins have high bioactivity, which is associated with their ability to be absorbed. The tannin is broken into its gallic acid monomers upon acid hydrolysis. Gallic acid with a triphenolic group is a very good scavenger of free radicals. Gallic acid has also showed anti-inflammatory and antimicrobial effects, and has shown some activity against cancer cell lines.[36–38]

Condensed tannins are able to form complexes with metal ions and macromolecules. This is mostly due to the properties of the tannin phenolic nuclei.[39] The ability of condensed tannins to chelate metal ions such as iron, copper, and zinc, can give potent health benefits. By binding to copper ions to prevent their absorption, the likelihood of copper binding to select polyphenols is lowered, which can lead to an increase in DNA cleavage.[40] Preventing the absorption of iron in the form Fe^{3+} leads to the prevention of iron being reduced to Fe^{2+} in cells via the Haber-Weiss/Fenton reactions, which then form the hydroxyl radical, and radical oxygen, which leads to oxidative stress. Ortho-dihydroxyl groups, the presence of a 5-OH and/or 3-OH with a C4 keto group and larger numbers of hydroxyl groups are important in the iron chelation process.[41] Condensed tannins contains numerous such organic functional groups, and so are well suited to iron chelation.

Potential antioxidant activity can be assessed through various test procedures. Antioxidants react through either free radical or molecular oxygen quenching, which delays or inhibits the oxidation process. Spectrophotometric techniques, such as the DPPH and ABTS assays, measure the reaction of a radical molecule with an antioxidant that can donate a hydrogen atom.

The DPPH (2,2-dephenyl-1-picrylhydrazyl) assay uses $DPPH^{\cdot}$, a stable free radical which does not dimerise. This is due to the delocalisation of the spare electron over the entire molecule. It has an absorption band with a maximum at 515 nm, presenting as a purple colour. When the free radical reacts with the antioxidant hydrogen donor, it is reduced to the stable form DPPH, leading to a change in colour to yellow. The change in absorption is linearly dependent upon the antioxidant concentration.[42]

The ABTS (2,2'azino-bis(3-ethylbenzthioazoline-6-sulfonic acid)) assay uses $ABTS^{\cdot+}$, a blue-green cation radical that absorbs at 743 nm. It is formed by the loss of an electron on the nitrogen atom by oxidation of ABTS by potassium persulfate or manganese dioxide. When $ABTS^{\cdot+}$ reacts with a hydrogen donating antioxidant, the solution decolourises.[42]

1.4.3 Phenolic antimicrobial properties

The antimicrobial activity of polyphenols has been investigated at length. Flavan-3-ols, flavonols, and tannins have received more attention that other polyphenols due to their higher activity level and wider spectrum activity against microorganisms.[43] Catechins have shown activity against *Vibrio chloreae*, *Streptococcus mutans*, *Campilobacter jejuni*, *Clostridium perfringes*, and *Escherichia coli*.[44–48]

Flavonols have shown good activity against both Gram-positive and Gram-negative bacteria. Although different methods of action have been identified, the most accepted is causing an aggregatory effect on the cells of the bacteria.[43,49] As for stilbenes, resveratrol has shown activity against *Aspergillus brasiliensis* and *Penicillium expansum*.[50]

Condensed tannins, or proanthocyanidins have well established antimicrobial activity. Condensed tannins have better activity against foodborne pathogens than their monomeric constituents. In a recent study of grape seed extract with high levels of condensed tannins, the

observed inhibition zone against fourteen bacteria ranged from 2.4 to 15.5 cm, whereas catechin ranged from 1.6 to 8.4 cm at an equal dose against only five bacteria.[51]

In a study by Mayer et al., oligomeric units of catechin and epicatechin were active against *S. aureus*, *P. aeruginosa*, *St. pneumonia*, *St. pyogenes*, *Klebsiella* sp., *E. coli*, *H. influenzae*, *S. epidermidis*, *En. faecalis* (VRE) and *En. casilliflavus* (VRE), while a fraction containing only monomers such as catechin and epicatechin only showed antibacterial activity against *P. aeruginosa*. The oligomeric fraction showed activity even at concentrations four times lower than that of the monomeric fraction.[52] This demonstrates the common trend, where antibacterial properties improve with the degree of polymerization.

Scalbert summarized the antimicrobial properties of tannins and demonstrated that there were three likely mechanisms for the inhibition of growth of microorganisms.[9] Firstly, the astringent character of tannins can cause the inhibition of extracellular enzymes. Tannins can also act directly on the microorganism membranes or form complexes with metal ions that affect microbial growth. Condensed tannins can potentially inhibit key enzymes by binding with proteins via hydrogen bonds between the carbonyl group of the peptide in the protein and the phenol group of the tannin. As the degree of polymerization increases, more phenol groups can form hydrogen bonds with the protein, maximizing this 'cross bridging effect'. However, as condensed tannins become too large, the bulk of the tannin prevents most of the phenol groups from forming hydrogen bonds with the protein. Thus, the compound can become too large to access key sites in the microorganism, causing it to lose toxicity. According to Scalbert,[9] oligomeric tannins are the most effective at inhibiting microorganisms, as they are large enough to ensure effective cross-linking, but small enough to reach microbial proteins.

Grape tannins are also active against the bacteria *Listeria monocytogenes*, which is a foodborne pathogen that can result in listeriosis, which has a 20-30 % mortality rate. Along

with *E. coli* O157:H7, *Listeria monocytogenes* is one of the main food borne pathogens that cost billions of dollars each year in healthcare and lost productivity.[53] In one study, phenolic fractions were extracted with ethanol and separated from the skin, seed and pulp of *Vitis vinifera* variety Ribier grapes.[54] The extracts and the polymeric phenolic fractions were assayed against *L. monocytogenes* ATCC 35152, *S. aureus* ATCC 35923, and *E. coli* ATCC 25922. The skin and seed extracts showed strong antilisterial activity, while the pulp extract showed no inhibition of the bacteria. The polymeric phenolic fractions were slightly inhibitory to *S. aureus* and *E. coli*, but this was only a small effect compared to that exhibited towards *L. monocytogenes*. The skin, seed and pulp of *Vitis vinifera* grapes were also tested for antimicrobial activity. Individual polyphenols, including procyanidin B2 and epicatechin gallate, showed inhibition against *L. monocytogenes* bacteria. However, the individual polyphenols had a significantly lower effect than the respective total extracts. The extracts were very active against the strain, especially at a 1% *w*/*v* concentration, while the procyanidin fractions showed much lower activity.[55] This suggests that there are possible synergistic effects taking place within the total extracts that are not seen with individual polyphenols.

1.5 Grape marc

Grape marc is the skin, seeds, and stems of grapes remaining after the wine making process. In New Zealand, there is over 50,000 tonnes of grape marc produced each year, most of it in the Marlborough region. Typically, of the grape harvest, grape marc consists of about 25% by weight.[56,57] The production of 6 L of wine is estimated to produce approximately 1 kg of grape marc, which can be extrapolated to 10.5 to 13.1 Mtons worldwide each year.[58] Grape marc can be used as a compost, for animal feed, to produce bioethanol, and for extraction of phenolic compounds.[59–63]

There are associated environmental hazards involved in the storage of grape marc, which can restrict its usage for wineries.[64] Prior to the 1990s, it was most economical for wineries to pay a disposal fee in order to remove grape marc. However, the fees and fines for unauthorised disposal have increased significantly and it is now important for wineries to look for alternative methods of disposal and to make better use of the waste stream.[65]

1.5.1 Grape marc extract

Grape marc extract is the polyphenol-rich powder that results from various extraction processes undertaken upon grape marc. Both red and white grape marc can be used to produce grape marc extract.

1.5.2 Extraction methods

The extraction of polyphenols from grape marc is dependent on a range of factors, including the properties of the marc, solvent type, temperature, pressure, and exposure time.[66] The particle size of the marc is an important property and can have a significant effect on polyphenol extraction. Smaller particle sizes allow the solvent to come into contact with a greater surface area of the marc. This is especially important in conventional extraction methods.

There are several different methods of extraction. Conventional extraction methods involve solvent contact with the grape marc, with polyphenols extracted into the solvent phase. Solid-liquid extraction is the most common conventional method of extracting polyphenols from grape marc. This is where the grape marc is mixed with the chosen solvent at a particular temperature for a defined time period. This method requires a large amount of solvent, however, as well as a long extraction time.

Non-conventional methods may involve solvent extraction in combination with techniques that can improve the efficiency of the extraction. Non-conventional extraction methods include

microwave-assisted extraction (MAE), ultrasound assisted extraction (UAE), pulsed electric field assisted extraction, enzyme-assisted extraction, pressurised liquid extraction (PLE), supercritical CO2 assisted extraction and supercritical fluid extraction (SFE). In general, non-conventional methods are more environmentally friendly, giving an extract with better yield and quality, while needing less operation time and chemicals.[67]

Microwave-assisted extraction uses microwave energy to extract polyphenols into the solvent. The rapid heating of the solvent can cause cell wall disruption, leading to the release of polyphenols. The solvent used in MAE must be chosen carefully due to this rapid heating. Suitable solvents for MAE should have a high selectivity towards the compounds of interest, as well as a high dielectric constant and dissipation factor.[67,68] Water has a high dielectric constant but a low dissipation factor and so alone should not be considered as a suitable solvent for MAE.[68] MAE has been shown to successfully extract polyphenols from grape marc and grape seeds.[69,70] It provides similar or better extraction when compared with other extraction methods, but over a much shorter time scale.[70]

Ultrasound-assisted extraction is another environmentally friendly technique. Ultrasounds pass through media via cycles of compression and expansion, which causes a phenomenon called cavitation, where microbubbles are created, which enlarge and implode within the liquid medium. These bubbles implode during the compression phase after reaching a critical point, creating a transitory hot spot. When this occurs close to the surface of plant material, a microjet is created, which aids in the extraction of compounds.[71]

Pulsed electric field assisted extraction (PEF) operates by destroying cell membrane structures in order to increase extraction efficiency. An electric potential is passed through the membrane of the cell, and molecules are separated according to their charge, due to the dipole nature of membrane molecules. Repulsion between these charge carrying molecules causes

pores to form in weak areas of the membrane, dramatically increasing the permeability. PEF treatment can be modified extensively by controlling the many process parameters, which include field strength, specific energy input, pulse number, and treatment temperature. Pulsed electric field extraction has been used successfully to extract phenolic compounds from grape waste. Corrales et al. (2008) compared PEF to other extraction techniques and found that phenolic compounds were best extracted by PEF, especially anthocyanin monoglucosides.[72]

Enzyme assisted extraction (EAE) involves pre-treatment of the sample with enzymes in order to recover phytochemicals that are not accessible using standard solvent extraction. Specific enzymes such as cellulose, pectinase, and α-amylase break down cell walls and hydrolyse polysaccharides. As EAE uses water as a solvent, it is an environmentally friendly extraction technique. Meyer et al. used EAE to extract polyphenols from grape marc and found that there was a correlation between the degree of cell wall breakdown by enzyme and the total phenolic yield. Pectinolytic enzymes were found to catalyse the degradation of grape marc polysaccharides, while cellulolytic enzymes did not.[73] Another study by Maier et al. also used EAE on grape marc to extract bioactive compounds. Using a mixture of pectinolytic and cellulolytic enzymes, they found that yields were higher with EAE than with sulphite-assisted extraction.[67,74] Gomez-Garcia et al. used the enzymes celluclast, pectinex, and novoferm in grape marc and found that novoferm had the strongest effect on phenolic release from the marc, followed by pectinex and then celluclast.[75]

Pressurised liquid extraction (PLE), is also known as pressurised fluid extraction, accelerated fluid extraction, enhanced solvent extraction, and high-pressure solvent extraction. PLE functions by applying high pressures to the system so that the solvent stays liquid above its normal boiling point. A combination of high pressure and temperature means that only small amounts of solvent are needed for the PLE processes. The extraction rate is improved by the higher temperature increasing the solubility and mass transfer rate, as well as decreasing the

viscosity and surface tension of the solvent. Due to the lower solvent use and faster processing, PLE is a popular extraction method to replace conventional solvent based extraction and is recognised as a green extraction technique.

Supercritical fluid extraction is performed using supercritical fluid as the extraction solvent. Supercritical fluid is defined as a substance above its critical temperature and pressure, where there is no clear distinction between liquid and gas states.[76] They can be used in place of organic solvents as a more environmentally friendly choice. Two key advantages of this technique are selective extraction, which is achieved by varying the temperature and pressure, and speed, which is due to the ability of supercritical fluids to diffuse rapidly into matrices. A common solvent used for the extraction of natural compounds is supercritical CO_2, which is non-toxic and can extract at relatively low pressure and temperature. As CO_2 is non-polar, addition of ethanol or methanol can allow the extraction of polar compounds. Casas et al. found that the addition of 5% ethanol, temperature of 35 ˚C, and pressure of 400 bar gave the optimal resveratrol extraction from grape marc.[77]

1.6 Global food spoilage

Up to one third of all food produced globally each year is lost or wasted before it is consumed. This amounts to around 1.3 billion tons of food annually.[78] Food waste has a negative environmental effect, contributing to excess consumption of fossil fuels and fresh water.[79] From 1974 to 2009, the per capita food waste in the United States increased by approximately 50% to 150 trillion kcal annually.[79] Most of the spoiled food, intended for human use, is caused by microbial contamination. Packaging is used to prevent microorganisms from gaining access to the food, and sterilisation techniques such as irradiation or high temperature treatment can kill the microbes.[80] In the last few decades, advances in

technology have led to the development of active packaging to assist in preserving food against spoilage.

1.6.1 Active packaging

There are two main types of packaging available today – passive and active packaging. Passive packaging acts as a barrier and a source of information to consumers, protecting the contents from the outside world while having a minimum interaction with the contents.[81] By contrast, active packaging responds to its environment in some way, providing further protection for its contents. Variations include materials sensitive to pH, UV light, temperature, and edible films.[82] Active packaging, and in particular green active packaging, is a popular draw card for consumers in light of modern environmental concerns. In the specific case of food packaging, active packaging materials have several major themes. These include using ethylene-, O_2- and CO_2-scavengers, moisture regulators, and the release of antioxidant and antimicrobial compounds.[82]

Oxidation of food and spoilage of food by moulds in the presence of oxygen are some of the main causes of food deterioration. Oxidative damage in foods includes rancidity of oils and fats, discolouration and loss of taste in both meat and plants, and nutrient loss. Removal of O_2 from packaging via vacuum packaging or modified atmosphere packaging is possible, but this does not prevent permeation of O_2 through the packaging, or indeed remove O_2 completely. Instead, O_2-scavengers can be added to packaging, which can prevent the growth of aerobic bacteria and moulds. By keeping the O_2-level below 0.1 %, the growth of many moulds can be avoided. However, O_2-free atmospheres can help the growth of anaerobic microorganisms.[82]

The level of moisture present in food can have a large impact on its shelf life. The presence of condensation or excess water in food packaging can cause many problems, including microbial spoilage and the decrease of consumer interest. Water evaporation can also favour

lipid oxidation or drying out of the food. Current strategies in this area include the use of desiccants and drip-absorbent sheets. Humectants can also be placed between layers of water permeable plastic to trap excess moisture. Common desiccants used include silica gel, CaO, natural clays and molecular sieves.[82]

The coating or incorporation of antimicrobial and antioxidant substances into packaging is another active packaging option. While there are many synthetic options available, in recent years consumers have indicated a preference for naturally sourced compounds.[83] Another way active packaging can be made more attractive for environmentally concerned consumers is through the incorporation of natural extracts. Natural extracts that have been investigated for this use include natural oils, such as rosemary and oregano, fruit extracts such as citrus and grapefruit, green tea, and barley husks.[84–90] However, there is a wide range of natural extracts that have still not been fully investigated as potential additives to active packaging, grapes among them.

1.6.2 Polyolefins

A polyolefin is any polymer that is produced from a monomer that is a simple alkene (formula C_nH_{2n}). Polyolefins can be both thermoplastics, which become mouldable upon heating and solidify upon cooling, and elastomers. Major polyolefins include polyethylene and polypropylene, which are both thermoplastics. Polyolefins first began to be developed in the early 1930s, when low density polyethylene was produced using free radical initiators. New discoveries through the 1950s led to the production of high-density polyethylene (HDPE) and better control of the production of new polymers. Polyolefins are now the most extensively used commercial polymers in the world, ranging in use from rubbish bags and containers, to tires and bullet proof jackets.[91] Polyolefins are low cost, easy to process and have good recyclability.[91] Most polyolefins are able to be processed using a wide range of methods,

including injection moulding and blow moulding, compression moulding and extrusion. They can also be processed into a variety of forms, such as films, fibres, sheets and pipes.

Due to their overall popularity, polyolefins have been widely used in active packaging. When considering natural compounds for incorporation, it is important to establish how the compound will respond to heat, due to the melt blending process.

1.6.3 Bio-based polymers

Bioplastics, or bio-based polymers, are made from renewable biomass sources and not all are inherently biodegradable. Some important classes of biobased polymers are starch-based, cellulose-based, polylactic acid, and polyamide 11. There are three pathways to produce bio-based polymers: i) extraction and modification of natural polymers like cellulose and starch, ii) polymerisation of bio-based monomers, and iii) extraction of polymers made by microorganisms.[92,93]

Cellulose is the most abundant of the bio-based natural polymers, making up 40-50% of wood and 90% of cotton. Crystalline packing of the cellulose polymer chains gives plants strength. Lignin and hemicelluloses are the other bio-based polymers produced by plants. Cellulose is a significant part of the cell wall of plants, allowing for easy harvesting through processes such as pulping. It can also be produced by fermentation of bacteria. Modification of cellulose gives a wide range of products, including cellulose nitrate, ethyl cellulose, and cellulose nitrate.[93]

Polylactic acid (PLA) is an example of a polymer produced from the polymerisation of bio-based monomers. PLA is generated from lactic acid, which is made from fermentation of agricultural by-products. The physical properties of PLA depend primarily on the ratio of R and S enantiomers of lactic acid. The mechanical and barrier properties of PLA are not as

effective as the polyolefins, but it is biodegradable under certain conditions. PLA is used in the food packaging industry and in agriculture, primarily due to its biodegradability.[92]

Polyhydroxyalkanoates (PHAs) are a family of polyesters that are produced via fermentation of bacteria. There are more than 150 different monomers that have been discovered in this family. Poly-3-hydroxybutyrate (PHB) is the most used PHA in industry. Different bacteria can be used to produce PHAs from renewable waste such as gas, vegetable oils, and fatty acids. However, PHAs in general have poor mechanical properties, which has limited its use in most applications.[92]

Protein films are also an option for environmentally conscious manufacturers. Gelatin and soy protein are low-cost, highly available, and abundant, making them an attractive alternative to polyolefins.[94] They are also food-grade and biodegradable, making them attractive for food packaging applications. Fish gelatins contain mostly glycine (31 – 37%), proline-hydroxyproline (14 – 20%), and alanine (10 – 12%). Soy protein contains mostly glutamic acid (20%), aspartic acid (12%) leucine (8%) and arginine (8%).[95] Their tolerance to heat is generally poor, with a thermal denaturation temperature of 35 °C for fish gelatin, and 75 °C for soy protein.[96,97] Although protein films have traditionally been produced via solution casting, it is also possible to use dry techniques such as extrusion or compression. In these cases, the protein is heated to above its glass transition temperature - a plasticiser can be added to decrease this temperature and help the thermoplasticity.[98–100]

Gelatin is made from the hydrolysis of collagen and two types can be produced, depending whether the pre-treatment conditions are acidic or alkaline.[101] Although the majority of gelatin produced is made from bovine and porcine origins, fish gelatin is becoming increasingly popular.[94] Fishery processing industries create a large amount of waste; the solid waste can make up 20-60% of the starting raw material.[102] Unfortunately, this waste can end up dumped

at sea or incinerated.[103] Other methods of waste disposal include fermentation for animal feeds, composting, and the production of fish oil supplements.[104–106] Fish gelatin has been used for antioxidant and antimicrobial active packaging. Polyphenols from tea, henna, and olives were successfully incorporated into fish gelatin films and extended the shelf life of fish, meat, and fruit.[107–109] Essential oils, including thyme, oregano, peppermint, and citronella have been used to produce antimicrobial fish gelatin films which showed some activity against *S. aureus* and *E. coli*.[110–112]

Soy protein is produced from soybeans, as a secondary product of soy oil extraction. In 2018/2019, over 360 million metric tons of soybean was produced worldwide.[113] During the soy oil extraction, other products are generated, including soy flour, soy protein concentrate, and soy protein isolate (SPI).[114] SPI contains about 90% protein, the highest of the by-products.[115] SPI is an attractive bio-sourced polymer for food packaging as it is readily available, is biodegradable, and has a high protein content. However, films made from SPI have been found to have inadequate mechanical properties and are not water resistant compared to polyolefins.[94] SPI films have also been used for antioxidant and antimicrobial packaging. Catechin has been successfully incorporated into SPI films, and showed very effective free radical scavenging ability.[116] A mixture of nisin, ethylenediaminetetraacetic acid (EDTA), and grape seed extract was also added to SPI films, showing inhibitory activity against *Listeria monocytogenes* and *E. coli*.[117]

1.6.4 Bread spoilage and packaging

Bread is recognised as one of the staple foods globally and its spoilage causes significant financial consequences for consumers and producers.[83] The spoilage of bread and other baked goods are mainly due to fungi, where the main contaminants include *Aspergillus*, *Fusarium*, and *Penicillium*. In wheat bread, the fungi present include *P. commune, P. solitum,*

P. corylophilum, and *A. flavus*. In rye bread *P. roqueforti*, *P. corylophilum*, and *Eurotium* species are present. Up of sixty percent of bread spoilage is due to *Penicillium* and *Aspergillus* species.[118]

The most important factors for the growth of fungi on food are pH, water activity, temperature, and oxygen. Sliced, pre-packaged and wrapped breads are most vulnerable to fungi as the packaging prevents moisture loss.[119] The most common reason for consumers to throw away bread is the presence of mould.[120] Therefore, it is of economic importance to investigate how the shelf life of bread products could be improved. One way in which this can happen is through the incorporation of antifungal natural products into the packaging.

Several different compounds have been incorporated into packaging to improve the shelf life of bread. The most common of these are organic acids, fungicides, alcohols, and antibiotics.[121] Focusing on natural compounds, essential oils have shown the most promise against bread-spoilage microorganisms. Clove and oregano essential oils were each added to methyl cellulose films, and improved the shelf life of sliced white bread by inhibition of *A. brasiliensis* and *P. citrinum*.[122] However, the incorporation of clove and cinnamon powders to starch films had no positive impact on the shelf life of bread.[123] Cinnamaldehyde, a key component of cinnamon essential oil, incorporated into gliadin films improved the shelf life of bread by 23 days.[124]

Although there have been multiple studies undertaken on the use of natural compounds to inhibit mould growth on bread using biodegradable films, there are a limited number using linear low-density polyethylene (LLDPE). Chitosan was blended with LLDPE to form films which inhibited the growth of microorganisms on white bread over 7 days.[125] LLDPE has also been blended with oxygen scavengers, namely zinc, iron, and ascorbic acid, which gave acceptable sensory data for bread over five days, compared to two days for the control.[126] It is

important to know more about the interactions between different natural compounds and LLDPE and how this can affect the application of active packaging using LLDPE.

1.6.5 Antifungal and antimicrobial additives for packaging

A potential solution for bread spoilage is the development of antifungal packaging, where additives showing antifungal and antimicrobial activity are utilised. A variety of natural extracts and essential oils have been identified as exhibiting antifungal properties. The natural extracts include both phenolics from natural sources and crude natural extracts.

1.6.5.1 Natural extracts

For centuries, folk medicine has used plant extracts to treat fungal infections among other diseases. It is estimated that only 1 – 10% of the 250,000 - 500,000 terrestrial plant species are used by humans.[44] This section will give an overview of plant compounds with known antifungal properties and some plant extracts that have exhibited the same.

Phenolic compounds extracted from plants have shown some antifungal activity, with their relative toxicity depending on the site(s) and the number of hydroxyl groups involved.[127] Increased hydroxylation may lead to increased fungal toxicity. Examples include flavonoids isolated from *Piper solmsianum* which show good antifungal activity, and coumarins which are known to have anti-inflammatory properties.[128] The furanocoumarin angelicin and its derivatives have been shown to be non-toxic and also active against fungal species including *Candida*, *Aspergillus* and *Saccharomyces*.[129] Xanthones isolated from *Securidaca longepedunculata* also show activity against *A. niger, A. fumigatus*, and *Penicillum*.[130]

Grapefruit seed extract (GSE) is obtained from the seeds and pulp of grapefruit and has been widely used in antifungal applications. A 0.5% liquid solution of GSE was highly effective against *Candida, Geotrichum, Aspergillus,* and *Penicillium* sp., as well as inhibiting Gram-positive and Gram-negative bacterial growth.[131] GSE has also been used in the

disinfection of instruments in hospitals, decontaminating water, and treating influenza.[132] Exposure of 0.5% GSE also efficiently inhibited the growth of *Botrytis cinerea* in table grapes, reducing the spore germination rate to 14%. A 1% chitosan film containing 0.1% GSE also reduced the spore germination rate to 25%.[133] An extract was produced from a mixture of pea, lentil, and fava bean flours which inhibited *Aspergillus parasiticus*, *Penicillium carneum*, *Penicillium paneum*, and *Penicillium polonicum*. This activity was attributed to the active native proteins nsLTP, ubiquitin alpha-1 chain, wound induced basic protein, defensin-1, and defensin-2, as well as a mixture of peptides. When this extract was incorporated as an ingredient in wheat bread, it increased the shelf life compared to the control.[134]

Galenia africana, a shrub which is known locally in South Africa as "kraalbos", has shown some antimicrobial activity. Historically its leaves have been used to alleviate toothache and to treat skin diseases. Flavonoids isolated from its leaves showed moderate antituberculosis activity. There are some reports of activity against *Fusarium*, but in that study the leaf extract showed no activity against *Penicillium*.[135]

Cinnamaldehyde is the major component of cinnamon, which can be extracted from cinnamon tree bark. Gliadin films containing cinnamaldehyde were highly effective against both *Penicillium* and *Aspergillus*. The fungi were completely inhibited after storage *in vitro* for 10 days with films containing 3% cinnamaldehyde. Films containing 5% cinnamaldehyde increased the shelf life of sliced bread from 4 days to 27 days.[136]

Hops are the flowers of the plant *Humulus lupulus*. They are used in the production of beer as a stabilising and flavouring agent, giving a bitter or citric taste. Hops are also used as an antibacterial agent in beer, as they are active against the beer-spoiling lactic acid bacteria. The iso-α-acids in hops are active against gram-positive but not gram-negative bacteria.[137] The hop bitter acids are active against inflammatory and metabolic disorders, and show potential

anticancer activity.[138] Xanthohumol from hops showed inhibition against two *Trichophyton* species, pointing to some antifungal activity.[139]

1.6.5.2 New Zealand based natural extracts

Horopito extract, *Pseudowintera colorata*, a shrub that is endemic to New Zealand, is rich in a sesquiterpene dialdehyde, polygodial. Tannins and eugenol may also be present in the extract. It. Due to the peppery taste of the leaves, it is also known as the New Zealand pepper tree. Historically, it has been used as an analgesic and antiseptic, and research has shown that it has anti-inflammatory, antifungal, antibacterial, and analgesic properties. Horopito extract has shown good activity against *Candida* and has been used to treat yeast infections.[140] It has also shown activity against *Penicillium marneffei*.[141]

Harakeke, *Phormium tenax,* is more commonly known as New Zealand flax. It is native to New Zealand and Norfolk Island and has cultural and historical importance through its use in Māori weaving. A potential antifungal compound, musizin, has been isolated from the roots of *Phormium tenax* and *Phormium cookianum*.[142] Researchers from Victoria University have also discovered that harakeke contains coumarin, a compound that smells like freshly mown grass, as well as hydroxycoumarin, and substituted hydroxycoumarins. Coumarins are thought to play an antimicrobial role within plants, protecting against microbial attack.[143]

Akeake, *Dodonaea viscosa,* is a flowering plant that is found in Africa, southern Asia, the Americas, and Australasia. The name ‘akeake’ means ‘forever and ever’. Historically, its wood was used by Māori to make clubs and other weapons. It has antifungal properties, with its extract showing activity against *Candida albicans,* as well as against *Aspergillus niger* and *A. flavus*.[144,145]

Manuka essential oil contains triketones, specifically leucospermone and isoleucospermone, which are considered its active components.[146] There are differences in

composition between essential oils distilled from manuka plants grown in the North and South Islands of New Zealand. Those grown in the East Coast of the North Island have higher levels of triketones, while those from the South Island have higher levels of sesquiterpene hydrocarbons and oxygenated hydrocarbons.[146] Manuka essential oils are active against gram-negative and gram-positive bacteria, as well as against the yeast *Candida*.[147] While manuka honey also contains active compounds and exhibits good antimicrobial activity, it would not be suitable for the active packaging application explored here.

1.6.5.3 Essential oils

Essential oils are volatile secondary plant metabolites, which have been isolated from plants, most commonly via steam distillation. They have been used for centuries to treat illnesses and to preserve and flavour food.[148] Though essential oils are currently mostly used in the perfume and food industries, there is potential for their antifungal properties to be better utilised. The most important part of the antimicrobial activity of essential oils lies in the structure of the compounds within it. The lipophilicity of the hydrocarbon skeleton and the hydrophilicity of the functional groups are important. The activity rank of essential oil components is in the order phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons.[148]

Phenolic compounds such as thymol, eugenol, and carvacrol oils have demonstrated the greatest antimicrobial activity with the broadest spectrum. Other oils in this group include thyme, oregano, and clove oils, as well as cinnamon oil, which contains cinnamaldehyde. Ketones are found in sage and peppermint oils. Oils containing alcohols, which are slightly less active, include tea tree, geranium, peppermint, and lavender. Ethers such as anethole and 1,8-cineole are found in fennel, eucalyptus, and rosemary oils. Hydrocarbons are a major compounds present in citrus and conifer oils.[148]

Essential oils are attractive for use in food applications due to their natural origin and volatile nature, which ensures only small concentrations are required. They exhibit low mammalian toxicity and environmental effects compared to commercial fungicides.[148–151] Most essential oils are generally recognised as safe by the US FDA, and major components such as carvacrol, cinnamaldehyde, p-cymene, eugenol, and thymol are registered by the EU as flavouring agents for food use.[151]

Some of the more common essential oils studied include thyme, sage, rosemary, cinnamon, clove, tea tree, and sugi. Thyme essential oil is extracted from the thyme plant *Thymus vulgaris*. Thyme has been found to be active against a range of fungi, including *A. niger*, *A. flavus* and *A. parasiticus*. Thyme essential oil was found to be more effective against *A. flavus* than *A. niger* using the agar dilution method.[152] Thyme essential oil showed antifungal activity in a wheat flour agar, where it completely inhibited the growth of *E. amstelodami*, *E. herbariorum*, *E. repens*, *E. rubrum*, *A. flavus*, *A. niger*, and *P. corylophilum* at water activities of 0.8, 0.85, and 0.9.[153] Thyme essential oil also showed activity against *A. flavus* in a liquid medium, inhibiting growth at 350 ppm.[154] Thymol is a component of thyme essential oil, making up from 33% to 54% of the oil.[153,155,156] It is a terpene derivative with carvacrol as its isomer, which is also obtained from the oil. However, thymol has a strong odour, which may affect its suitability in food packaging applications. It is active against *A. flavus*, *A. fumigatus*, *C. albicans*, and *Cryptococcus neoformans*.[148] Klaric et al found that pure thymol showed a three-fold stronger inhibition than thyme essential oil against mould genera collected from damp houses, which included both *Aspergillus* and *Penicillium* genera. However, the MICs of both thymol and thyme essential oil were below 20 mg L^{-1} for the two genera.[155] As essential oils are volatile compounds, their stability must be considered therefore to minimise the degradation of thyme essential oil, encapsulation has been used. A self-assembled polymer of chitosan and benzoic acid nanogel (CS-BA) was used to encapsulate the essential oil. Under

sealed conditions, the CS-BA encapsulated oil had an MIC of 300 mg L^{-1} against *A. flavus*, while the MIC for the free essential oil was 400 mg L^{-1}.[157]

Sage essential oil was tested against *Aspergillus brasiliensis*, *Penicillium chrysogenum* and *Rhizopus* spp. The essential oil was not active against *Rhizopus* spp. and had a growth limiting effect against *Aspergillus* and *Penicillium*, with no total inhibition.[158] Rosemary essential oil contains 1,8-cineole, α-pinene, camphor, and camphene.[159,160] Rosemary essential oil is more effective against bacteria and fungi than the individual components of 1,8-cineole and α-pinene. It has historically been used to treat renal colic, dysmenorrhea, and anxiety related conditions.[159] It shows inhibitory effects against *Aspergillus brasiliensis*, as well as against gram-positive and gram-negative bacteria.[160] Rosemary essential oil showed some inhibitory effects on the radial growth and conidial germination of *Penicillium digitatum*.[161]

Clove essential oil comes from the *Syzgium aromaticum* plant. It shows greater inhibition against *A. niger* compared to *A. flavus*. Against maize seeds infected with *A. flavus*, clove essential oil completely inhibited nine isolates of the fungus at a concentration of 10 µL L^{-1}. Against the one remaining isolate, *A. flavus* PSRDC-2, the oil inhibited 58.7% at 100 µL L^{-1}.[162]

Cinnamon essential oil is extracted from bark from trees from the genus *Cinnamomum*. Cinnamaldehyde is a major component and is more effective than other compounds in the oil.[163] Cinnamon essential oil was encapsulated in a starch foam in concentrations of 500 to 1500 ppm and postponed the spoilage of bread by 3 to 6 days.[118] Cinnamaldehyde has activity due to its high reactivity with nucleophiles. Films, coatings, and nanoparticles have been created incorporating cinnamaldehyde, including starch, polypropylene, cellulose, and chitosan.[136] Cinnamon essential oil was incorporated into wax paraffin as a paper coating for bread and strongly inhibited growth of *Rhizopus stolonifer*.[164] Gliadin films containing

cinnamaldehyde were highly effective against both *Penicillium* and *Aspergillus*. The fungi were completely inhibited after storage *in vitro* for 10 days with films containing 3% cinnamaldehyde. Films containing 5% cinnamaldehyde increased the shelf life of sliced bread from 4 to 27 days.[124]

Tea tree essential oil is extracted from a tree or tall shrub that is native to Australia. The indigenous people used the leaves to treat coughs and colds as well as wounds.[165] Its main components are monoterpenes, 1-terpinen-4-ol, cineole, and other hydrocarbons[166] and it showed activity against *Aspergillus flavus* and *A. niger*.[167] Tea tree essential oil has also been incorporated into chitosan films, and tested against *Penicillium italicum*. Compared to the control, the films containing tea tree essential oil inhibited the growth of the mould.[168]

Sugi essential oil is sourced from the Sugi tree, which originates from Japan. However, it is found globally, including in New Zealand. It mainly contains terpenoids, sesquiterpenes, and triketones. Its antifungal activity has been tested by Chen et al. against four wood decay fungi and six tree pathogenic fungi and was strongly active against all types. The essential oil used in their study contained 82.56% sesquiterpene hydrocarbon compounds.[169]

1.7 Research Objectives

This research evaluates the extraction of polyphenols from winery waste and the characterisation of the resulting extract. It is important to fully understand the antioxidant and antimicrobial properties of the grape marc extract to be able to fully see what suitable applications can be considered for the extract. In addition, the impact of exposure to heat on these properties is vitally important. There are many applications where inadequate thermal stability will have a significant negative effect. In addition, the inclusion of the extract into bio-based polymers can be investigated.

This focus extends from winery waste to an important food presentation application, that of antifungal active packaging targeting the fungi that affect bread. As one of the staple foods worldwide, bread is an important foodstuff. A breakthrough in active packaging using natural extracts or essential oils that inhibit these problematic organisms could have immense positive consequences for both consumers and producers. A thorough investigation into the antifungal properties of different natural compounds and the properties associated with the proposed application can be conducted.

2 Methods and Materials

2.1 Materials

2.1.1 Chemicals/reagents

2, 2´-Azobis (2-methylpropionamidine) dihydrochloride, (AAPH), (Sigma-Aldrich Corp., St. Louis, MO, USA)

2, 2-Diphenyl-1-picryhydrazl, (DPPH), (Sigma-Aldrich Corp., St. Louis, MO, USA)

2,2´-Azinobis-(3-ethylbenzothiazotine-6-sulfonic acid), (ABTS), (Sigma-Aldrich Corp., St. Louis, MO, USA)

2,2-diphenyl-1-picrylhydrazyl (DPPH), (Sigma-Aldrich Corp., St Louis, MO, USA)

Acetic acid, (Merck Ltd., New Jersey, USA)

Acetonitrile, (Scharlau, Sentmenat, Spain)

Amberlite FPX-66, (Dow Chemicals Ltd., Auckland, NZ)

Ascorbic acid, (Sigma-Aldrich Corp., St Louis, MO, USA)

Copper sulfate, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Diatomaceous earth, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Dimethyl sulfoxide (DMSO), (J. T. Baker, New Jersey, USA)

Disodium hydrogen phosphate, (Scharlau, Sentmenat, Spain)

Ethyl alcohol, (Scharlau, Sentmenat, Spain)

Folin-Ciocalteu reagent, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Formic acid, (ECP Ltd, Auckland, NZ)

Glycerol, (ECP Ltd., Auckland, NZ)

Lithium chloride, (Sigma-Aldrich Corp., St Louis, MO, USA)

Methyl alcohol, (Scharlau, Sentmenat, Spain)

Potassium iodide, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Potassium persulfate, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Potassium Sodium Tartrate, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Rochelle salt, (J. T. Baker, New Jersey, USA)

Saline, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Sodium carbonate, (Chem-Supply, Gillman, SA, Australia)

Sodium chloride, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Sodium dihydrogen phosphate, (Scharlau, Sentmenat, Spain)

Sodium hydroxide, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Sodium thiosulfate, (Ajax Finechem, Ltd PTY, Sydney, NSW, Australia)

Tween 80, (Thermo Fisher Scientific, Waltham, MA, USA)

Starch, corn, (Cream, A.E Staley Manufacturing Company, Decatur, IL, USA)

2.1.2 Media

Agar, (Hach Pacific, Penrose, Auckland, New Zealand)

RPMI-1640 broth, (Gibco, USA)

Sabouraud dextrose agar (SDA), (BD Difco, USA)

Tryptic soy broth (TSB), (Hach Pacific, Penrose, Auckland, New Zealand)

Yeast Extract Peptone-Dextrose (YPD), (Thermo Fisher Scientific, Waltham, MA, USA)

2.1.3 Polymers

Ethyl cellulose (22cps) 48% ethoxy content, (Acros, Geel, Belgium)

Fish gelatin bloom 240, (Healan Ingredients, East Yorkshire, UK)

Polyethylene, linear-low density (LLDPE) Dowlex 2517, (Dow Chemical Company, Midland, MI, USA)

Soy protein isolate, (ADM, The Netherlands)

2.1.4 Standards

(-)-Epicatechin, (Sigma-Aldrich Corp., St. Louis, MO, USA)

(+)-Catechin, (Sigma-Aldrich Corp., St. Louis, MO, USA)

3, 4-Dihydroxycinnamic acid (caffeic acid), (Sigma-Aldrich Corp., St. Louis, MO, USA)

4-Hydroxy-3-methoxycinnamic acid (ferulic acid), (Sigma-Aldrich Corp., St. Louis, MO, USA)

4-hydroxycinnamic acid (p-coumaric acid), (Sigma-Aldrich Corp., St. Louis, MO, USA)

6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (Sigma-Aldrich Corp., St Louis, MO, USA)

Fructose, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Gallic acid, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Glucose, (Merck Ltd., New Jersey, USA)

Kaempferol, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Malvidin-3-O-glucoside, (Sigma-Aldrich Corp., St Louis, MO, USA)

Quercetin, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Resveratrol, (Sigma-Aldrich Corp., St. Louis, MO, USA)

Rutin, (Sigma-Aldrich Corp., St. Louis, MO, USA)

2.2 Methods

2.2.1 Grape marc samples

White grape marc was obtained from the Marlborough region in the 2015 vintage. The samples were collected after pressing and were identified as Chardonnay. The grape marc was stored at -4 ˚C until it was processed. A red grape marc mix was obtained from the Auckland region in the 2016 vintage. The samples were collected after pressing and stored at -4 ˚C until it was processed.

2.2.2 Conventional solvent extraction

A large-scale extraction was carried out, along with a small-scale extraction. For the large-scale extract, approximately 50 kg of white grape marc was homogenised in a vertical food processor (Robot Coupe Blixer 60) and washed with 50 °C water in approximately equal parts. The resulting slurry was then extracted while stirring for approximately 48 hours at room temperature. The filtrate was then removed using a water press and stored at -4 °C. For the small-scale extract, 5 kg of white grape marc was homogenised using a Kensington food processor model 219708 at maximum speed for approximately 5 minutes and washed with hot water in approximately equal parts. The resulting slurry was extracted while stirring for

approximately 24 hours. The filtrate was then removed by filtration using a muslin cloth and stored at -4 °C.

The large scale and small-scale extracts (labelled as bulk and small scale, respectively) were then subject to the same conditions. The filtrates were passed through a column containing Amberlite FPX-66 resin purchased from Dow Chemicals Ltd. The column was then washed with three volumes of deionised water. The phenolic compounds were released from the resin by passing three volumes of ethanol through the column. The ethanolic fraction was then collected and concentrated using a rotary evaporator (Savant, Thermo Fisher Scientific, Waltham, MA) and dehydrated using a speed vacuum (Thermo Savant, Waltham, MA). The resulting brown powder was homogenised using a mortar and pestle.

Bulk grape marc waste from the 2013 vintage was obtained, consisting of white grape skin, seeds, stems, and pulp. The grapes were predominantly Chardonnay. Five kg of white grape marc was homogenised using a Kensington food processor model 219708 at maximum speed for approximately five minutes and mixed with hot water at 100 °C in approximately equal parts. The resulting slurry was left at room temperature for the extraction to occur for approximately 24 hours. The filtrate was then removed by filtration using a muslin cloth and was then passed through a column containing Amberlite FPX-66 resin purchased from Dow Chemicals Ltd. The column was then washed with three volumes of deionised water. The phenolic compounds were released from the resin by passing three volumes of ethanol through the column. The ethanolic fraction was then collected and concentrated using a rotary evaporator (Savant, Thermo Fisher Scientific, Waltham, MA) and dehydrated using a speed vacuum (Thermo Savant, Waltham, MA). The resulting brown powder was homogenised using a mortar and pestle.

2.2.2.1 Balance

The large-scale weighing of grape marc was performed using a Mettler model SW60 top-loading balance, with units of kg to two decimal places. Smaller scale weighing of compounds was performed using one of two balances: a Shimadzau model UW2200h top-loading balance in g to two decimal places, and a Mettler model XS204 analytical balance in mg to one decimal place.

2.2.2.2 Rotary Evaporator

GME solutions were concentrated using a BUCHI roto-vap model R210 (Postfach, Switzerland) after extraction and purification. The BUCHI vacuum pump model V700 was set to the maximum vacuum of 10 mBar and the BUCHI water bath model B491 was set to a temperature of 45 ˚C. The solvent was then reused in the purification procedure.

2.2.2.3 Speed Vap

Once concentrated, a Savant Speed Vap model SPD131-DDA (Thermo Fisher, Waltham, MA, USA) was used to dry the extracts to powders. The temperature was set to 45 ˚C. The removed solvent was collected in a Thermo refrigerated solvent trap model RVT4104 (Thermo Fisher, Waltham, MA, USA) set to -80 ˚C.

2.2.2.4 Freeze dryer

Freeze-drying was conducted using an Alpha 2-4 LSC freeze-dryer (Christ, Osterode, Germany) at 0.070 mbar for 3 days with a condenser temperature of −90 °C. Freeze drying consists of freezing the sample and then lowering the pressure, which removes the ice by sublimation.

2.2.3 Accelerated solvent extraction

The red and white grape marc were freeze dried before the extraction. The yield from this process was around 38% for both varieties. Grape marc polyphenols were then extracted with

a method adapted from Heffels et al., using an accelerated solvent extractor model ASE 350 (Thermo Scientific, Inc., Braunschweig, Germany).[170] 0.2 g of freeze dried grape marc was mixed with diatomaceous earth and packed into a 10 mL stainless-steel extraction cell. The cells were prepared with two cellulose filters on the bottom and top of the cells. The four extraction solvents used were (A) deionised water, (B) 20% ethanol, (C) 80% ethanol, and (D) 80% ethanol with 1% acetic acid. Each sample was extracted in triplicate. Extraction parameters were 5 min static time, 3 cycles, 40 °C, and 100% flush volume. Finally, all extracts were adjusted to 25 mL with water and used for UHPLC analysis after membrane filtration with regenerated cellulose.

2.2.3.1 Accelerated solvent extractor

Phenolic compounds from red and white grape marc were extracted using an accelerated solvent extractor model ASE 350 (Thermo Scientific, Inc., Braunschweig, Germany). Conditions were temperature of 40 ˚C and pressure of 1500 psi. The cells were packed with diatomaceous earth and grape marc. Its operation is based on the use of high temperature and pressure, where the high pressure keeps the solvent in a liquid state at temperatures above its boiling point, and the high temperature increases the extraction efficiency.

2.2.4 Ultrasound assisted extraction

Grape marc (6 g) was filled in centrifuge tubes in three replicate samples and treated with 20 mL of extraction solvent. The solvents used were (A) 100% deionised water, (B) 20% ethanol, (C) 80% ethanol, and (D) 80% ethanol with 1% acetic acid. The suspension was homogenized with an Ultra-Turrax (IKA 3720000 T) and, afterward, placed upright into a sonication bath (1100 W) for 10 min. Subsequently, the samples were centrifuged at 10,947 g for 10 min, and the supernatant was decanted. The residue was extracted again under the same conditions, excluding the homogenization step, with 10 mL of the extraction solvent. Finally,

the supernatants were pooled and made up to 50 mL with water. Each extraction solvent was repeated five times. This solution was used for ultra-high-pressure liquid chromatography (UHPLC) analysis.

2.2.5 Extract heating

Samples of the GME prepared as in 2.2.2 above were heated to temperatures similar to those experienced in the melt blending process. The bulk and small-scale extracts were weighed into approximate 2 g portions in 20 mL glass vials. One vial of each extract was then placed into an oven preheated to the specified temperature for a period of either 10 minutes (for all temperatures) or one hour (for 100 and 150 ˚C). The extracts were exposed to temperatures of 100 ˚C, 150 ˚C, 200 ˚C, and 250 ˚C. Each sample was allowed to cool to room temperature before being homogenised using a mortar and pestle. They were then stored at -4 ˚C for further analysis.

2.2.5.1 Oven

A Contherm oven model Thermotec 200 (Contherm Scientific Ltd., Hutt City, NZ) was used for leachability studies, heat tolerance testing, and drying of samples as required.

2.2.6 Film preparation

2.2.6.1 Ethyl cellulose films

Ethyl cellulose films were prepared via solution casting based on the method described by Olejar et al. with minor modifications.[171] First, GME was added to 70 mL ethanol and mixed until fully dissolved at concentrations of 0.5, 1, and 2% by ethyl cellulose weight. Then, 6 g of ethyl cellulose was slowly added and mixed until dissolved in the absence of light. Dibutyl sebacate at 10% (w/w) was added as plasticiser. The resulting solution was poured into glass petri dishes and kept at -4 ˚C for 48 hours, or until the films were dry.

2.2.6.2 Soy protein isolate films

Soy protein isolate (SPI) films were prepared by compression moulding following a method by Garrido et al.[172] GME was dissolved in 4 mL deionised water at a concentration of 2% by SPI weight. Glycerol as a plasticiser was added at a concentration of 30% by SPI weight. 20 g of SPI was then added, and the resulting powder was homogenised. The powder was placed between two aluminium plates preheated to 120 ˚C and pressed at 12 MPa for 2 minutes. The resulting films were allowed to cool and conditioned in a controlled environment chamber at 25 ˚C and 50% relative humidity before testing. Finished films measured approximately 10 cm diameter.

2.2.6.3 Fish gelatin films

Fish gelatin films were also prepared by compression moulding, following a method by Uranga et al.[173] GME was dissolved in 4 mL deionised water at a concentration of 2% by gelatin weight. Glycerol as a plasticiser was added at a concentration of 20% by gelatin weight. 10 g of fish gelatin was added and the resulting mixture was homogenised. The mixture was then placed between two aluminium plates preheated to 60 ˚C and pressed at 0.8 MPa for 2 minutes. The resulting films were allowed to cool and conditioned in a controlled environment chamber at 25 ˚C and 50% relative humidity before testing.

2.2.6.4 LLDPE film preparation

A Brabender model DSE 25 was used to blend linear low-density polyethylene (LLDPE) with active compounds identified as having antifungal properties. The LLDPE pellets were mixed for 1 minute at 200 ˚C, before the plant extract was added, and mixed for a further 2 minutes at 200 ˚C. The total mass of all components within each batch was 37 g. The measured torque range was 100 Nm. After mixing the polymer mixture was removed and cut into pellets. These were subsequently used to prepare thin films.

Thin films were prepared with a 20-ton LabTech hydraulic press model LP-S-20 (Samutprakarn, Thailand) with heated platens. Pressing of thin films was undertaken at 200 ˚C, with a 3.5-minute pre-heating cycle, a 30 second pressing cycle at 1000 psi, and a 4-minute cooling cycle.

2.3 Analytical tests

2.3.1 Antioxidant assays

The DPPH radical scavenging assay was performed as described by Olejar et al. with slight modifications.[174] A DPPH˙ solution was made up to a concentration of 63.4 µmol L^{-1} using 80% methanol. Triplicate samples of each film with total surface area of 10 cm^2 were weighed and rinsed with Milli-Q water and placed in 20 mL vials. Following this, 20 mL of the DPPH˙ solution was added to each vial. The solutions were kept at room temperature for 24 hours in the absence of light. The absorbances of the solutions were then read at 515 nm using 80% methanol as a blank. Controls containing no film and a film with no GME additive were also measured. Standard solutions of DPPH˙ were made up of concentrations of 63.4, 31.7, 15.9, and 7.9 µmol L^{-1} to construct a standard curve.

The ABTS radical scavenging assay was performed as described by Olejar et al. with minor modifications.[171] A stock solution of ABTS˙ was prepared by mixing equal volumes of ABTS at 7 mmol L^{-1} and potassium persulfate at 2.45 mmol L^{-1} aqueous solutions and allowing it to stand at room temperature for at least 16 hours in the absence of light. The resulting solution was diluted with ethanol to prepare an ABTS˙ solution with an absorbance of 1.40 ± 0.2. Triplicate samples of each film with a total surface area of 10 cm^2 were weighted and rinsed with Milli-Q water and placed in 20 mL vials. Following this, 20 mL of the prepared ABTS˙ solution was added to each vial. The samples were kept at 30 °C for one hour and then stood at room temperature for an additional hour in the absence of light. The absorbance of each

sample was then read at 734 nm using ethanol as a blank. A control sample with no film was prepared and measured, along with a control with a film with no GME additive. Standard solutions of ABTS˙ were made up to concentrations of 140, 70, 35, 17.5, and 8.75 µmol L^{-1} to construct a standard curve.

2.3.1.1 DPPH Assay

The DPPH radical scavenging antioxidant assay was adapted from the procedure described by Villano et al. (2007). DPPH free radical solution at a concentration of 63.4 µmol L^{-1} was prepared by dissolving 12.5 mg DPPH in 0.5 L 80% methanol. A 1:2 (DPPH:80% methanol) dilution series of the DPPH free radical solution was used to give a standard curve.

For the powdered GME analysis, 0.1 mL of DPPH dilutions, diluted GME or blank solution was added to 3.9 mL of DPPH free radical solution. The solutions were shielded from light for 30 minutes at room temperature. The absorbances of the samples were measured on a Thermo Helios gamma spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) at 515 nm. All UV/Vis analysis was performed on a Shimadzu spectrophotometer model UV 1700 (Kyoto, Japan).

In Excel, a standard curve was created by plotting the absorbance of the diluted DPPH solution against the DPPH concentration. The curve was then used to find the % of DPPH free radicals scavenged by the GME solutions.

For analysis of both films, a 20 cm^2 total surface area of each sample was prepared in triplicate. The films were rinsed with distilled water to remove surface particulates. The film was placed in a vial and 20 mL of DPPH free radical solution at a concentration of 63.4 µmol L^{-1} was added. The vials were shielded from light for 24 hours at room temperature. A dilution series of the DPPH free radical scavenging solution was prepared as previously described to

give a standard curve. The absorbances of the test solutions were used to find the % of DPPH free radicals scavenged by the films.

2.3.1.2 ABTS Assay

The ABTS radical scavenging method was used to establish the antioxidant activity of ethyl cellulose films. This method is based on that described by Re et al.[175] A 7 mM solution of ABTS solution was reacted with potassium persulfate solution to give a final concentration of 2.45 mM potassium persulfate and mixed well. The solution was protected from light at room temperature for 16 hours to allow production of the ABTS radical cation, $ABTS^{+}$. The absorbance of the solution was then measured at 734 nm and diluted until an absorbance of 0.7 ± 0.1 absorbance units was established. A 2:1 ($ABTS^{+}$:distilled water) series dilution was performed to establish a standard curve.

Polymer films were cut to 20 cm^2 total surface area pieces and rinsed with distilled water to remove surface particulates. The films were placed in vials and 20 mL of $ABTS^{+}$ solution was added. The vials were then incubated at 30 ˚C for 60 minutes and shielded from light. After incubation, the absorbance of the solutions was measured at 734 nm. The recorded absorbances were analysed in Excel to find the % of $ABTS^{+}$ scavenged by the films.

2.3.1.3 Spectrophotometer

All UV/Vis analyses were performed on a Shimadzu spectrophotometer model UV 1700 (Kyoto, Japan). Absorbance readings were obtained at the specified wavelengths as described in the individual assays.

2.3.2 Antimicrobial assays

2.3.2.1 Minimum bactericidal concentration (MBC)

Antimicrobial testing was conducted against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 using the standardised broth microdilution protocol.[176,177]

An overnight culture of the bacteria was prepared by placing one bacterial colony in 10 mL of TSB broth and incubating overnight in a shaker at 37 ˚C and 1500 RPM. The 10^6 CFU mL^{-1} inoculum was prepared from the overnight culture by a series of 10-fold dilutions. The CFU mL^{-1} of the inoculum was confirmed using the Miles and Misra method. The neat solution was serially diluted seven times by adding 100 µL suspension to 900 µL saline. Three TSB agar plates were divided into eight sectors and labelled with the dilutions. Then, 20 µL of each dilution was dropped onto a sector of each agar plate and allowed to dry. The plates were then inverted and incubated at 37 ˚C for 24 hours.

The powders were dissolved in tryptic soy broth (TSB) to a concentration of 80 mg mL^{-1}. These were then used for doubling dilutions in sterile flat bottomed 96-well polystyrene microtiter plates and inoculated with approximately 1 x 10^6 organisms. After incubation at 37 ˚C overnight, the results were read. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of GME with no visible turbidity, as determined by the naked eye.

The minimum bactericidal concentration (MBC) was then determined by drop plating 20 µL of each well onto TSB agar plates. These were again incubated at 37 ˚C overnight, after which the results were read. The MBC was defined as the lowest concentration of GME with no visible bacterial colony growth.

2.3.2.2 Minimum fungicidal concentration (MFC)

The minimum fungicidal concentration of the extracts/essential oils was attained using the standardised broth microdilution protocol issued by the Clinical and Laboratory Standards Institute (CLSI), documents M38-A and M38-A2.[178,179] Plant extracts (Table 2.1) were tested against *A. brasiliensis* and *P. citrinum*. Liquid cultures were grown in RPMI-1640 broth (Gibco, USA). Agar cultures were grown on Sabouraud dextrose agar (SDA) (BD Difco, USA). The broth was made up from powder purchased from Sigma Aldrich and was buffered and pH adjusted to 7.0 before use. Plant samples needed to be prepared by dilution in different solvents before they were able to be tested for Minimum Fungicidal Concentration (MFC). Plant samples that were able to be dissolved in broth were diluted down to 4% (*w/v*) working solution, using the volumes of 60 µL plant sample in 1440 µL of broth (Table 2.1). Other plant samples were required to be dissolved in DMSO or ethanol, then diluted with broth. In cases where a solvent with antimicrobial activity was used, solvent-only controls were included to confirm effects were due to the extract.

Table 2.1. Plant extracts and essential oils with dilution details for antifungal assay.

Sample	Dilution details
G. africana extracts, Sugi essential oil, manuka essential oils, tea tree oil	1440 µL broth + 60 µL extract
Totarol, Thyme essential oil, Sage essential oil, Cinnamon bark essential oil, Clove essential oil, Rosemary essential oil, Hops extract, Cinnamaldehye, *G. africana* (raw), grapefruit seed extract	0.1 g extract dissolved in 1 mL ethanol 80 µL ethanol solution + 920 µL broth
Pre-dissolved in 65% ethanol, Akeake, Kawakawa	80 µL extract in 920 µL broth
Pre-dissolved in 45% ethanol, Harakeke, Horopito	110 µL extract in 890 µL broth

DMSO and ethanol controls were used at a working concentration of 8% to ensure that the solvents were not the source of any antifungal activity found. A negative control containing saline was also used to show that the microorganisms can grow in the broth.

To prepare the inoculum, 15 mL RPMI-1640 broth was added to a fungal plate to collect the spores. The number of spores in the neat solution was measured through optical density reading at 600 nm; once the number of Colony Forming Units (CFU) per mL of the neat solution was known, this was diluted down to give 10^4 CFU mL^{-1} of inoculum.

Once the extracts were dissolved, they were used for doubling dilutions in 96-well microtitre plates and were inoculated with 50 µL of inoculum. Each well contains a total volume of 100 µL. The microtitre plates were incubated at 28 ˚C in a shaking incubator for 48 hours, after which the minimum inhibitory concentration (MIC) was, where MIC is no visible growth. Many plant extracts were coloured, making the accuracy of this reading inconsistent. Therefore, the minimum fungicidal concentration (MFC) was used as a more reliable measure of antifungal potency. To find the MFC, 10 µL from each MIC microtitre plate well was drop plated onto SDA plates. These were then incubated at 28 ˚C for 48 hours, after which the MFC was recorded, where MFC is no visible growth at the dilution. Each

biological replicate contained three technical replicates. Each plant extract had three biological replicates.

2.3.2.3 MFC of heated extracts

Extracts that were selected for heating were put into individual glass vials and placed separately into an oven set at 200 ˚C for a period of 10 minutes. This was to mimic the conditions of extract incorporation into a polyethylene film. At the end of the heating period, the glass vial was removed and allowed to cool before being stored at -4 ˚C. The heated extracts were then tested against *A. brasiliensis*, *P. citrinum*, and *Pichia burrtonii* using the AST protocol as described above for *A. brasiliensis* and *P. citrinum*. The protocol was modified slightly for testing against *P. burrtonii*, as it is a yeast. The broth used was Yeast Extract Peptone-Dextrose (YPD) broth and RPMI-1640 broth. The agar used remained SDA. The inoculum was prepared via overnight culture in YPD broth at 28 ˚C for 17 to 20 hours. The YPD broth was then washed off as the remainder of the AST was carried out in RPMI-1640 broth. The inoculum of 10^3 CFU mL^{-1} was prepared from the neat via dilution.

2.3.2.4 Antibacterial testing of films

The antibacterial activity of ethyl cellulose, soy protein isolate, and fish gelatin films containing GME was established using the Japanese Industrial Standard (JIS) Z 2801:2000 with some modifications. The broth used was tryptic soy broth (TSB) and the agar used was tryptic soy agar (TSA). The inoculum was prepared by an overnight culture of *S. aureus*. The neat solutions were prepared to contain 10^8 CFU bacteria using ten-fold dilutions in saline.

The CFU mL^{-1} of the inoculum was confirmed using the Miles and Misra method. Briefly, the neat was serially diluted seven times by adding 100 µL suspension to 900 µL saline. Three TSB agar plates were divided into eight sectors and labelled with the dilutions. Then, 20 µL of

each dilution is dropped onto a sector of each agar plate and allowed to dry. The plates were then inverted and incubated at 37 ˚C for 24 hours. The CFU mL^{-1} of the neat was calculated by counting the colonies present at the different dilutions.

To establish the antimicrobial properties of the films, each film was cut to 5 x 5 cm and inoculated with 100 µL inoculum, after which a 4 x 4 cm square of polyethylene was transferred to cover the inoculum. The inoculum was made to cover the film squares by manipulation of the polyethylene film with sanitised tweezers. The samples were stored in petri dishes in a humid environment at 36 ˚C for 24 hours. After incubation, the films were each collected into a stomacher bag and washed with 10 mL TSB broth containing 1% (v/v) Tween 80 by stomaching twice for a period of 30 seconds, with one minute rest in a Seward Stomacher 400 Lab Blender (London, England). The wash was collected, diluted, and spread on TSB agar plates at the concentrations estimated to contain 10^2, 10^4, and 10^6 CFU if the CFU on the surface remained unchanged. After drying, the plates were incubated at 36 ˚C for 24 hours. After incubation, the colonies on each plate were counted and recorded. Plates containing colonies of 300 or more were recorded as 'too many to count' (TMTC).

2.3.2.5 Antifungal testing of films

The antifungal activity of the polyethylene films was established using the Japanese Industrial Standard (JIS) Z 2801:2000 protocol with some modifications for testing against filamentous fungi. The broth used was YPD broth and the agar used was YPD agar. The inoculum was collected by washing a cultivated plate of the respective fungi with 15 mL YPD broth and collecting approximately 10 mL of spore suspension. The absorbance of the neat culture was recorded to establish the number of spores, after which the neat culture was diluted in saline to give an inoculum of 10^5 CFU mL^{-1}.

Films containing plant extract were cut to 5 cm x 5 cm squares, with associated polyethylene film covers cut to 4 cm x 4 cm squares. Each film containing plant extract was inoculated with 100 µL inoculum, after which a 4 cm x 4 cm square of polyethylene was transferred to cover the inoculum. The inoculum was made to cover the film squares by manipulation of the polyethylene film with sanitised tweezers. The samples were stored in petri dishes in a humid environment at 28 ˚C for 48 hours. After incubation, the films were collected into a stomacher bag and washed with 10 mL YPD broth containing 1% (*v/v*) Tween 80 by processing twice for a period of 30 seconds, with one-minute rest in a Stomacher. The wash was collected, diluted, and spread on YPD agar plates at the concentrations estimated to contain 10^1 CFU mL^{-1}, 10^2 CFU mL^{-1} and 10^3 CFU mL^{-1} if the CFU on the surface remained unchanged. After drying, the plates were incubated at 28 ˚C for 48 hours.

After incubation, the colonies on each plate were counted and recorded. Plates containing colonies of 300 or more were recorded as TMTC. Each film was tested as duplicate technical replicates, on three separate occasions as biological replicates.

2.3.3 Stomacher

A Seward Stomacher 400 Lab Blender (London, England) was used to wash the inoculum from films after incubation using the Japanese Industrial Standard process. Paddles are used to homogenise the solution.

2.3.4 Incubator

For incubation of bacteria overnight cultures and 96-well microtitre plates, a New Brunswick model Innova 44 shaking incubator (Eppendorf Inc., Hamburg, Germany) set to 200 RPM and 37 ˚C was used. Agar plate cultures were incubated in a Steridium incubator set to 37 ˚C for bacteria and 26 ˚C for fungi.

2.3.5 High performance liquid chromatography (HPLC)

High performance liquid chromatography was performed to establish the phenolic profile of the grape marc extracts. It was performed using the method described by Olejar et al. (2015).

Standards were prepared by dilution in methanol to concentrations of 100 µg, 50 µg, 10 µg, 5 µg, 1 µg, 500 ng, 50 ng, and 10 ng mL^{-1}. These were then filtered through a 0.2 µm syringe filter into amber glass vials and sealed. GME samples were prepared by dilution in methanol to a concentration of 1 mg mL^{-1}. The solutions were then filtered through a 0.2 µm syringe filter into amber glass vials and sealed.

Chromatography was performed on an Agilent 1100 HPLC equipped with an ESA CouloChem electrochemical detector. The flow rate was 1.0 mL min^{-1} for 30 minutes at 40 ˚C on a Supelco Ascentis RP-amide column with the specifications 3.0 x 100 mm, 3 µm. The mobile phases used were A: pH 2.6, 30 mmol phosphate buffer, and B: pH 2.6, a mix (30:10:60) of 100 mmol phosphate buffer, methanol, and acetonitrile. The gradient used was 1-10 min 12% B, 10-15 min 30% B, 15-17.5 min 55% B, 17.5-21 min 55% B, 21-23 min 100% B, and 23-25 min 0% B. Components were detected at 280, 305, 320, and 365 nm, and 450 and 750 mV.

The HPLC method used for the samples described in Chapter 3 was performed at the University of Bonn, Germany. The flow rate was 0.4 mL min^{-1} at 40 ˚C. The autosampler temperature was 10 ˚C. The mobile phases used were A: 0.1% formic acid, and B: 0.1% formic acid in acetonitrile. The gradient used was 0-2 min 20% B, 2-6.5 min 40% B, 6.5-9.5 min 99% B, 9.5-11.5 min 99% B, 11.5-12 min 20% B. The PDA recorded from 190 to 600 nm and the RF detector had an extinction at 286 nm and emission of 316 nm. Linear calibration curves were established using standards of malvidin-3-O-glucoside (oenin) for the PDA detector and catechin for the RF detector.

2.3.6 Gel Permeation Chromatography (GPC)

Gel permeation chromatography was performed in Scion, Rotorua, NZ to establish the molecular weight distribution of GME samples. GPC was carried out on a Polymer Laboratories PL-GPC-50 integrated GPC system with a Knauer K-301 refractive index detector, using two PolarGel-L columns (Varian, 300 x 7.5 mm) connected in series and protected by a guard column (50 x 7.5 mm) of the same material. The system was run at 50 °C with the eluent 0.05 mol L^{-1} LiCl in DMSO. Flow rate was maintained at 1 mL min^{-1}. The system was calibrated using pullulan saccharide standards (PSS, 180 – 47,000 MW). Samples were dissolved overnight on a shaker at a concentration of 3 mg mL^{-1}. Samples that did not fully dissolve were filtered through a 0.45 µm syringe filter. The injection volume was 100 µL.

2.3.7 Folin-Ciocalteu assay

This assay was used to establish the total phenolic content and was used as described by Bajcan et al.[180] The extract was dissolved in 50% ethanol to a concentration of 1 mg mL^{-1}. Gallic acid standards were dissolved in 50% ethanol to the desired concentrations. In a 4 mL cuvette, 1.58 mL deionised water and 20 µL of the GME solution, standard or blank (50% ethanol) was added and mixed well. To this solution 100 µL of Folin-Ciocalteu reagent and 300 µL of 20% sodium carbonate were added and mixed well. The solutions were shielded from light at room temperature for 90 minutes. The absorbance of the samples was then measured at 765 nm.

A gallic acid concentration curve was established by plotting the absorbance against the concentration of gallic acid. This was then used to establish the total phenolic content of the GMEs as gallic acid equivalents (GAE).

2.3.8 Fourier Transform Infrared (FT-IR)

Fourier Transform Infrared (FT-MIR) spectroscopy was conducted on the bulk and small-scale powders. Spectra were taken using a diamond ATR Smart Orbit™ accessory with a refractive beam at incidence angle 45˚ using a deuterated triglycerine sulfate (DTGS) detector with a KBr window, operating in the Mid-IR. The powders were scanned over the range of 4000–600 cm^{-1} with a resolution of 4 cm^{-1} and averaged over 128 scans. Omnic software was used for data acquisition and analysis.

2.3.9 Fourier Transform Raman (FT-Raman) Spectroscopy

Raman spectra were collected in triplicate using a MultiRam FT-Raman spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a liquid nitrogen cooled Ge detector (D418-T) at the University of Otago, Dunedin. The spectra were collected using OPUS 6.5 (Bruker Optics, Ettlingen, Germany) with 4 cm^{-1} resolution, the power settings (11 to 200 mW). The number of spectral accumulations (200 to 400 scans) varied between samples depending on how thermally emissive the samples were. The spectra were pre-processed using a linear baseline correction and SNV transformation over the spectral region 520 to 1800 cm^{-1}. Principal Component Analysis (PCA) was then calculated using full cross validation. All spectral pre-processing and data analysis were carried out using The Unscrambler X 10.3 (CAMO, Norway) and Origin(Pro) 2019b (OriginLab Corporation, USA).

2.3.10 Leachable Solids

Leaching of plant extracts from polyethylene films were measured following the protocol in *The United States Pharmacopeia*, monograph 661.[181] Polymer films were cut to 120 cm^2 surface area and rinsed twice with deionised water. The films were then placed in vials containing 20 mL of either ethanol or deionised water. The vials were then placed in an oven at 70˚C for 24 hours, after which 20 mL of liquid was removed and placed in a weighed petri

dish. The petri dishes were then placed in a 70˚C oven to evaporate the liquid and allowed to cool before reweighing. The film was also dried and reweighed. Films containing 1% and 5% plant extract were used. The analyses were performed in triplicate. A polyethylene control film was used as a control.

2.3.11 Sugar determination

2.3.11.1 High performance liquid chromatography

The concentrations of fructose and glucose in the GME samples were determined at the University of Bonn, Germany. A Smartline HPLC system (Knauer, Berlin, Germany) equipped with two pumps, RI detector 2300, column oven, autosampler (Midas, Spark Holland, Emmen, Netherlands), and a Nucleodur 100-5 NH2-RP column (150 × 4.6 mm, particle size 5 µm) from Macherey-Nagel (Düren, Germany) was used. Isocratic elution with acetonitrile:water (85:15, v/v) at 40 °C with a constant flow of 1.0 mL min^{-1} was used for analysis. The injection volume was 10 µL.

2.3.11.2 Rebelein method

Residual sugar content in the GME powder was obtained using the Rebelein method described in Production Wine Analysis.[182] In this method, copper is reacted with the reducing sugars present in the sample. The excess copper that remains after the reaction is then reduced with excess iodide ion to make an equivalent amount of iodine. This is then titrated with sodium thiosulfate.

$$2Cu^{2+} + 2I^{-} \rightarrow 2Cu^{+} + I_2 \qquad (2.1)$$

$$I_2 + 2S_2O_3^{2-} \rightarrow 2I^{-} + S_4O_6^{-2} \qquad (2.2)$$

GME powder was dissolved in Milli Q water at a concentration of 2 mg mL^{-1}. In a 200 mL Erlenmeyer flask, 10.0 mL of 0.168 mol L^{-1} $CuSO_4$-$5H_2O$ in 0.01 Nor H_2SO_4, 5.0 mL 0.886 M

Rochelle salt in 2 M NaOH, and 2.0 mL of sample. The mixture was then heated rapidly until steam was evolved and maintained for 1.5 min, after which it was rapidly cooled in an ice bath. This step permitted the reaction of copper and the reducing sugar. Once cool, 10 mL of 1.81 M KI in 0.1 Nor NaOH, 16% H_2SO_4, and 10 mL of a 1% starch solution in 0.120 M KI in 0.01 Nor NaOH were added and mixed. This step allows for the excess copper to be reduced with the iodide ion to form iodine, which was then titrated using 0.056 M sodium thiosulfate in 0.05 Nor NaOH to a creamy white endpoint. The amount of sugar was then calculated using the equation:

$$RS = \frac{\left(\left[28-28\left(\frac{V_S}{V_B}\right)\right]\times 1000\right)}{S} \qquad (2.3)$$

where V_S is the amount of titrant used for the sample, V_B is the amount of titrant used for the blank, S is the sample concentration in g L^{-1}, and RS is the sugar concentration as mg g^{-1} of extract.

2.3.12 Thermogravimetric analysis (TGA)

TGA was carried out on a TA Instruments model Q5000 (New Castle, DE, USA) using Q series software 2.8.0.34 release 5.0.1. The mass of each extract was measured to determine the change in mass as a function of temperature. The TGA consists of a precision balance which supports a sample pan. This is then placed into a furnace with controlled temperature and atmosphere. As the temperature is increased, the sample can undergo decomposition, leading to a change in mass.

The samples were placed into tared pans and then into the autosampler of the instrument and heated at a constant rate of 10 ˚C min^{-1} from 25 ˚C to 500 ˚C. Results were recorded as a graph of change in mass vs temperature. Analysis was performed using TA Instruments Analysis software (version 4.5A, build 4.5.0.5).

2.3.13 Mechanical strength testing

The tensile properties of the films was evaluated using an Instron 5567 universal testing machine (Norwood, MA, USA), using the ASTM D882 standard, with modifications.[183] The films were cut according to the ASTM D412 standard, using a Dumbbell die.[184] The thickness of the film was measured in three places and averaged for use in calculations. The films were placed in the grips at a distance of 60 mm from each other. The gauge length was 25 mm. The top clamp was tightened first to ensure alignment of the samples. The samples were pre-tensioned to 0.3 mPa to remove slack from the load string. A cross head speed of 5 mm min^{-1} and a load cell of 1000 N was then used. Tension continued to be applied until the sample broke or there was a decrease in the load of 40%, which indicates sample failure. Each sample type was repeated five times. Statistical analysis was performed using Tukey-Kramer.

2.3.14 Colorimeter

Colour parameters (L*, a*, b*) were determined using a CR-400 Konica Minolta Chroma-Meter (Multi-Gloss 268 Plus) (Konica Minolta, Tokyo, Japan). Films were placed on the surface of a white standard plate (calibration plate values: L* = 97.39, a* = 0.03, and b* = 1.77). Colour parameters were measured using the CIELAB colour scale. Six measurements were taken for each sample.

2.3.15 Water Vapour Permeability

Water vapour permeability of SPI films was measured at the University of San Sebastian, Spain using the procedure described by Etxabide et al.[185] The controlled humidity environment chamber PERME W3/0120 (Labthink Instruments Co. Ltd., Shandong, China) was used, which uses the cup method. Triplicate samples of each film were cut into circles with a diameter of 7.4 cm and a test area of 33 cm^2. As per ASTM E96-00, the films were kept at a temperature

of 38 ˚C and relative humidity of 90%. The water vapour permeability of the films was determined gravimetrically until constant weight.

The water vapour transmission rate (WVTR) was calculated using

$$WVTR = \frac{G}{t \times A} \qquad \text{Equation 2.4}$$

where G is the change in weight (g), t is time (s), and A is the test area (cm^2).

The water vapour permeability (WVT) was calculated using

$$WVT = \frac{WVTR \times L}{\Delta P} \qquad \text{Equation 2.5}$$

where L is the thickness of the sample (cm) and ΔP is the partial pressure difference of the water vapour across the sample (Pa). The WVT for each sample was calculated in triplicate.

Table 2.2. Film thickness (mm) for SPI films containing GME used to calculate the water vapour permeability.

Sample	Film thickness (mm)
SPI control	0.320 ± 0.01
SPI + 0.5% GME	0.470 ± 0.05
SPI + 1% GME	0.300 ± 0.07
SPI + 2% GME	0.320 ± 0.04

3 Evaluation of Grape Marc Extraction

This chapter describes different methods of extracting phenolic compounds from grape marc. The resulting extracts are characterised using high performance liquid chromatography and sugar determination.

3.1 Introduction

Grape marc is a waste product from the wine-making industry. There are different laws and regulations worldwide about its further use and disposal, but in some parts of the world the environmental impact of improperly stored grape marc can be significant. In Marlborough, New Zealand, the District Council is sufficiently concerned with the wide-reaching impacts of this waste to prosecute users who allowed leachate from grape marc to infiltrate a nearby stream. In the 2016 grape harvest, Peter Yealands, a prominent figure in the New Zealand wine industry, attempted to store 5,000 tons of grape marc in an unlined pit for further composting. The Biological Oxygen Demand (BOD) downstream of the pit was 1080 g m^{-3}, which is significantly higher than that typically seen for raw sewage.[186] The stream had grey clouds of leachate in the water which were burning grass, as well as a black fungus. Yealands was fined $18,000 in 2019. This is an example of how even a life-long environmentalist who was committed to solving the grape marc challenge can mismanage the problem.

Grape marc can be disposed of or used in various methods after the wine-making process is over. Grape marc has been used to make nutraceuticals and cosmetics.[187] A common use for grape marc is composting, but the marc must be handled correctly to avoid situations like that described in Marlborough. Another major option for grape marc disposal is as an additive in animal feed. This is despite the presence of condensed tannins which can negatively affect the digestibility of the feed.[188] However, tannins may also reduce methane emissions and nitrogen excretion, therefore ameliorating the greenhouse gas emissions of animal farming.[58] Another

option is the use of grape marc to produce bioethanol – an alternative to fossil fuels.[63] Biogas production is another potential output, using anaerobic digestion. White grape marc is best suited for biomethane production. It has been estimated that this could generate up to 1245 GWh per year of electricity that wineries could then use.[63]

Polyphenols can be removed from the grape marc using different methods, which are described in 1.5.2. Ideally, extraction methods give high extraction rates, can be undertaken quickly, and are cost-effective.[189] More modern technologies are being used more frequently in recent times, as industry looks to be more 'green' in their approach. The two alternative extraction methods evaluated in this chapter are ASE and UAE. Accelerated solvent extraction is an automated technique that uses solvents at high pressure and temperatures above their boiling points. The high pressure keeps the solvent in a liquid state at the elevated temperatures, while the higher temperature increases the efficiency of phenolic compound extraction.[190] Ultrasound assisted extraction increases the fragmentation of the raw material, increasing the solid surface area, which then results in increased extraction rate and yield.[191]

There are many important factors to consider with the different available extraction techniques. These include the time taken for the extraction, the energy consumption, solvents used, and the effectiveness of the extraction technique in producing the target compounds. While conventional solvent extraction can take hours to days to fully extract phenolic compounds, techniques such as UAE and ASE can perform the same extraction in minutes to hours. However, these techniques also require more energy consumption, specialised equipment, and additional sample preparation and treatment.

3.2 Methods

3.2.1 Extraction

Grape marc samples were obtained in New Zealand. White grape marc from the 2013 vintage was obtained in the Marlborough region, consisting of predominantly Chardonnay grape skin, seeds, stems, and pulp. Red grape marc was obtained from the Auckland region in the 2015 vintage. Grape marc was stored at -4 °C. Grape marc used for the accelerated solvent and ultrasound assisted extractions were transported from Auckland, New Zealand to Bonn, Germany at -4 °C. The grape marc was still frozen upon arrival. It was then stored at -4 °C until use.

3.2.1.1 Accelerated solvent extraction

Accelerated solvent extraction method is described in 2.2.3.

3.2.1.2 Ultrasound assisted extraction

Ultrasound assisted extraction method is described in 2.2.4.

3.2.1.3 Conventional solvent extraction

Conventional solvent extraction method is described in 2.2.2.

3.2.2 Phenolic determination

The HPLC method used for the different extraction methods is described in 2.3.5. Linear regression curves for malvidin-3-O-glucoside and catechin are presented in Figure 9.1 and Figure 9.2, respectively.

3.2.3 Sugar determination by HPLC

The concentrations of fructose and glucose in the grape marc extract (GME) samples were determined using the method described in 2.3.11.1. Linear regression curves are presented in Figure 9.3 and Figure 9.4.

3.2.4 Sugar determination by Rebelein method

Residual sugar content in the grape marc extract powder was obtained using the Rebelein method described in 2.3.11.2.

3.2.5 Statistical Analysis

All results came from a minimum of three replicates and statistical analysis including one-way ANOVA and post hoc Tukey-Kramer test was performed on Microsoft Excel 2011 for Windows.

3.3 Results and Discussion

3.3.1 Influence of solvent extraction type on phenolic compounds

The concentrations of malvidin-3-O-glucoside and catechin from red and white grape marc extracts, respectively, were determined by HPLC. The amount of phenolic compounds within the extract has a direct impact on its free radical scavenging ability and antimicrobial properties. Malvidin-3-O-glucoside and catechin are prominent representative compounds of anthocyanins and flavan-3-ols, respectively.

The GME powder had a concentration of catechin of 0.063 ± 0.021 mg mL^{-1}, which is comparable to the levels seen in the UAE white grape marc. UAE gave higher concentrations of catechin and M3G compared to ASE as an extraction method. It has previously been reported that UAE gave higher yields of phenolic products compared to ASE or conventional solvent extraction.[192] This may be due to the 'cavitation effect' seen with UAE, disrupting cell membranes and allowing better penetration of the solvent into the plant matter. The mean particle size distribution after extraction with UAE was less than that after extraction with ASE.[192]

Table 3.1. Concentration of malvidin-3-O-glucoside (M3G) in mg mL^{-1} of ASE and UAE extracts of red grape marc. Values not connected by the same letter are significantly different ($p < 0.05$).

	Concentration of M3G (mg mL^{-1})	
Extraction type / Solvent	ASE	UAE
H_2O	0.033 ± 0.002^{a}	0.123 ± 0.024^{c}
20% EtOH	0.068 ± 0.013^{b}	0.329 ± 0.023^{d}
80% EtOH	0.067 ± 0.003^{b}	0.508 ± 0.044^{e}
80% EtOH + 1% acetic acid	0.069 ± 0.005^{b}	0.634 ± 0.089^{f}

Table 3.2. Concentration of catechin in mg mL^{-1} of ASE and UAE extracts of white grape marc. Values not connected by the same letter are significantly different ($p < 0.05$).

	Concentration of catechin (mg mL^{-1})	
Extraction type / Solvent	ASE	UAE
H_2O	0.003 ± 0.001^{a}	0.050 ± 0.013^{e}
20% EtOH	0.008 ± 0.001^{b}	0.081 ± 0.017^{f}
80% EtOH	0.015 ± 0.002^{c}	0.084 ± 0.009^{f}
80% EtOH + 1% acetic acid	0.012 ± 0.001^{d}	0.094 ± 0.026^{f}

The concentration of malvidin-3-O-glucoside was dependent on the extraction solvent used. UAE with water showed the lowest yield, followed by 20% ethanol, 80% ethanol, and 80% ethanol with 1% acetic acid. All values were significantly different from each other. Pressurised liquid extraction with water showed significantly less anthocyanidin content compared to using a solvent of ethanol with or without acetic acid was used. There was no significant difference based upon the levels of solvent used with the ASE. While the extraction

of anthocyanins increases initially with increasing ethanol concentration, it has been reported that the highest level of extracted anthocyanins is found at lower concentrations.[193] It has been found that increased ethanol concentration increased diffusivity up to a maximum.[194] Further increased ethanol concentration then decreased diffusivity. This diffusivity is directly correlated to anthocyanin yields. This implies that the diffusivity at 20% ethanol is higher than at 80% ethanol when using ASE, leading to less extraction of M3G. When using UAE, this diffusivity effect is not seen. This may be due to the increased kinetic energy, which allows the anthocyanins to solubilise into the ethanol more easily.[195]

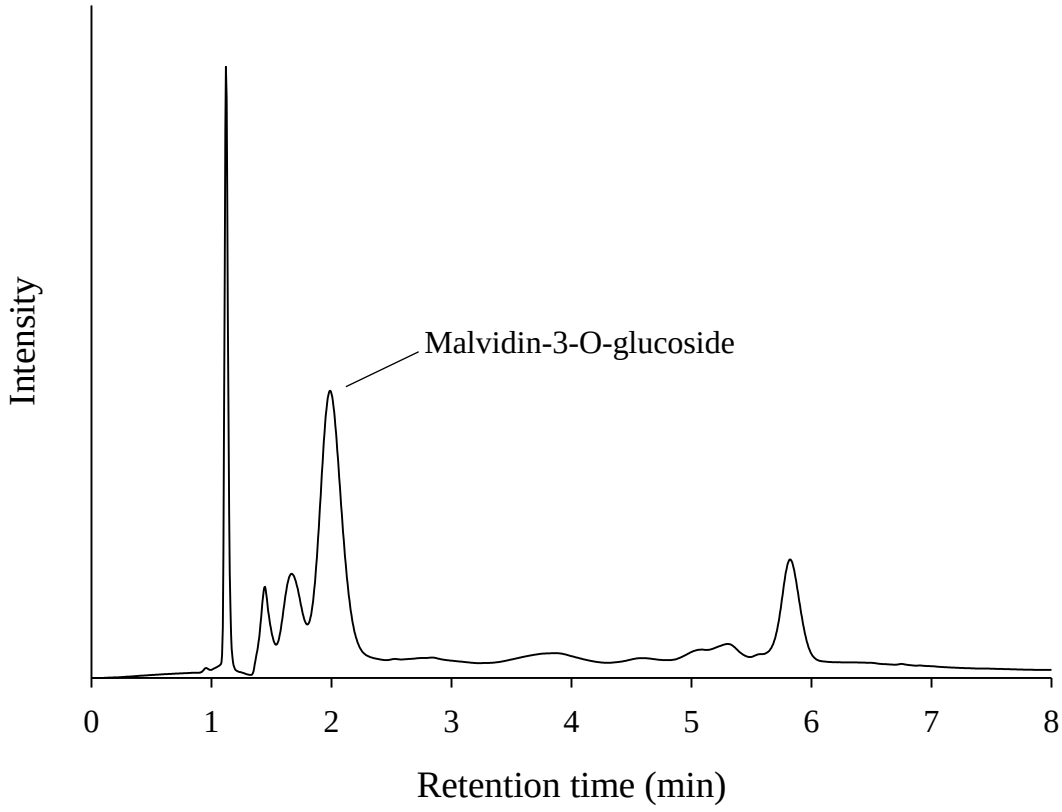

Figure 3.1. HPLC chromatogram of red grape marc extracted via UAE with solvent of 80% ethanol and 1% acetic acid. The detector used was PDA at 520 nm.

For white grape marc, the extraction solvent did affect the catechin concentration yield for ASE. The extraction solvents of water and 20% ethanol did not show a significant difference, but the catechin concentration increased significantly for the solvents 80% ethanol and 80% ethanol with 1% acetic acid. The solubility of catechin in water is less than that in ethanol.[196] Therefore, as the concentration of ethanol increased, the amount of catechin also increased.

However, for UAE, the level of solvent did not significantly change the catechin content of the extracts, except for water which had a lower value than any other solvent. This may be caused by the greater kinetic energy from the UAE allowing the catechin to solubilise into the ethanol.[195] The higher concentration of ethanol did not improve on this effect.

This gives information on the changes in extraction of anthocyanins and flavan-3-ols from the grape marc using malvidin-3-O-glucoside and catechin as representative phenolic monomers. Other phenolic compounds may not follow these same trends, so it is important in future work to identify other compounds that are present and quantify them. Example chromatograms of red and white grape marc extracted with 80% ethanol and 1% acetic acid are presented in Figure 3.1 and Figure 3.2, respectively. The peaks of malvidin-3-O-glucoside and catechin are labelled.

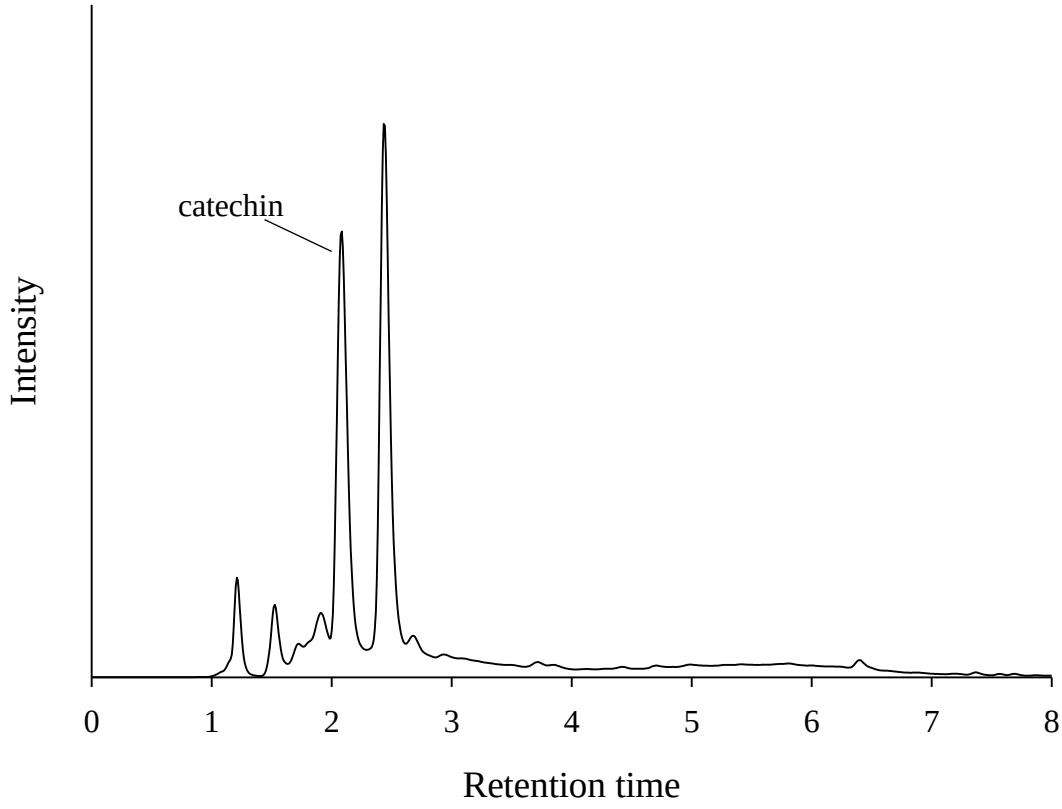

Figure 3.2. HPLC chromatogram of white grape marc extracted via UAE with solvent of 80% ethanol and 1% acetic acid. The detector used was RF.

3.3.2 Sugar determination

The glucose and fructose concentrations of the grape marc extracts were determined via HPLC. The residual sugar concentration of GME powder was determined by the Rebelein method. The amount of sugar in an extract can affect its potential applications in packaging – too much sugar and it can discolour the packaging if exposed to high temperatures.

Table 3.3. Sugar concentrations in g L^{-1} of ASE grape marc with associated solvents. Values in the same column not connected by the same letter are significantly different ($p < 0.05$).

Grape type / Solvent	Red		White	
	Fructose	Glucose	Fructose	Glucose
H_2O	0.958 ± 0.037^{a}	0.949 ± 0.021^{a}	1.39 ± 0.03^{a}	$1.52 \pm 0.09^{a,b}$
20% EtOH	0.842 ± 0.094^{b}	0.871 ± 0.071^{b}	1.25 ± 0.05^{b}	1.59 ± 0.09^{a}
80% EtOH	0.724 ± 0.040^{b}	0.727 ± 0.044^{c}	1.13 ± 0.03^{c}	1.43 ± 0.04^{b}
80% EtOH + 1% acetic acid	0.741 ± 0.061^{b}	0.752 ± 0.051^{c}	1.02 ± 0.09^{d}	1.33 ± 0.08^{c}

Table 3.4. Sugar concentrations in g L^{-1} of UAE grape marc with associated solvents. Values in the same column not connected by the same letter are significantly different ($p < 0.05$).

Grape type / Solvent	Red		White	
	Fructose	Glucose	Fructose	Glucose
H_2O	3.48 ± 0.69^{a}	3.36 ± 0.77^{a}	5.53 ± 0.50^{a}	$6.22 \pm 1.59^{a,b}$
20% EtOH	4.03 ± 0.34^{a}	3.67 ± 0.50^{a}	6.21 ± 0.30^{b}	7.95 ± 0.47^{a}
80% EtOH	3.46 ± 0.24^{a}	3.18 ± 0.36^{a}	5.98 ± 0.14	7.18 ± 0.28
80% EtOH + 1% acetic acid	3.80 ± 0.43^{a}	3.59 ± 0.65^{a}	5.52 ± 0.27^{a}	6.41 ± 0.42^{b}

In general, the white grape marc contained higher sugar concentrations than the red grape marc. The white grape marc contained approximately 1.5 times more fructose and 2 times more glucose than the red grape marc. This is consistent with other findings in literature.[197] Red grape marc may contain less carbohydrates than white grape marc due to wine making processes. Red grape marc typically stays in contact with the juice for the maceration period in

order to improve the sensory qualities of the wine. Over this time, phenolics from the grape marc may be extracted into the juice and sugars are fermented during this time.[197]

The GME extracts produced via UAE contained significantly higher levels of both glucose and fructose than those produced via ASE. This is because UAE gave better extraction of all compounds from the grape marc, as seen in the difference in concentrations in the phenolic compounds.

Red grape marc extracted with ASE showed some difference in fructose and glucose levels with solvent type. Red grape marc extracted with water had significantly higher levels of both fructose and glucose compared to the other solvents, while white grape marc extracted with water had significantly more fructose than with the other solvents. Red grape marc extracted with 20% ethanol also had significantly higher levels of glucose than those extracted with 80% ethanol. The solubility of glucose in water is 909 g L^{-1}, while in ethanol it is only 1.96 g L^{-1}. The solubility of fructose in ethanol is 17.4 g L^{-1} and it is very soluble in water.[198] The difference in sugar concentration could be due to the difference in solubility between solvents. As glucose and fructose are both very soluble in water, but significantly less soluble in ethanol, the sugars would be more easily extracted into the water solvent. This phenomenon was not seen in the UAE results. This may be because the UAE released more sugar by breaking the cell walls.[195] The increased kinetic energy from the UAE may also have allowed the sugars to solubilise into the ethanol more easily.

The GME powder contained 70 ± 33 mg g^{-1} residual sugars. When analysed for fructose and glucose content using the same method as for the ASE and UAE extracts, there was no detectable level. This is due to the GME powder undergoing a purification step using Amberlite resin, which was intended to remove free sugars. This shows that the resin is successful at

removing the sugars from the extract. The detected residual sugars could be due to hydrolysable tannins, which have sugar molecules within their structure.

3.4 Conclusion

Both red and white grape marc were successfully extracted using UAE, ASE and conventional solvent extraction. UAE was the most effective of the extraction techniques, which increased the yield in the phenolic compounds malvidin-3-O-glucoside and catechin for red and white grape marc, respectively. Water was the least effective solvent, as the addition of just 20% ethanol significantly increased the extraction of phenolic compounds. The purification of the conventional solvent extract significantly lowered the sugar content without lowering the catechin content. This has shown that grape marc can be extracted with different techniques and solvents.

4 Bio-based films containing GME

This chapter will examine how GME can be incorporated into biodegradable and environmentally sourced films that are processed at low temperatures (defined as less than 150 ˚C). The antioxidant and antimicrobial properties of the films incorporated with GME will be assessed.

4.1 Introduction

Currently, the majority of food packaging produced worldwide is made of non-biodegradable polymers such as polyethylenes.[199] It has been estimated that, as of 2015, approximately 6300 million metric tons of virgin plastics had been globally produced. Although some of these can be recycled, the majority end up in landfills or in the environment. Around 9% of the plastics produced had been recycled, 12% incinerated, and 79% accumulated in landfills or otherwise discarded. If there is no change to this situation, it is estimated that by 2050 approximately 12,000 million metric tons of plastic will be in landfills or thrown away elsewhere.[200] Due to the increasing public awareness about environmental concerns, it is important to investigate other types of films that can be used for food packaging or other active packaging uses.

Ethyl cellulose is one potential alternative to current packaging. It is a plant-derived polymer that can be processed at room temperature, removing the need for additional heating during the processing and manufacturing of films. Unlike cellulose, ethyl cellulose is hydrophobic.[201] However, plasticisers are required to be used in manufacturing to improve its thermo-plasticity and flexibility.[202] Natural additives have been added into cellulose polymers to increase the active packaging properties of the films, including GME, bayberry polyphenols, and clove essential oil.[171,203,204] Cellulose polymers are also widely used in the pharmaceutical industry, mainly as a coating material for tablets and drug microcapsules.[202]

Gelatin and soy protein are two other alternatives to polyolefins.[94] Their tolerance to heat is a weak point, with a thermal denaturation temperature of 35 °C for fish gelatin, and 75 °C for soy protein.[96,97] Another issue is their hydrophilicity. To improve soy protein films, it is possible to incorporate lipids into the film forming solution, or crosslink the protein chains to increase hydrophobicity.[205] Fish gelatin films have been modified with oxidised linoleic acid successfully to form a hydrophobic surface.[206]

In this chapter, ethyl cellulose, fish gelatin, and soy protein films are evaluated for their suitability for use as active packaging with the inclusion of GME. The antioxidant and antimicrobial capacity of the films are also investigated.

4.2 Methods

4.2.1 Phenolic Compound Extraction

Extraction method is described in 2.2.2.

4.2.2 Film Preparation

The methods used to prepare ethyl cellulose, soy protein isolate, and fish gelatin films are described in 2.2.6.

4.2.3 Colour determination

The method used for colour determination is described in 2.3.14.

4.2.4 Water Vapour Permeability

The method used to find the water vapour permeability of the films is described in 2.3.15.

4.2.5 Antioxidant Activity

The methods used to find the antioxidant activity of the films using DPPH and ABTS assays are described in 2.3.1.

4.2.6 Antimicrobial Activity

The antimicrobial activity of the films was found using a modified JIS method, which is described in 2.3.2.4.

4.2.7 Statistical analysis

Statistical analysis of ANOVA and post hoc Tukey-Kramer tests was performed using Microsoft Excel for Windows.

4.3 Results and Discussion

4.3.1 Film appearance

The CIELab colour coordinates are described by L*, a*, and b*. L* is a measure of lightness, where black is 0 and white is 100, a* is a measure of changes from green (negative) to red (positive), and b* is from blue (negative) to yellow (positive). All films decreased in lightness with addition of 2% GME (increase in L*) (Table 4.1). The red hue, denoted by a*, increased for all films when 2% GME was added. The films also all became more yellow, with a significant increase in b* for all films when 2% GME was added. The similar trends in all films is due to the identical GME that was added to each kind of film.

Table 4.1. CIELab colour coordinates for the EC, SPI, and FG films with and without 2% GME added. Values in the same column not connected by the same letter are significantly different ($p < 0.05$).

Sample	L*	a*	b*
EC	91.7 ± 0.7^{a}	5.41 ± 0.11^{a}	-7.71 ± 0.51^{a}
EC + 2% GME	76.9 ± 3.2^{b}	6.89 ± 0.63^{b}	14.7 ± 1.6^{b}
SPI	85.9 ± 0.1^{c}	0.312 ± 0.085^{c}	33.7 ± 0.2^{c}
SPI + 2% GME	73.4 ± 0.7^{d}	8.73 ± 0.30^{d}	43.8 ± 0.4^{d}
FG	96.3 ± 0.1^{e}	-0.113 ± 0.008^{e}	3.17 ± 0.14^{e}
FG + 2% GME	94.4 ± 0.2^{f}	0.138 ± 0.041^{f}	9.21 ± 0.31^{f}

Fish gelatin films needed to be stored with paper barriers between each film, as they were inclined to adhere strongly to other plastics. After pressing, SPI and fish gelatin films were pressed under a flat weight to avoid deformation while cooling. Ethyl cellulose films did not require this as they were produced via solvent casting. All films were malleable and not easily broken. The edges of the SPI films were powdery due to incomplete pressing.

4.3.2 Water Vapour Permeability

Water vapour permeability is an important property for packaging materials. Especially in the case of food packaging, the introduction of water into the packaged product can have a severely detrimental effect on the quality and the shelf life of the product.[207] The water vapour permeability measurements were performed only on soy protein isolate films. The fish gelatin and ethyl cellulose films were unable to be made to the required size for such testing.

Table 4.2. Film thickness (mm) for SPI films containing GME used to calculate the water vapour permeability.

Sample	Film thickness (mm)	
	1	2
SPI control	0.323	0.316
SPI + 0.5% GME	0.508	0.434
SPI + 1% GME	0.258	0.350
SPI + 2% GME	0.294	0.352

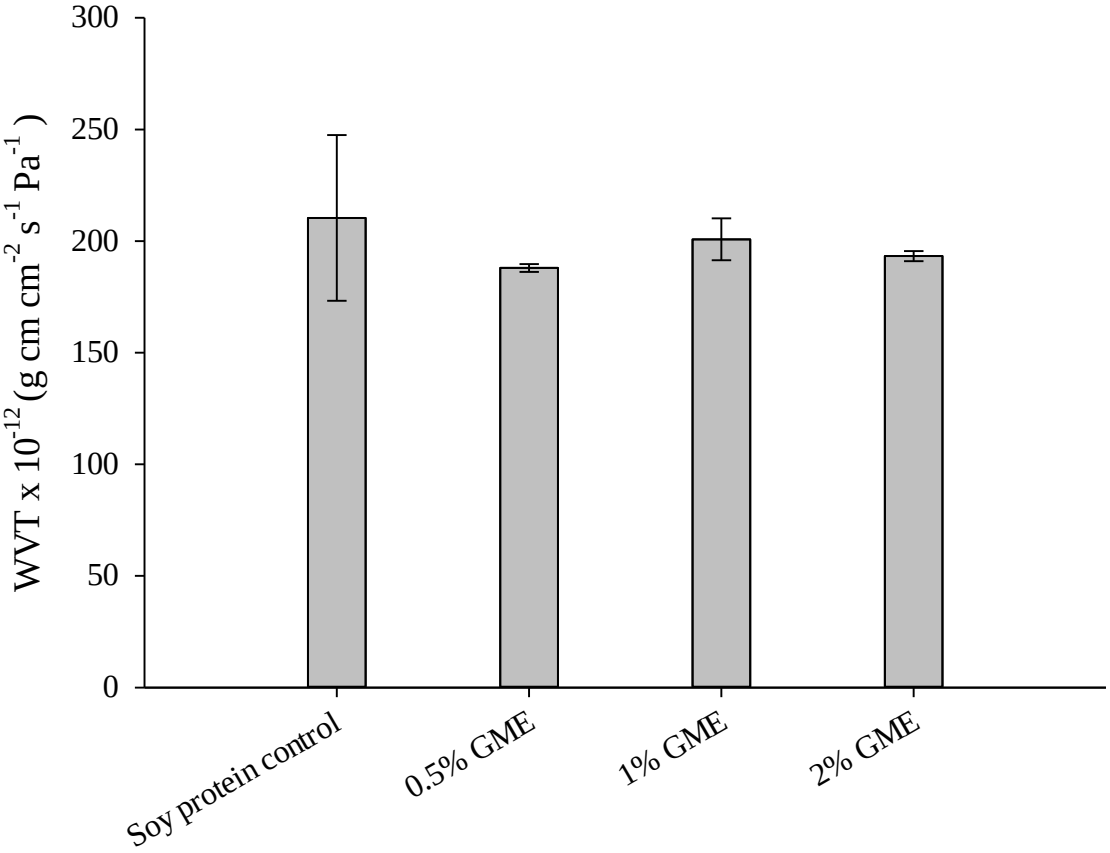

Figure 4.1. Water vapour permeability measurements for SPI films containing GME.

The inclusion of GME to the SPI films had no significant impact on the water vapour permeability of the films (Figure 4.1). Variability in film thickness was used when calculating the water vapour permeability (Table 4.2). Protein films in general have poor water vapour permeability qualities for packaging, which can limit their applications. In general, they have high water permeability, letting water through more easily than other polymers used for packaging. This can be mitigated by adding hydrophobic components like waxes, long chain fatty acids, or lipids.[208] Polyethylene, a widely used polymer in packaging applications, has a WVT of 1.14 x 10^{-16} to 6.74 x 10^{-16} g cm cm^{-2} s^{-1} Pa^{-1}.[209] Poly lactic acid (PLA), an alternative bio-sourced polymer, has a WVT of 46.1 x 10^{-13} g cm cm^{-2} s^{-1} Pa^{-1}.[210] However, PLA can

undergo hydrolysis so is not suitable as a replacement either. Alternative biobased films need additional work and modification before they are a viable replacement for polyolefins in the future.

4.3.3 Antioxidant capacity

The antioxidant activities of the films were evaluated through the DPPH and ABTS radical scavenging assays.

Table 4.3. DPPH radical scavenging assay results of fish gelatin and soy protein isolate films

	% Scavenging of DPPH$^{\cdot}$
Fish gelatin control	57.7 ± 7.6^{a}
Fish gelatin 2% GME	95.8 ± 4.2^{b}
SPI control	$72.3 \pm 7.2^{a,c}$
SPI 2% GME	84.6 ± 1.2^{c}

Table 4.4. ABTS radical scavenging assay results of ethyl cellulose films

	% ABTS$^{\cdot}$ Scavenged
Ethyl cellulose control	5.1 ± 1.1^{a}
1% GME	74.8 ± 1.5^{b}
2% GME	94.5 ± 4.9^{c}

Ethyl cellulose films were evaluated using the ABTS radical scavenging assay. The DPPH radical scavenging assay was not suitable as the ethyl cellulose films would have dissolved in the 80% methanol used as a solvent.

Both fish gelatin and soy protein isolate films showed inherent antioxidant activity present in the control films. The fish gelatin control scavenged 57.7 ± 7.6% while the SPI control scavenged 72.3 ± 7.2%. There have been some reports that fish gelatin shows antioxidant activity because of the presence of amino acids such as glycine and proline.[211–214] Similarly, the inherent antioxidant activity of the SPI films may be due to a peptide fraction of SPI.[215] The inclusion of 2% GME into the fish gelatin films increased the free radical scavenging ability of the film significantly. In contrast, SPI films containing 2% GME scavenged 84.6 ± 1.2% DPPH˙. Li et al. found that the addition of 1% grape seed extract to gelatin-based films gave a DPPH radical scavenging increase of over 70% compared to the control, where here the increase was 12.3%.[216] The addition of 2% red grape extract by Ciannamea et al. to soy protein films showed a radical scavenging ability increase of over 60% of DPPH˙, compared to 38.1% here.[217] The difference is due to the inherent high free radical scavenging ability of the control films, which may be explained by a different source of SPI and fish gelatin.

The control film for ethyl cellulose scavenged only 5.1 ± 1.1% of the ABTS˙. This small scavenging ability may be due to hydroxyl moieties being oxidised.[171] The ethyl cellulose results were higher than those reported previously, with Olejar et al. reporting ABTS˙ radical scavenging ability of approximately 45% at 2% GME.[171] This may be due to differences in the GME, as the grape extract used by Olejar et al. was purchased from a commercial supplier, while the GME used in this experiment was extracted from fresh grape marc. The experimental test parameters used were identical to those involved in this experiment.

4.3.4 Antimicrobial activity

Fish gelatin and SPI films showed no antimicrobial activity against *S. aureus*, at the tested GME concentrations. Fish gelatin and SPI films have shown no inherent antimicrobial activity in literature.[218,117] Fish gelatin films impregnated with clove essential oil at 6% did show some antimicrobial activity against *E. coli*, while SPI films with 1% grape seed extract did not inhibit the growth of *E. coli*. [117,219] However, in the present study, ethyl cellulose films did show some antimicrobial activity, including the ethyl cellulose control film with no added GME.

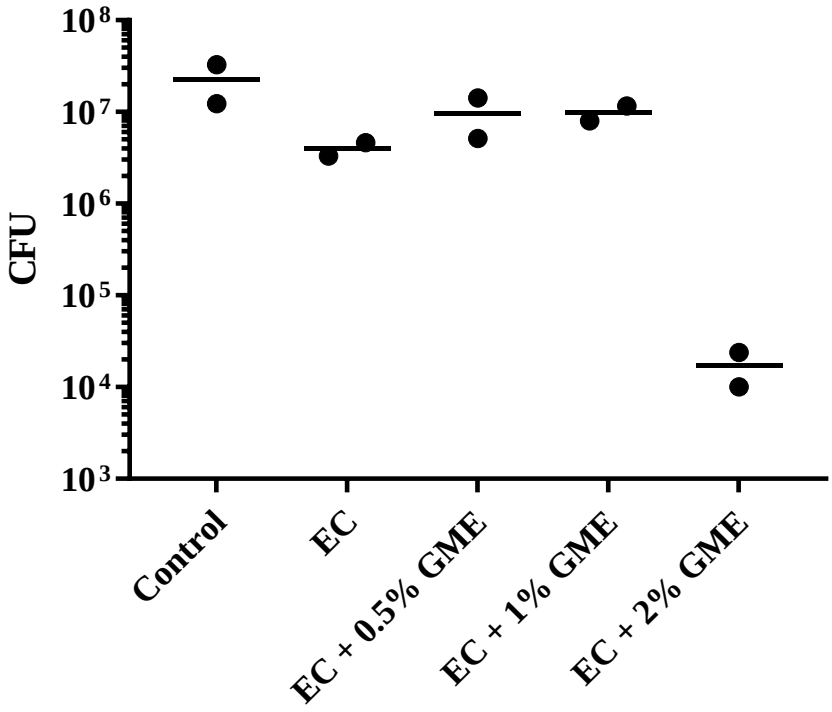

Figure 4.2. JIS results of ethyl cellulose films against *S. aureus* showing median values. The y-axis is shown as a $\log_{10}$ scale.

The ethyl cellulose control film did show some inhibition of *S. aureus* growth, displayed in Figure 4.2. The mean CFU value of the control was $2.25 \pm 1.44 \times 10^7$, and that of the ethyl cellulose film was $3.95 \pm 0.92 \times 10^6$. This is a reduction of almost one order of magnitude. There was no change in CFU for the films containing 0.5% or 1% GME. However, the film

containing 2% GME showed a significant decrease in CFU with a three order of magnitude difference compared to the polyethylene control.

As the ethyl cellulose films were produced using ethanol as a solvent, it was very important to ensure that all of the solvent was removed before testing. Any presence of ethanol could interfere with the bacterial growth and contaminate the results. This also meant that the films needed to be sterilised using UV light instead of the standard use of ethanol. These factors may have had an impact on the antimicrobial results presented here. Although ethanol testing strips were used to test for ethanol before starting, it is still possible that some residual ethanol was present. The use of UV light to sterilise the films may also have affected the grape polyphenols present in the films. Another factor that needs to be considered is the distribution of the GME within the films. When the GME is present at the surface of the film, the polyphenols will be in direct contact with the inoculum and will therefore have more effective inhibitory effects on the microbial growth. This is not a factor that affected the antioxidant testing, as the films were fully submerged in the solutions containing the free radicals. More testing needs to be done to ascertain how to better modify the JIS standard to evaluate more accurately the antimicrobial properties of the ethyl cellulose films.

4.4 Conclusion

GME has shown good potential for its use as an antioxidant and antimicrobial additive in biodegradable and bio-sourced films produced at low temperatures. The addition of GME at 2% to fish gelatin, soy protein isolate and ethyl cellulose films significantly increased the antioxidant activity compared to the control. In addition, ethyl cellulose films with 2% GME showed a three order of magnitude decrease in CFU against *S. aureus*. Ethyl cellulose with no additives also showed minor antimicrobial activity against *S. aureus*, with a one order of magnitude decrease in CFU compared to the polyethylene control. Fish gelatin and soy protein

isolate films showed no antimicrobial activity at the tested concentrations. The water vapour permeability of SPI films showed how bio-sourced polymers do have lesser mechanical properties than polyolefins, but as consumer awareness of environmental issues grows, these polymers will become more attractive to industry.

5 Influence of heating on GME

5.1 Introduction

While GME has shown promise in bio-based polymers such as ethyl cellulose, most of the packaging used worldwide traditionally makes use of petroleum-based polymers such as high-density polyethylene (HDPE), linear low-density polyethylene (LLDPE), and polypropylene (PP). These are processed through melt blending, where the material is subject to temperatures of 150 – 200 ˚C, and in this stage of the processing additives such as grape marc can be included. It is therefore important to know whether the antioxidant and antimicrobial properties of grape marc extracts are significantly affected by this exposure to higher temperatures.

Thermal treatment can be used during or prior to the extraction process; grape seeds heated before extraction gave a higher polyphenol yield.[220] Sólyom et al. found that the total phenolic content and antioxidant activity of red grape marc showed an initial increase when heat treated over 100 ˚C.[221] In studies that specifically looked at grape seed extract, there was no change in antioxidant activity, tannin content, and procyanidin components after being heat treated in a furnace at 100 ˚C for 15, 30, and 60 minutes.[222] When grape seed extract was heated to 150 ˚C for 40 minutes, the phenolic content increased by 50%, but increasing the temperature to 200 ˚C reduced both the phenolic content and antioxidant activity.[220] This indicates that phenolics in grape seed extract can be liberated by heat treatments. Studies on sesame meal extracts and citrus peel extracts showed that similar heat treatment converted insoluble phenolics to a soluble form.[223,224] Grape seed extract showed less thermal degradation than individual samples of gallic acid, catechin, and vanillic acid.[225] To the best of our knowledge, there has been no previous research on the effects of heat treatment on the antimicrobial activity of grape marc extract.

In this chapter, GME is evaluated for its suitability for use in melt blended films for active packaging. The structural properties, antioxidant, and antimicrobial capacity of the extracts after exposure to specific temperatures are also explored.

5.2 Methods

5.2.1 Phenolic Compound Extraction

The extraction method is described in 2.2.2.

5.2.2 Heating of extracts

The extracts were heated as described in 2.2.5.

5.2.3 Spectroscopic Analysis

FT-MIR and Raman spectroscopy were used using the methods described in 2.3.8 and 2.3.9, respectively.

5.2.4 Principal Component Analysis

Principal Component Analysis (PCA) was then conducted using full cross validation. All spectral pre-processing and data analysis were carried out using The Unscrambler X 10.3 (CAMO, Norway) and Origin(Pro) 2019b (OriginLab Corporation, USA).

5.2.5 HPLC for Monomeric Phenolics Determination

The HPLC method used is described in 2.3.5.

5.2.6 Gel Permeation Chromatography (GPC)

The GPC method was carried out as described in 2.3.6.

5.2.7 Total Phenolic Content

The total phenolic content of the samples was established using the Folin-Ciocalteu assay, described in 2.3.7.

5.2.8 Antioxidant Capacity

The antioxidant capacity of the samples was evaluated using the DPPH assay, described in 2.3.1.1.

5.2.9 Antimicrobial Activity

Antimicrobial testing was conducted against *E. coli* and *S. aureus*, using the method described in 2.3.2.1.

5.2.10 Statistical analysis

Basic data analysis was performed using Microsoft Excel 2015 for Windows. Statistical analysis included ANOVA and post hoc Tukey-Kramer test.

5.3 Results and Discussion

5.3.1 Structural analysis by FT-MIR spectroscopy

Heated extracts were analysed using FT-MIR to ascertain any impact the heating had on the structure of the compounds. The grape marc powders showed the typical bands observed for flavan-3-ol based compounds in the fingerprint region, presented in Table 5.1 and Figure 5.1. No major changes were seen between extracts heated to 100 ˚C for 10 minutes and one hour.

Table 5.1. Main peaks for bulk GME kept at room temperature (B-RT). (sh) denotes a shoulder.

Peak B-RT (cm^{-1})	Assignment[21]
1725/1708/1695 (broad)	C=O stretching, catechinic acid rearrangement during extraction process
1602-03	Aromatic C=C bending
1515	Stretching C=C aromatic compounds
1466 (sh)	Stretching C=C aromatic compounds
1447 (sh) – 1441	Stretching OH, stretching C-O-C, bending OH
1357	Combination C-O stretching C-OH bending vibrations
1310 (sh)	C-OH deformation of phenols
1280	Stretching C=C aromatic compounds
1243	C-O stretching and C-OH deformation
1202	C-OH bending
1150	Combination C-O stretching and OH bending vibrations
1091	Aromatic CH out-of-plane bending/CH out-of-plane deformation and OH out-of-plane deformation
1058-59	OH bending vibration
1036	Symmetric stretching C-O
1016	C-O stretching aliphatic secondary alcohol, stretching C-C and bending C-H
982 (sh)	Stretching CC and bending CC
887	Ring deformation/breathing
820	Deformation aromatic hydroxyl groups/CH deformation aromatic ring
778	CC, CH, C-O, and ring vibrations/CH_2OH bending
765	H-bonded OH stretching in carboxylic groups

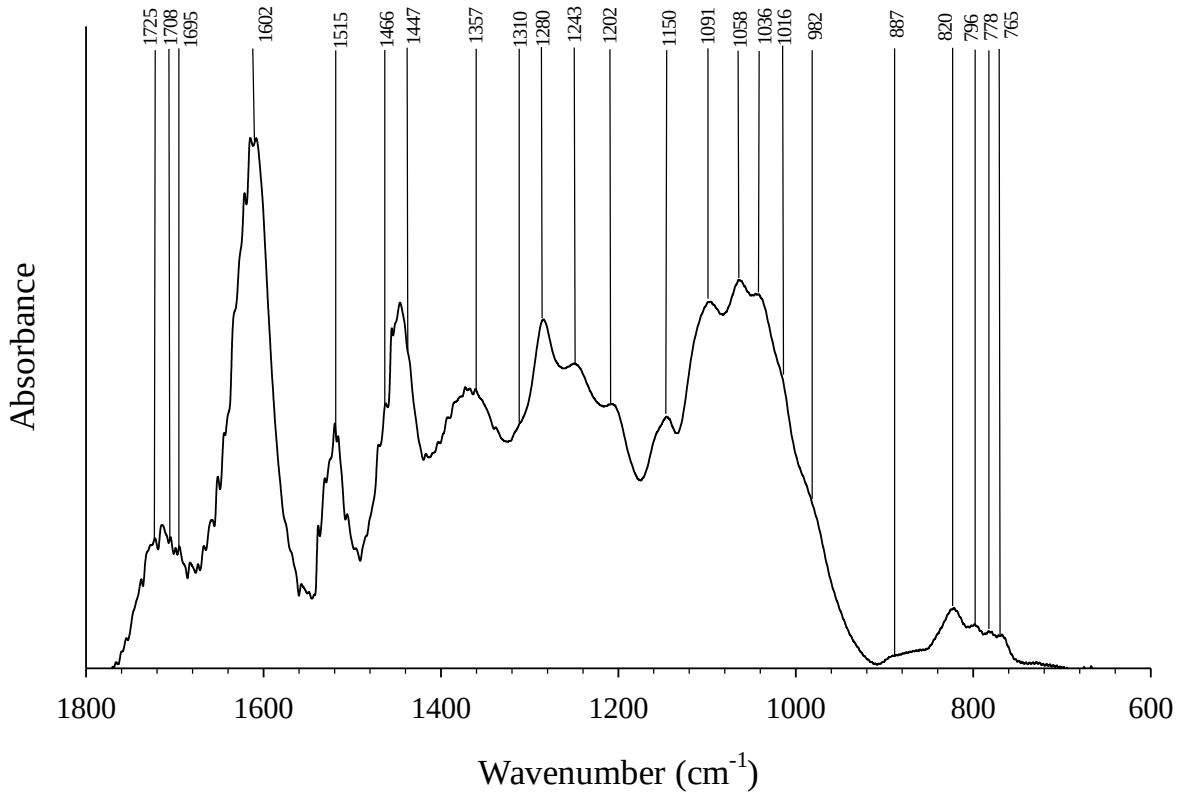

Figure 5.1. FT-MIR spectra of bulk GME kept at room temperature. Range 1800 – 600 cm^{-1}.

Outside of the fingerprint region, the other range of interest is from 3600 to 2500 cm^{-1}. This region can give information about the polymerisation of tannins.[21] The broad band from 3600 – 3000 cm^{-1} was deconvoluted into several peak components for the 100 ˚C treatment (Figure 5.2). The components at 3158, 3204, 3324, 3397, and 3523 cm^{-1} are the result of the complex network of -OH groups in the powder. The -OH groups are involved in inter- and intra-molecular hydrogen bond formation, which relate to H-bonded -OH and free -OH stretching motions, respectively.[21,226,227]

The presence of these -OH groups is a result of the coexistence of flavan-3-ol monomers such as catechin and epicatechin with proanthocyanidin oligomers and polymers. The -OH groups also crosslink and stabilise the proanthocyanidin structures by forming multiple hydrogen bonds. The compounds all interact to different extents, resulting in different constraints on the -OH stretching vibrations. The other three components at 2862, 2928, and 3050 cm^{-1} are assigned to C-H stretching vibrations of aromatic rings.[21,228,229]

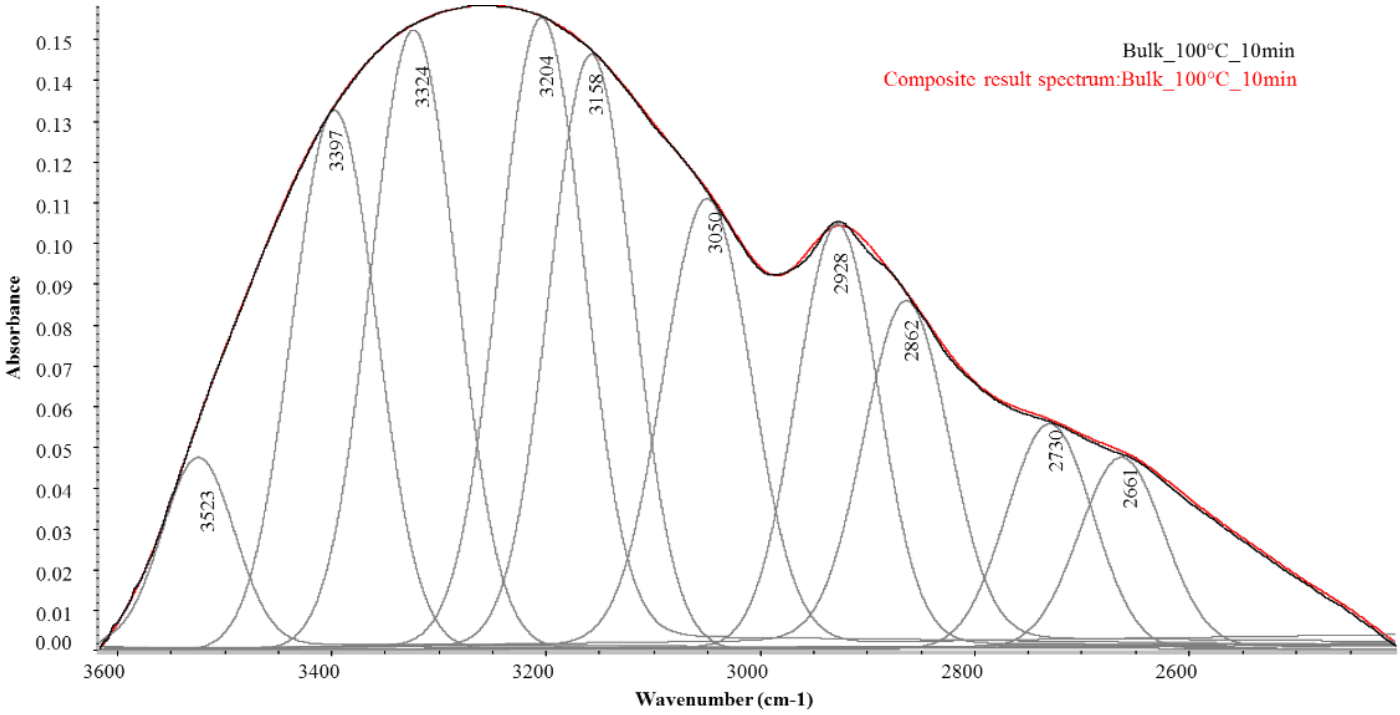

Figure 5.2. Deconvolution of bulk 100 ˚C treated GME for 10 minutes spectral region 2500 to 3600 cm^{-1}

The GME powder treated at 250 ˚C for 10 minutes showed significant differences in the same spectral region (Figure 5.3). The deconvolution of the broad peak gave peaks at 2863, 2920, 3060, 3141, 3209, 3311, 3408, and 3535 cm^{-1}. This shows a generic shift of vibrational frequencies to higher values, also known as a ‘blue-shift’. This is caused by the disruption of hydrogen bonding. Just as the formation of hydrogen bonds results in a shift in the stretching

vibrations of the proton donor group to lower frequency values, the disruption of hydrogen bonds results in a shift to higher frequency values, or a blue-shift, of the vibrational frequencies involved in the hydrogen bond linkage.[230,231] This shift implies that exposure to higher temperatures such as 250 ˚C may cause hydrogen bond network disruption in the GME powder.

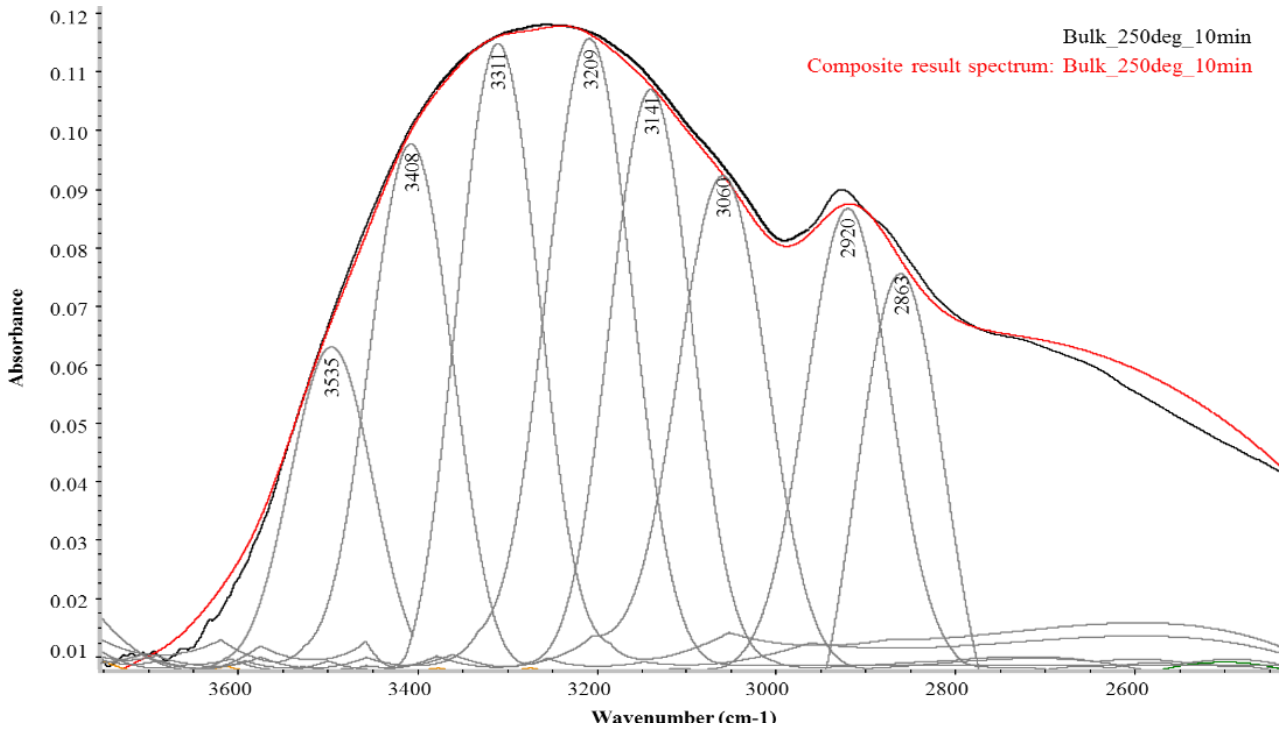

Figure 5.3. Deconvolution of bulk 250 ˚C treated GME for 10 minutes spectral region 2500 to 3700 cm^{-1}

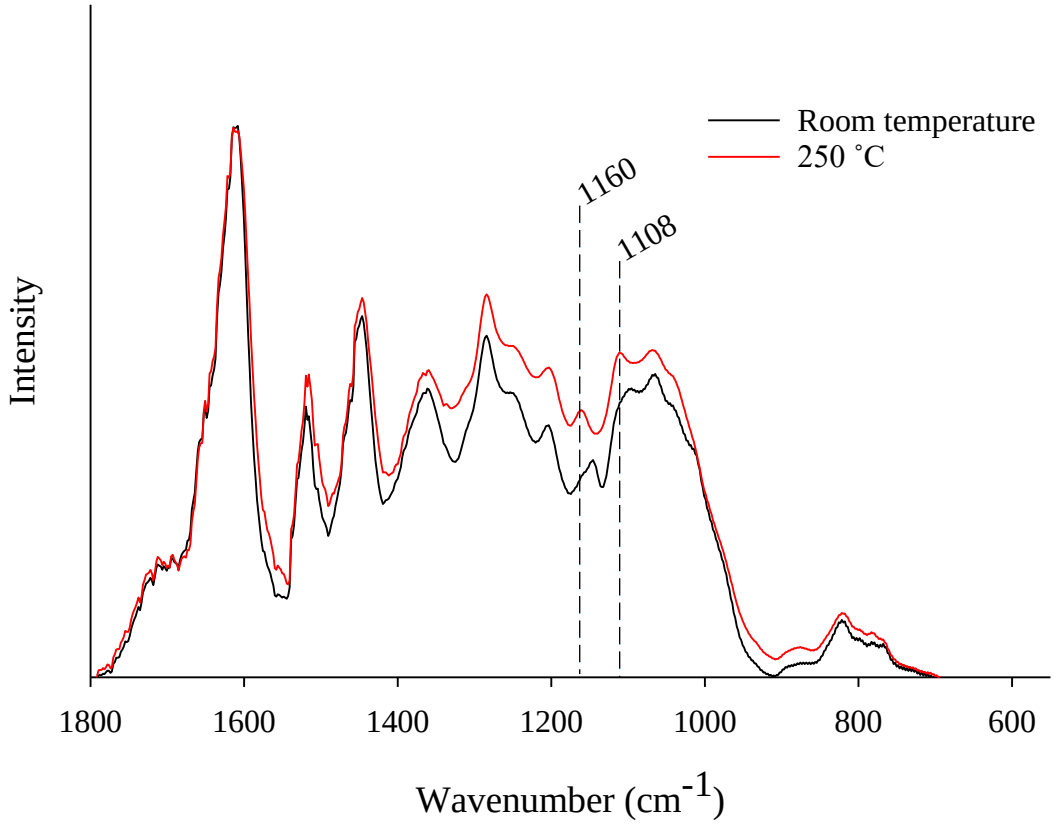

Figure 5.4. Comparison between room temperature and 10 minute, 250 ˚C heated small-scale extract in fingerprint region 1800 to 600 cm^{-1}.

The blue-shift seen in the deconvolutions in Figure 5.2 and Figure 5.3 is further evident in the fingerprint and combination band regions for the extracts. In Figure 5.4 the small-scale extracts treated with room temperature and heated to 250 ˚C for 10 minutes are compared. The peak that arises at 1108 cm^{-1} may be attributed to CH_2OH deformation or structural interactions with the extraction solvent.[232] Another possible attribution is a combination of C-O stretching and OH deformation. Compared to the literature and also the room temperature GME (1100 cm^{-1}), this peak is blue-shifted due to the release of some hydrogen bonds.[21] This blue-shift was again seen in the shift of the 1150 cm^{-1} peak to 1160 cm^{-1}; this peak is attributed to a

combination band for C-O and OH deformations.[21] From this we can hypothesise that the hydrogen networks within tannins are selectively modified by temperature.

Figure 5.5 and Figure 5.6 show the FT-IR spectra in the spectral region 1800 to 600 cm^{-1} of bulk and small-scale GME, respectively. The main structural elements seen in both GME persist across all treatments, including after 10 minutes at 250 ˚C.

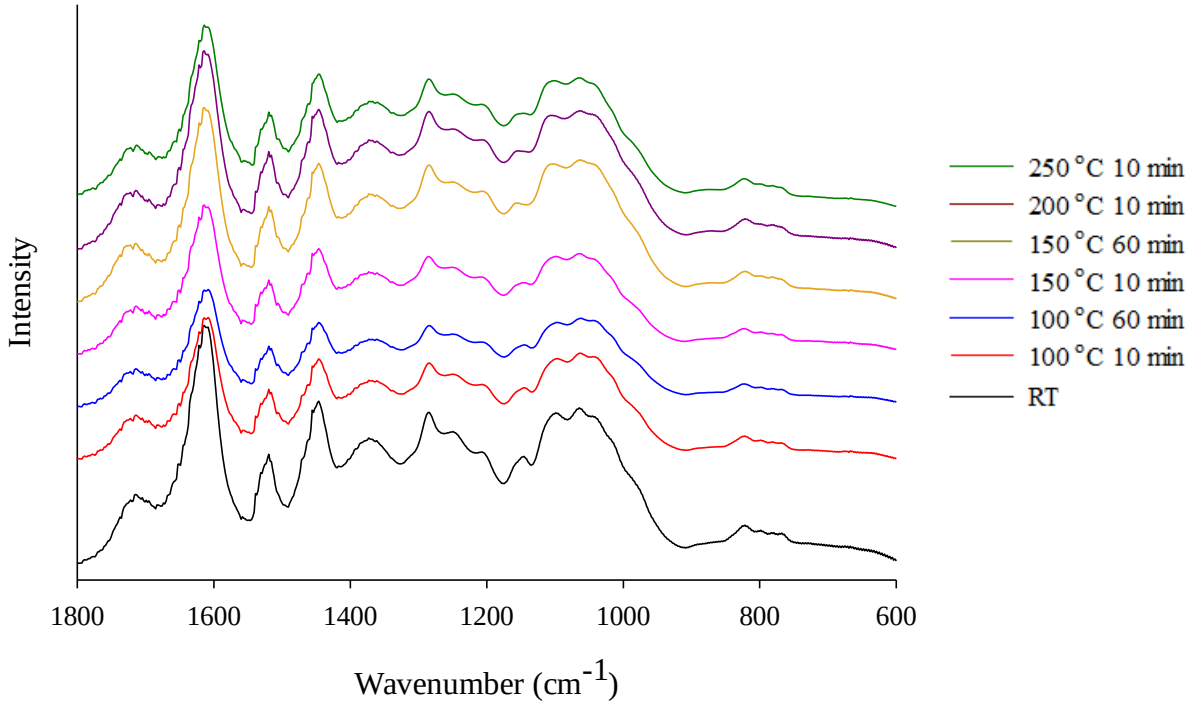

Figure 5.5. FT-IR spectra of all bulk GME in the spectral region 1800 to 600 cm^{-1}.

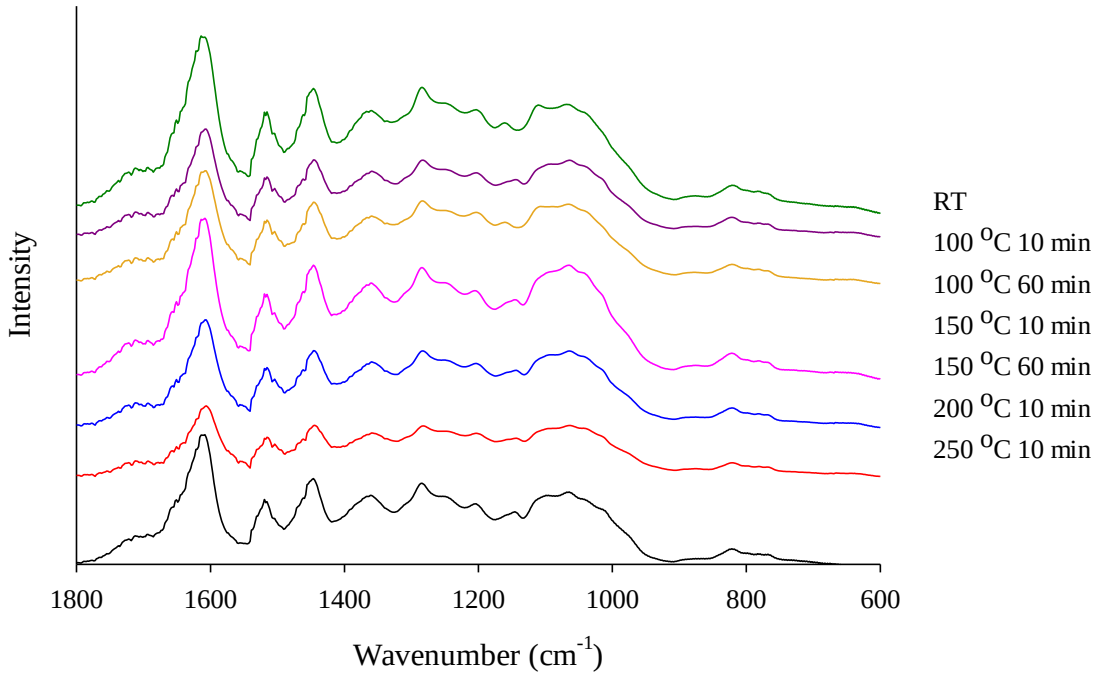

Figure 5.6. FT-IR spectra of all small-scale extracts over the spectral region 1800 to 600 cm^{-1}.

Principal component analysis (PCA) was carried out on the FT-MIR spectra. The spectra were all pre-processed using a linear baseline correction and SNV transformation over the region 520 to 1800 cm^{-1}. The scores plot for the two principal components (PCs) show separation between the bulk and small-scale extract (PC1 separation) and differences between low and high heat treatments for the small-scale extract (PC3 separation) (Figure 5.7). The second PC (PC2) showed inter-sample variation within the two groups but did not show any clear trends. The scores are not shown. PC1 accounts for 45% of spectral variance, while PC3 accounts for a further 16% of spectral variance.

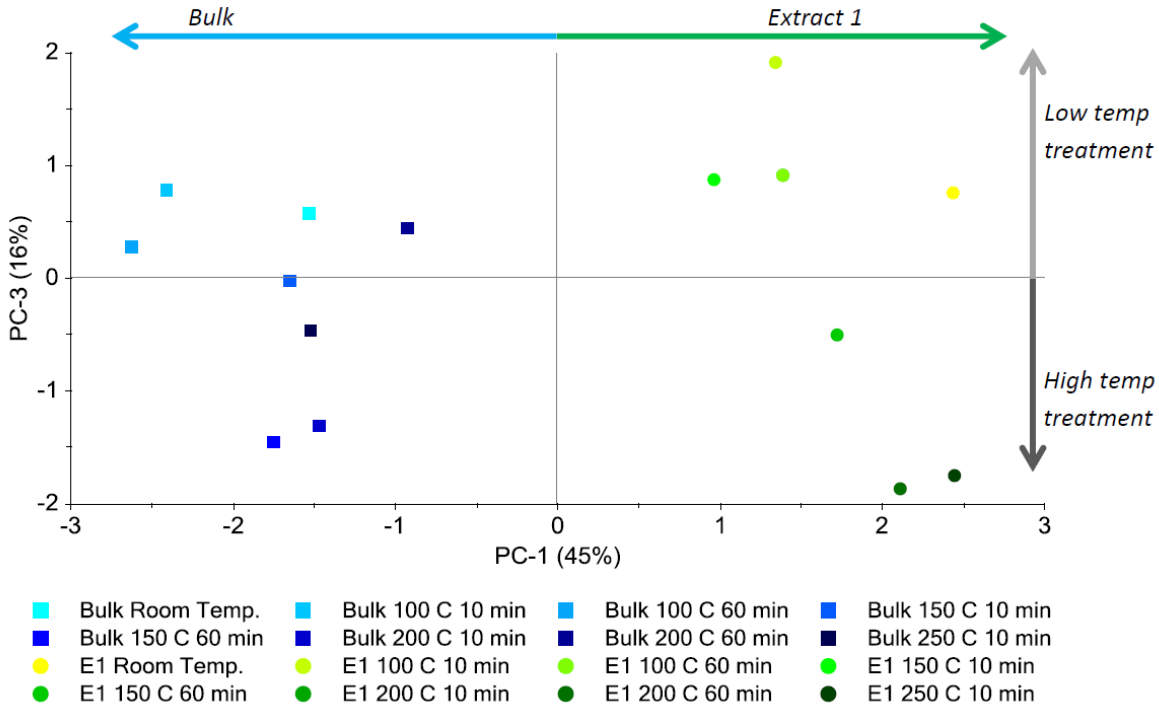

Figure 5.7. Scores plot for principal component 1 (PC-1) and principal component 3 (PC-3) from the principal component analysis of the FT-MIR data.

The associated loadings plot (Figure 5.8) shows which spectral features are significant to the separations. The spectra were analysed in transmission mode, so the negative features are associated with separation into positive PC space, and vice versa.

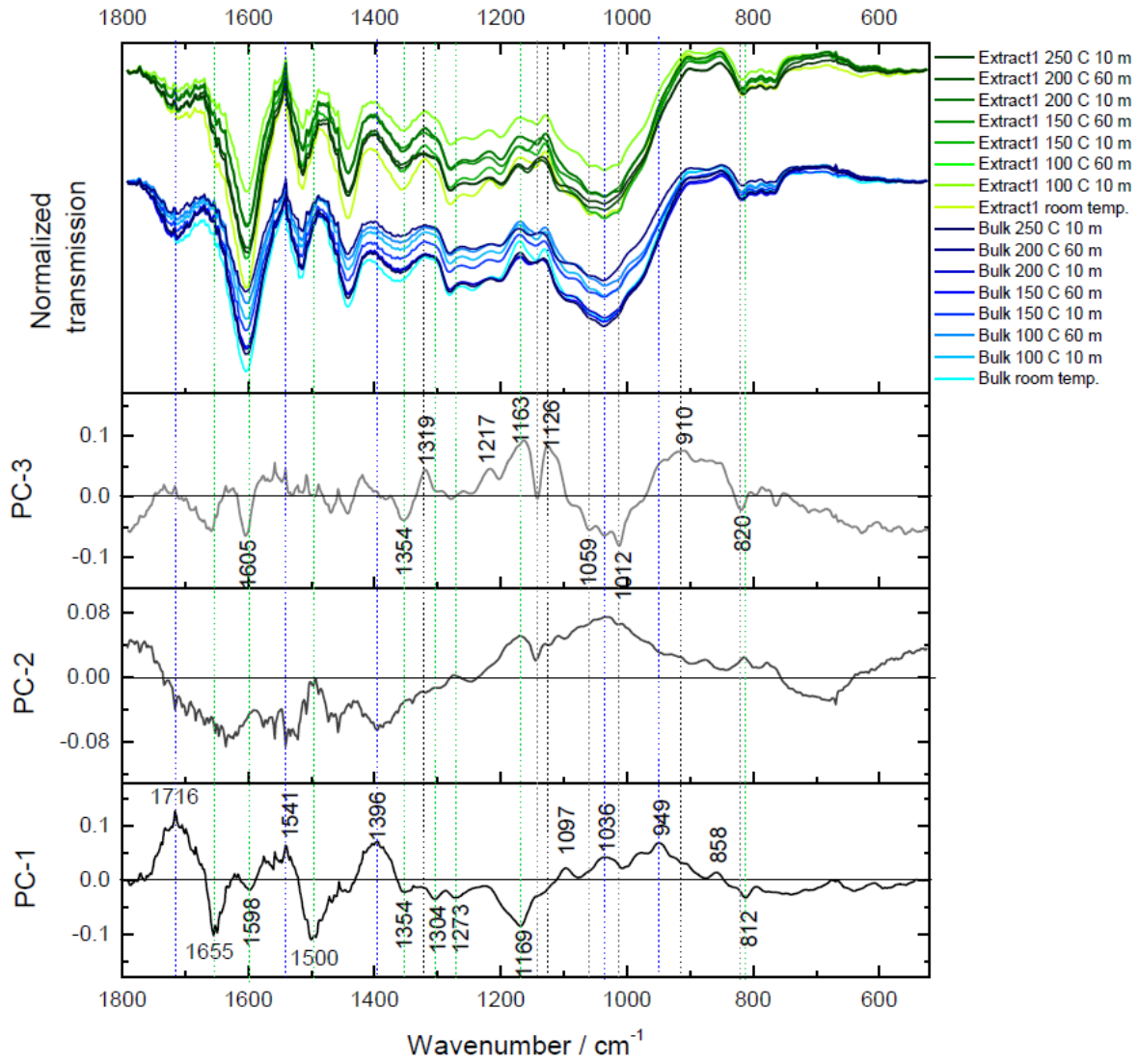

Figure 5.8. Loadings plot and spectra from the principal component analysis conducted on the FT-MIR spectra for small-scale GME ("Extract 1") and the bulk extract, subject to different temperatures for either 10 or 60 minutes ("m").

For PC1, the key bands associated with the small-scale extract are observed at 1655, 1598, 1500, 1354, 1304, 1273, 1169, and 812 cm^{-1} (Figure 5.8 and Table 5.2). The key bands associated with bulk GME are observed at 1716, 1396, 1097, 1036, 949, and 858 cm^{-1} (Figure 5.8 and Table 5.3).

Table 5.2. Key bands associated with the small-scale GME from PC1. (sh) denotes a shoulder feature.

Peak (cm^{-1})	Attribution[21]
1655 (sh)	Ring vibrations
1598	Stretching C=C aromatic compounds
1500 (sh)	Stretching OH, stretching C-O-C, bending OH
1354	C-O and CC stretching of flavonoids
1304	Stretching CC and bending CH
1273	Antisymmetric C-O-C deformation
1169	Stretching CC and bending CH
812	Ring breathing

Table 5.3. Key bands associated with the bulk GME from PC1.

Peak (cm^{-1})	Attribution[21]
1716	Stretching C=O groups
1396	C-OH deformation of phenols
1097	Aromatic CH in-plane bending
1036	Stretching C-O
949	CH_2OH deformation/bending
858	Aromatic CH out-of-plane bending

These differences between the two GMEs are based on the extraction size (bulk and small-scale). While the purification process was the same for both extract sizes, the extraction process did have slightly different features. The bulk GME was processed using large-scale industrial food processing equipment, while the small-scale extract was performed using benchtop equipment. In addition, the bulk GME was extracted for 48 hours, while the small-scale GME was extracted for 24 hours. These factors had an impact on the phenolic composition of the resulting GMEs.

In Figure 5.8, the peaks at 1655, 1500, and 1169 cm^{-1} are the strongest spectral features corresponding to the small-scale GME. These are associated with ring vibrations, stretching OH, stretching C-O-C, bending OH, and stretching CC and bending CH (Table 5.3).

The third PC has key bands associated with higher heat treatment at 1319, 1217, 1163, 1126, and 910 cm^{-1} (Figure 5.8 and Table 5.4). The key bands associated with lower heat treatment are 1605, 1354, 1059, 1012, and 820 cm^{-1} (Figure 5.8 and Table 5.5).

Table 5.4. Peaks associated with higher heat treatment for PC3.

Peaks (cm^{-1})	Attribution[21]
1319	C-O stretching
1217	Stretching vibration C-O bonds
1163	C-O-C asymmetric stretching vibrations
1126	C-C stretching
910	Aromatic out-of-plane bending

Table 5.5. Peaks associated with lower heat treatment for PC3.

Peaks (cm^{-1})	Attribution[21]
1605	C=C stretching aromatic ring
1354	Combination C-O stretching, C-OH bending vibrations
1059	OH bending vibration
1012	C-O stretching for OH on pyran ring of epicatechin
820	CH deformation aromatic ring/Deformation aromatic hydroxyl group

5.3.2 Structural analysis by Raman spectroscopy

The Raman spectra for all samples with no processing are shown in Figure 5.9. The GME powder samples were coloured and so tended to heat up and give off black body radiation. A consistent oscillation or sine wave was also observed as an artefact. The spectra therefore required pre-processing before further analysis.

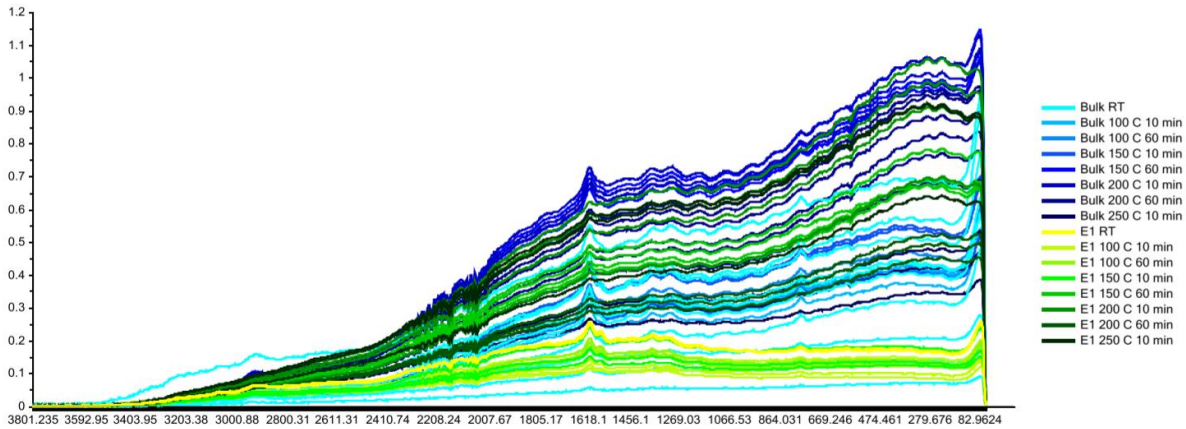

Figure 5.9. Raw FT-Raman spectra for grape marc extracts exposed to various treatments.

First, a linear baseline correction was performed for regions of interest (680 – 935 cm^{-1}, 1230 – 1730 cm^{-1}, and 2825 – 3115 cm^{-1}), followed by a standard normal variate (SNV) transformation to give Figure 5.10, Figure 5.11, Figure 5.12 and Figure 5.13. Principal component analysis (PCA) was then undertaken using leave-one-out full cross-validation.

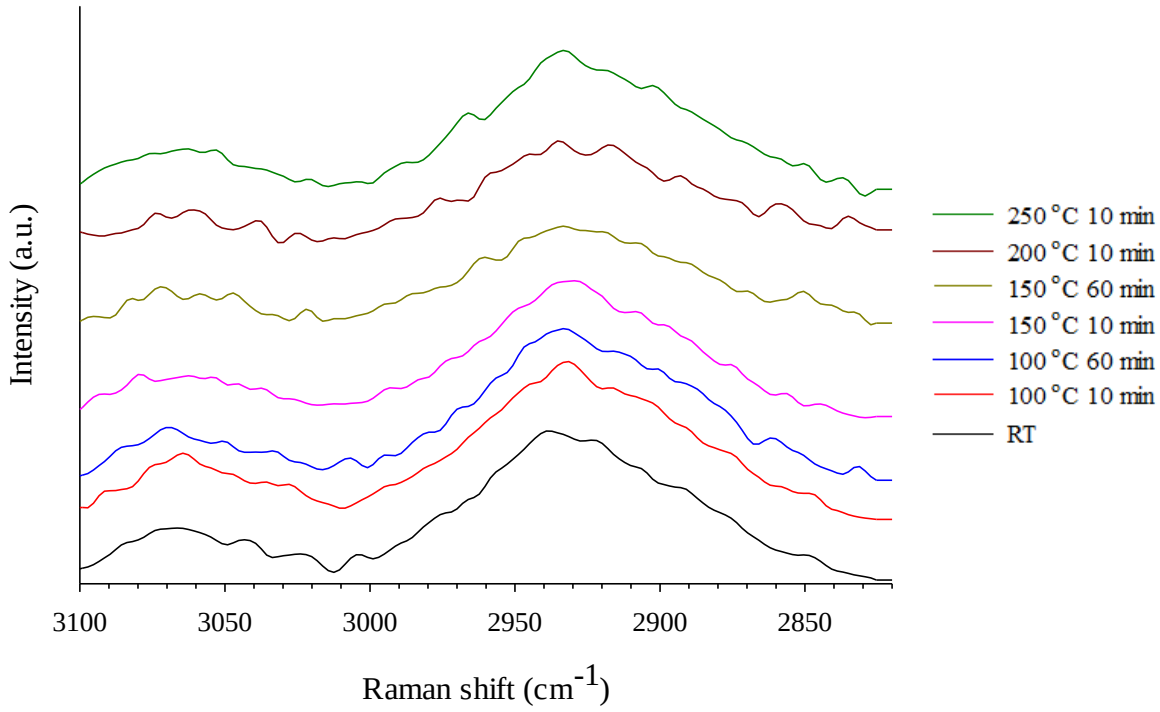

Figure 5.10. FT-Raman spectra over the region 3100 to 2800 cm^{-1} of bulk GME powder exposed to various treatments after linear baseline correction and SNV pre-processing.

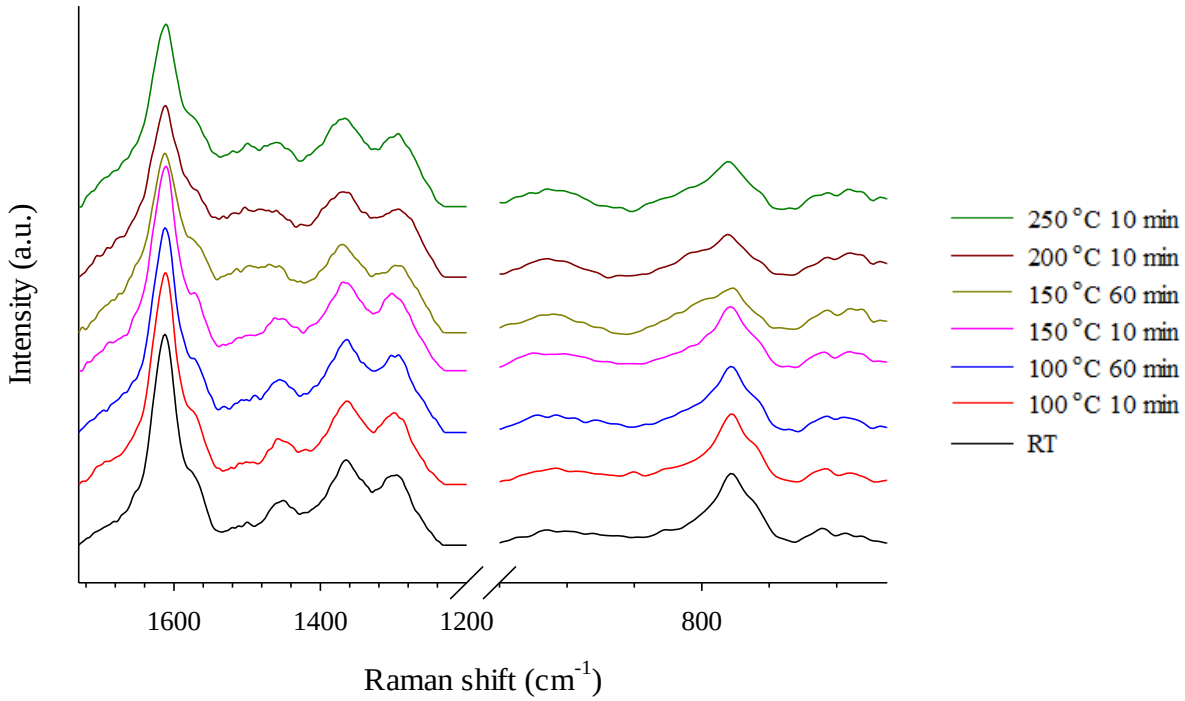

Figure 5.11. FT-Raman spectra over the region 1800 to 620 cm^{-1} of bulk GME powder exposed to various treatments after linear baseline correction and SNV pre-processing.

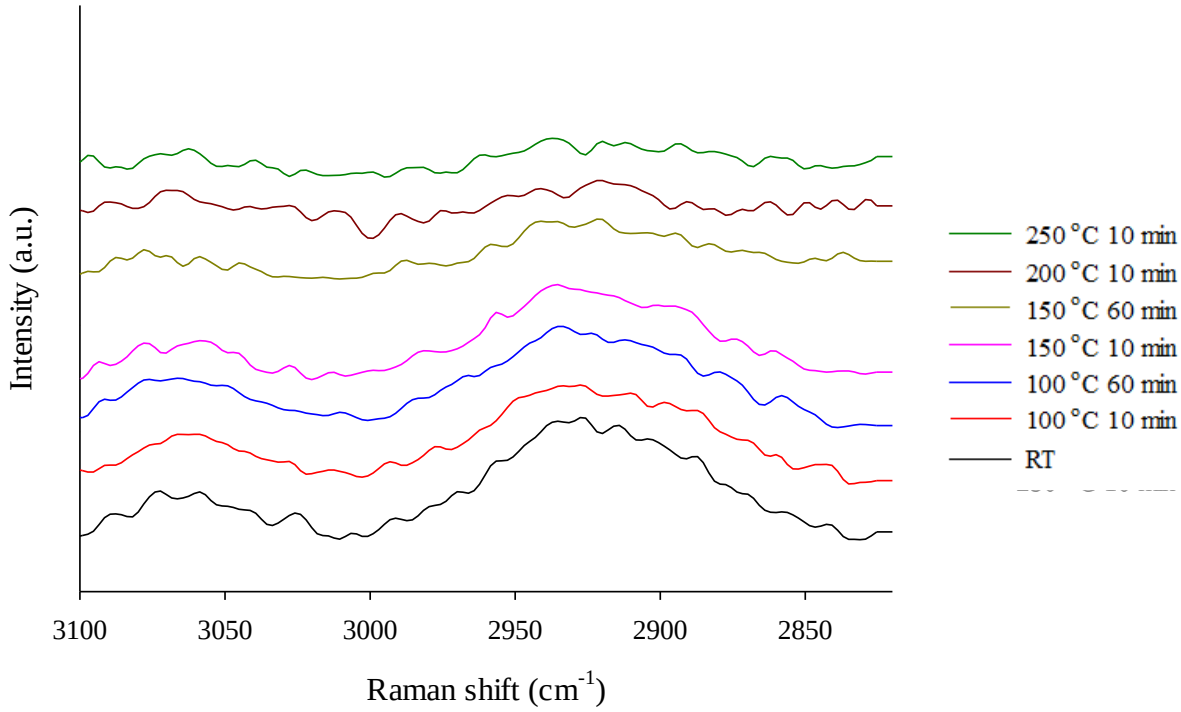

Figure 5.12. FT-Raman spectra over the region 3100 to 2800 cm^{-1} of small-scale GME powder exposed to various treatments after linear baseline correction and SNV pre-processing.

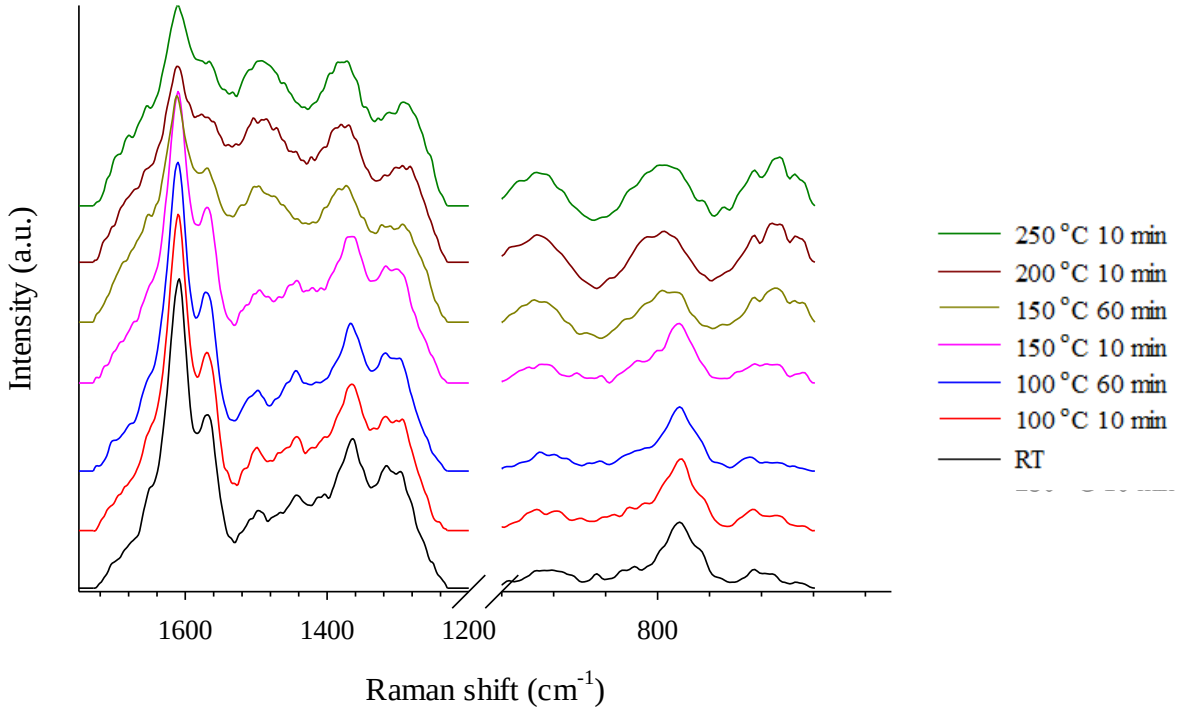

Figure 5.13. FT-Raman spectra over the region 1800 to 620 cm^{-1} of small-scale GME powder exposed to various treatments after linear baseline correction and SNV pre-processing.

The scores plot in Figure 5.14 shows each sample spectrum as a single point in the principal component (PC) space. The first PC, PC-1, separates samples based on treatment temperature and time and accounts for 87% of the overall spectral variance. Samples that were treated with lower temperatures tend to plot in positive PC-1 space, and higher temperature treated samples in negative PC-1 space.

The separation of lower temperature treatment samples is associated with the relative intensities of the bands observed at 783, 1304, 1363, 1572, 1612, 2928, and 3062 cm^{-1} (Figure 5.15). These bands are typical of flavonoids. The band at 783 cm^{-1} is probably related to CH out-of-plane deformation of aromatic rings.[233] The band at 1363 cm^{-1} is associated with a combination band between ring deformation and CC stretching of aromatic rings, while the band at 1304 cm^{-1} is the related vibration.[234] The peak at 1612 cm^{-1} is related to C-CH quadrant stretching modes, which indicates phenolic compounds.[235,236] The bands at 2928 and 3062 cm^{-1} can be attributed to CH stretching of aromatic rings.

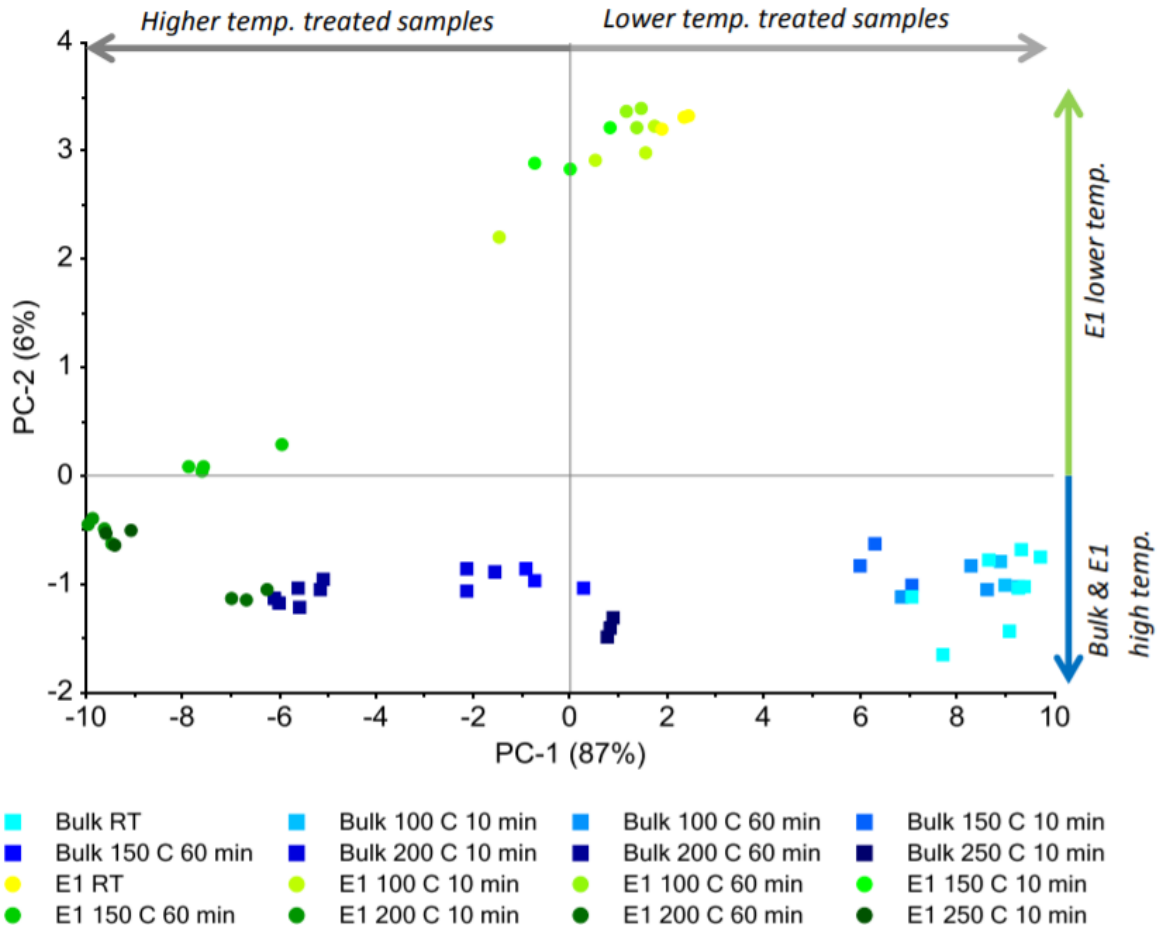

Figure 5.14. Scores plot for the first two principal components from the principal component analysis for the smaller regions. This accounts for 90% of the overall spectral variance observed in the sample set.

The second PC accounts for 6% of the overall spectral variance and separates lower temperature treated small-scale samples from the bulk and higher temperature treated small-scale samples. The associated loadings plot (Figure 5.15) gives the spectral features associated with the separation. The strongest positive features are 1321, 1364, 1421, 1441, 1566, and 1607 cm^{-1}, while the strongest negative features are 1269, 1611, and 2930 cm^{-1}. These are small changes in the spectra and not associated with definite peaks.

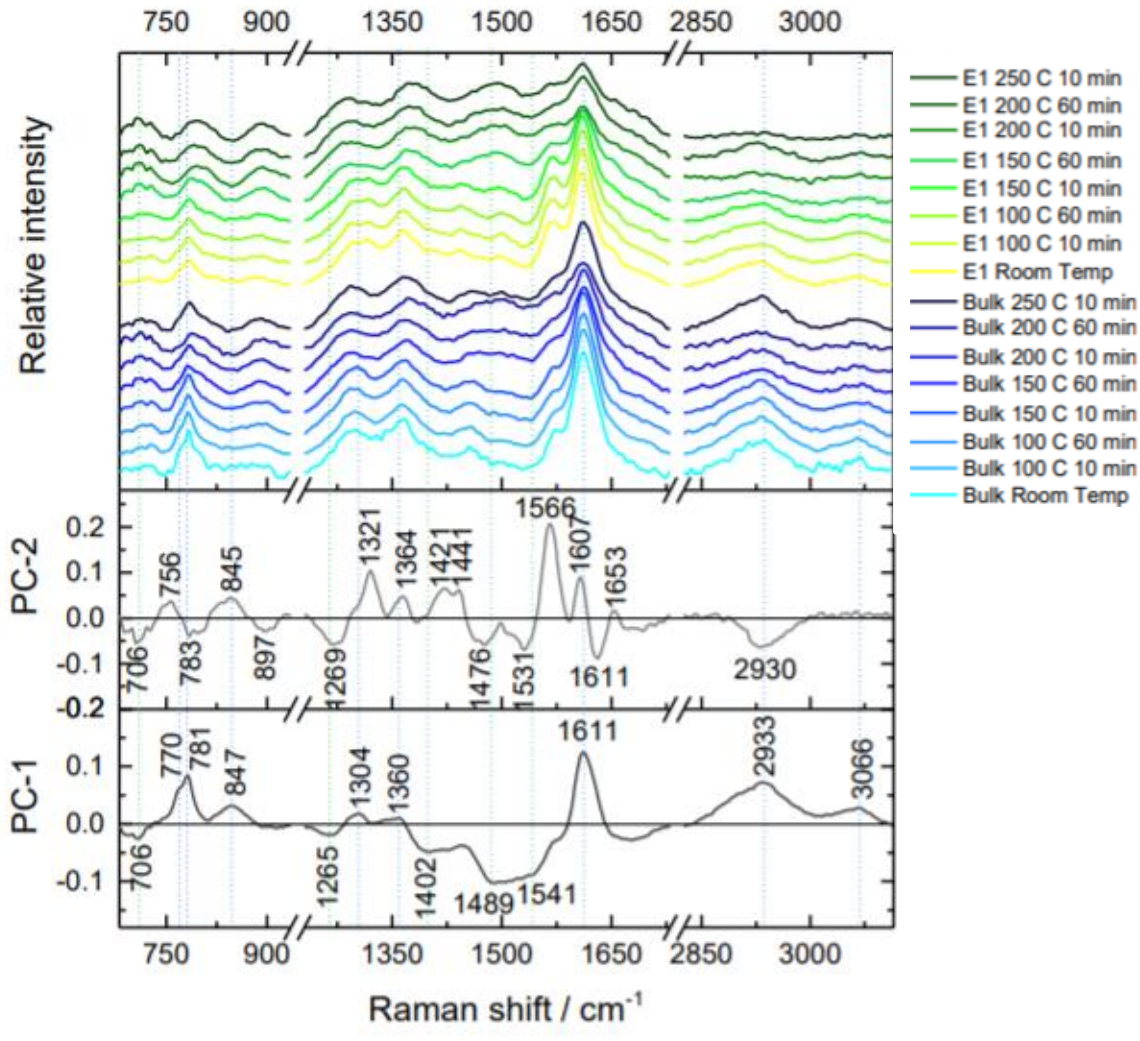

Figure 5.15. Loadings for principal component 1 (PC-1) and principal component 2 (PC-2) from the principal component analysis for smaller regions.

PCA was then carried out on the larger spectral region from 300 to 1800 cm^{-1} after linear baseline correction and SNV. The scores plot for the first two PCs from this larger spectral region analysis is shown in Figure 5.18.

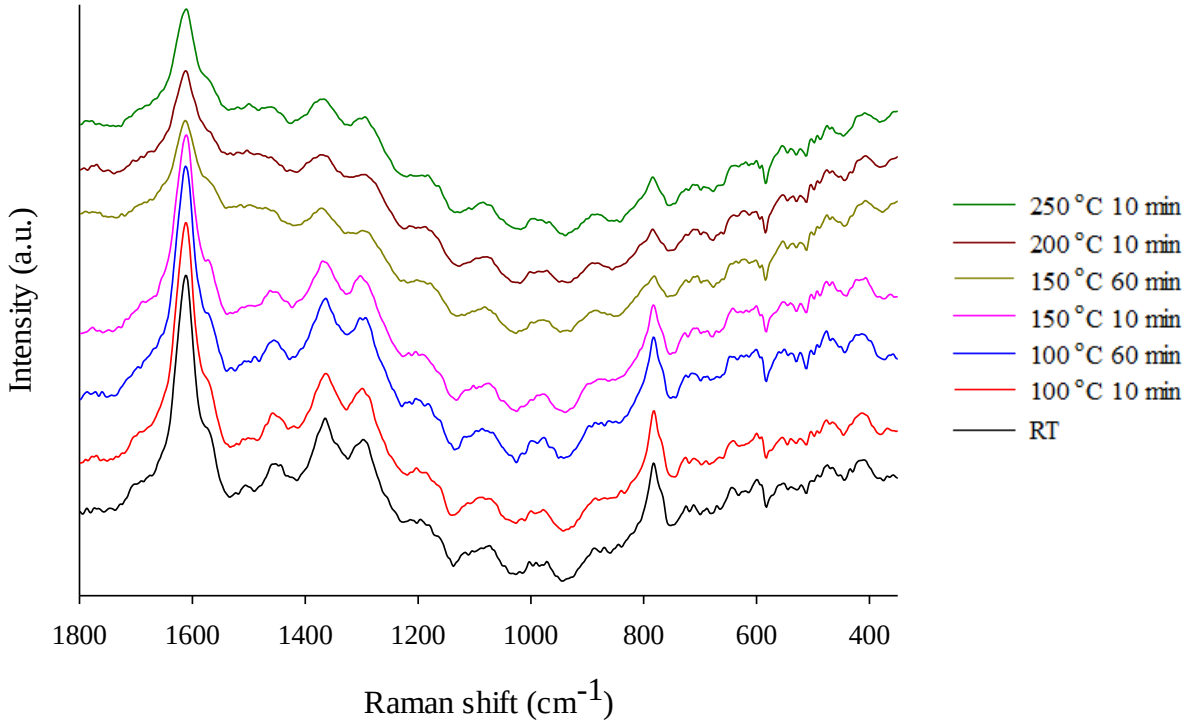

Figure 5.16. FT-Raman spectra of bulk GME exposed to various temperatures after pre-processing from 300 to 1800 cm^{-1}.

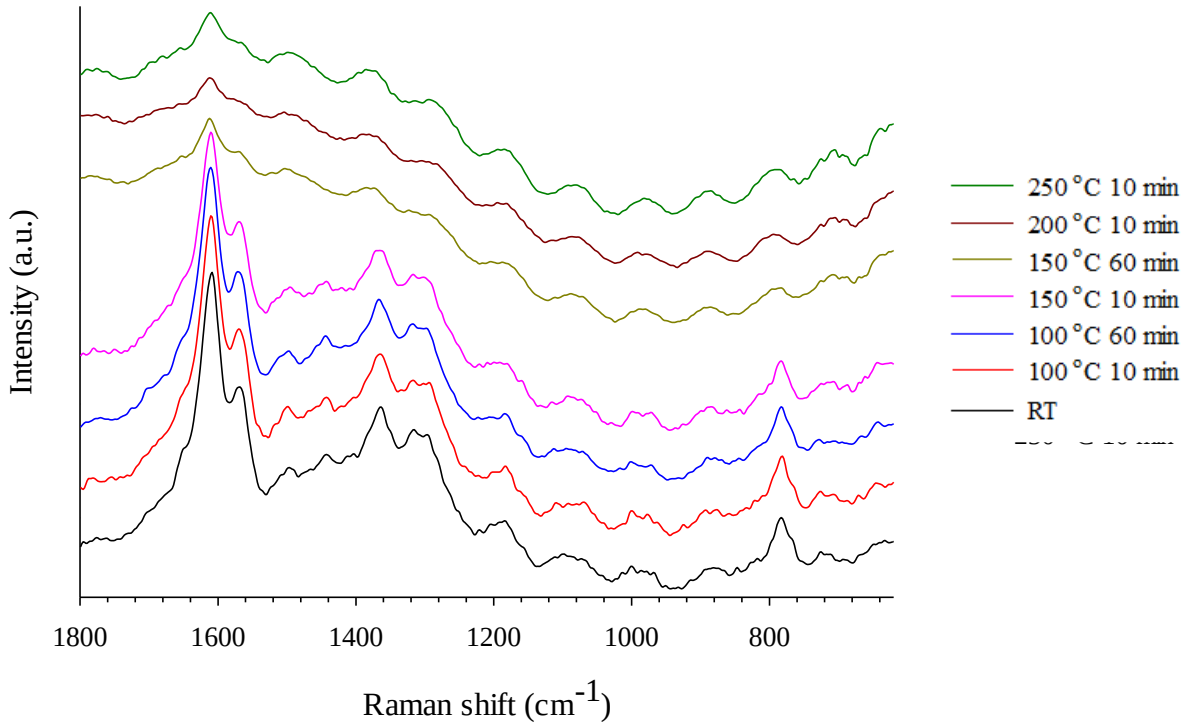

Figure 5.17. FT-Raman spectra of small-scale GME exposed to various temperatures after pre-processing from 300 to 1800 cm^{-1}.

The first PC shown in Figure 5.18 separates samples based on temperature. It accounts for 78% of the spectral variance. Samples kept at room temperature and exposed to temperatures of 100 ˚C (for 10 min and 1 hour) and 150 ˚C (for 10 min) are in positive PC1 space and all other samples are in negative PC1 space. The spectral features associated with this separation are presented in Figure 5.19. The strongest positive features are 595, 781, 1302, 1362, 1443, 1570, and 1610 cm^{-1}. The strongest negative features are 1485 and 1553 cm^{-1}. These bands have some overlap with the spectral features seen in the first PCA: 781, 1302, 1362, and 1610 cm^{-1} are all positive features for both PCs. The band at 1443 cm^{-1} is associated with bending of CH_2 groups.[237]

The second PC accounts for 20% spectral variance. It separates low temperature small-scale extract samples from the low temperature bulk samples, with all heat-treated samples in approximately neutral PC2 space. These features are small changes in the spectra and not definite peaks.

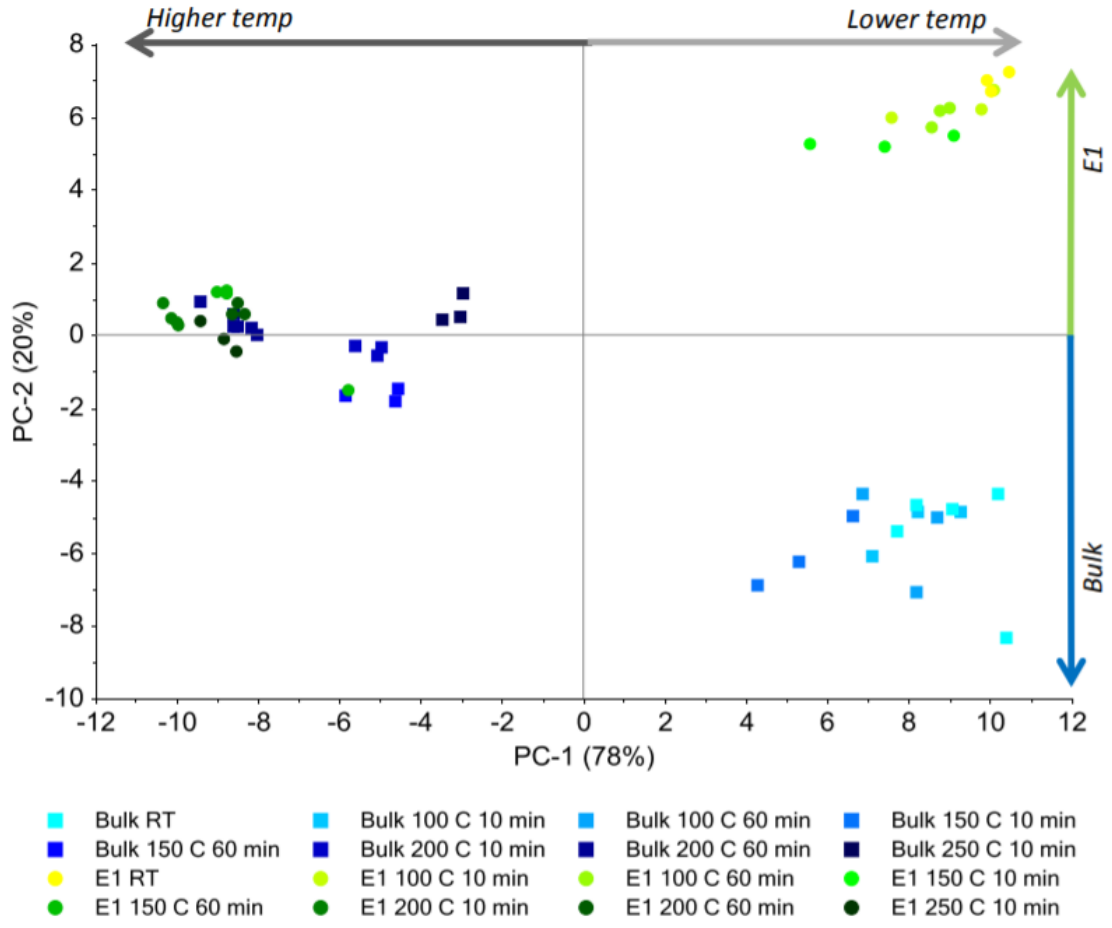

Figure 5.18. Scores plot for the first two principal components (PC-1 and PC-2) from the principal component analysis of the larger spectral region.

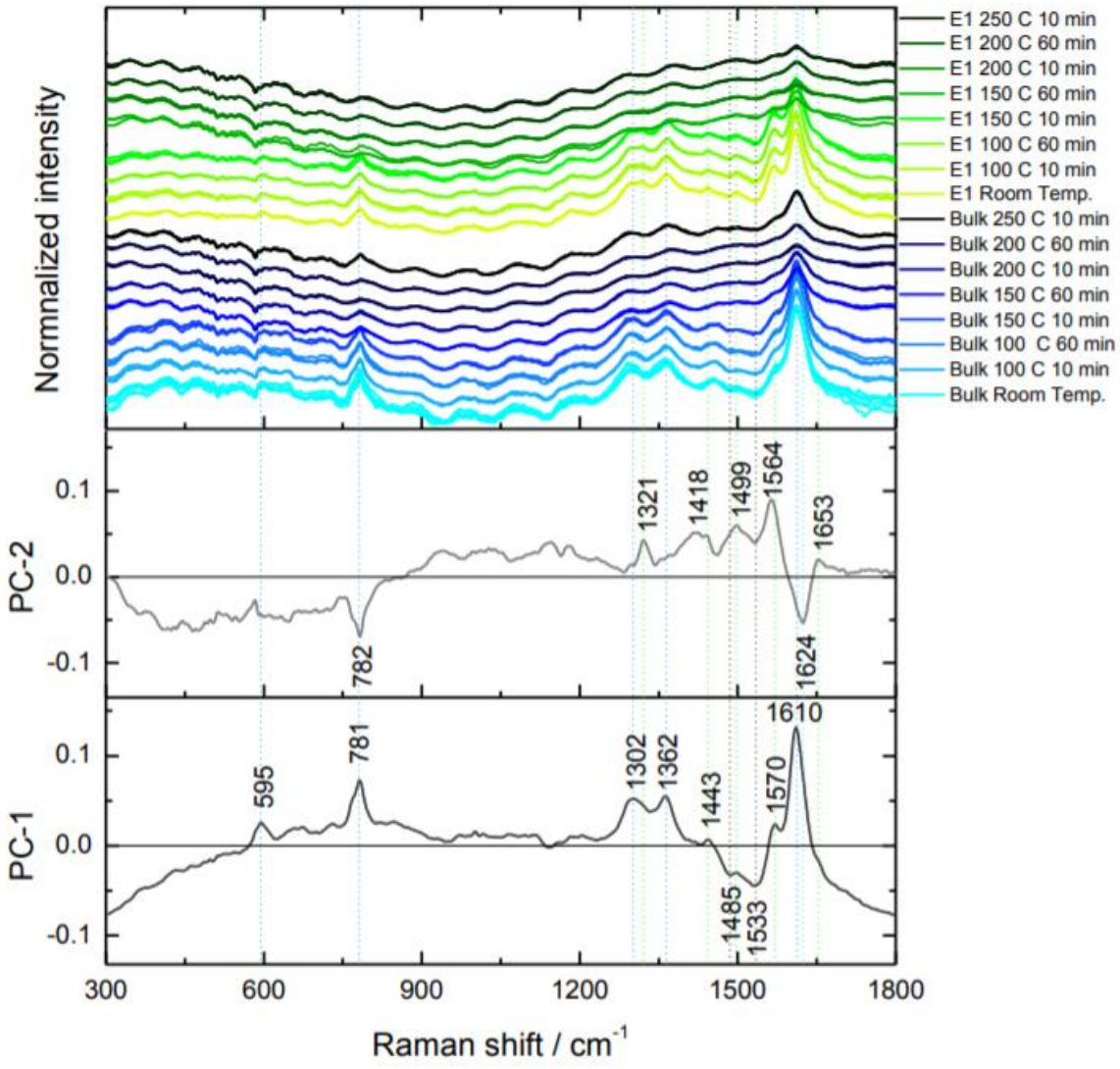

Figure 5.19. Loadings for principal component 1 (PC-1) and principal component 2 (PC-2) for the principal component analysis of the larger spectral region.

The FT-Raman spectra and PCA show some separation with heat exposure. The bands associated with the separation are mainly attributed to deformation and stretching of the aromatic ring. This may indicate that the aromatic ring has been affected by changes in sample treatment. This agrees with results from the FT-MIR analysis, which also shows some separation with heat exposure (Figure 5.4). In the case of FT-MIR, the higher temperature treated GME samples were more strongly associated with C-C and C-O-C stretching, and aromatic out-of-plane bending (Figure 5.8 and Table 5.4). FT-MIR and FT-Raman spectroscopy have different selection rules. For Raman, there must be a change in the polarizability of the molecule, while for IR, there must be a change in dipole moment.

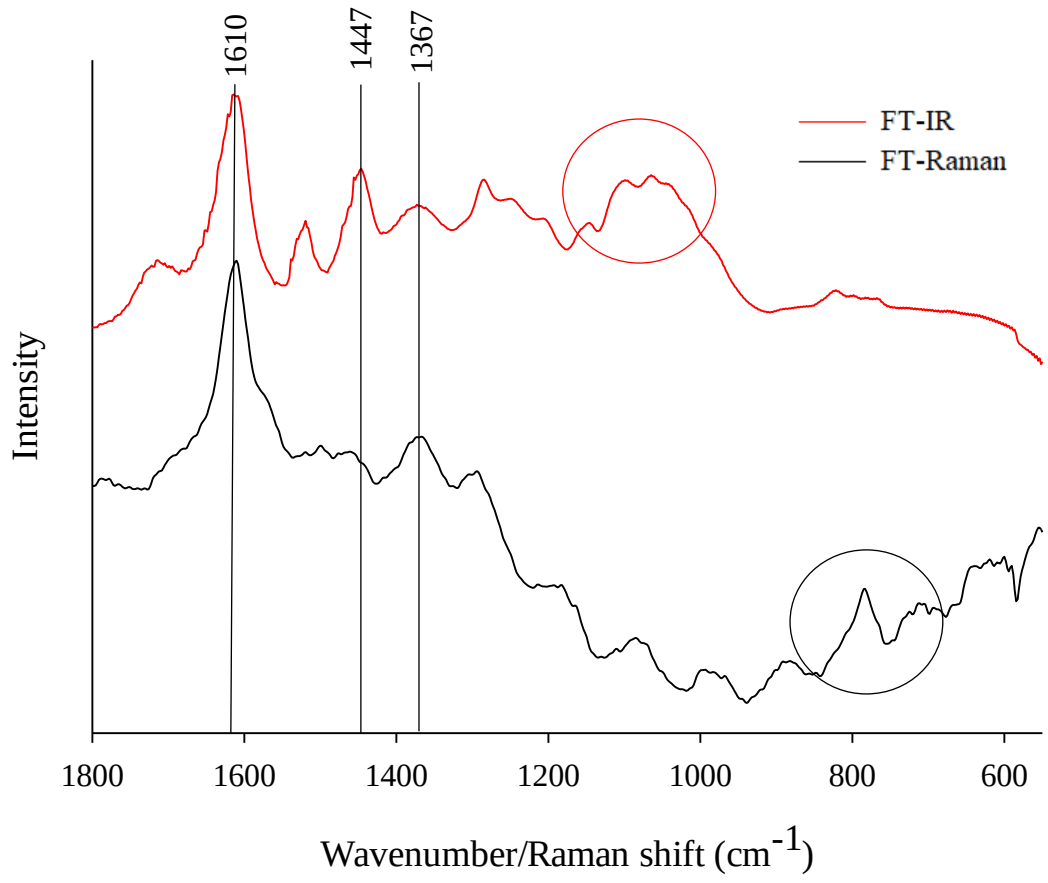

Figure 5.20. Comparison of FT-Raman and FT-MIR spectra for bulk grape marc extract kept at room temperature.

The FT-Raman and FT-MIR spectra do show some bands in common (Figure 5.20). In FT-Raman, the band at 1610 cm^{-1} is attributed to CC quadrant stretching modes, which indicates the presence of phenolic compounds. In FT-MIR this peak is associated with stretching vibrations of CC bonds and in-plane vibrations of the benzene ring for the catechol moiety.[21] The band at 1447 cm^{-1} in FT-Raman is associated with bending of CH_2 groups, while in FT-MIR it is associated with stretching OH, stretching C-O-C, bending OH and aromatic C=C bonds. Finally, the band at 1367 cm^{-1} is associated with a combination band between ring deformation and CC stretching of aromatic rings in FT-Raman. Whereas in FT-MIR, it is associated with CC, CH, C-O, and ring vibrations.[21] These peaks were not associated with separation of GME by heat with FT-MIR. However, the bands at 1362 and 1610 cm^{-1} were associated with this separation with FT-Raman.

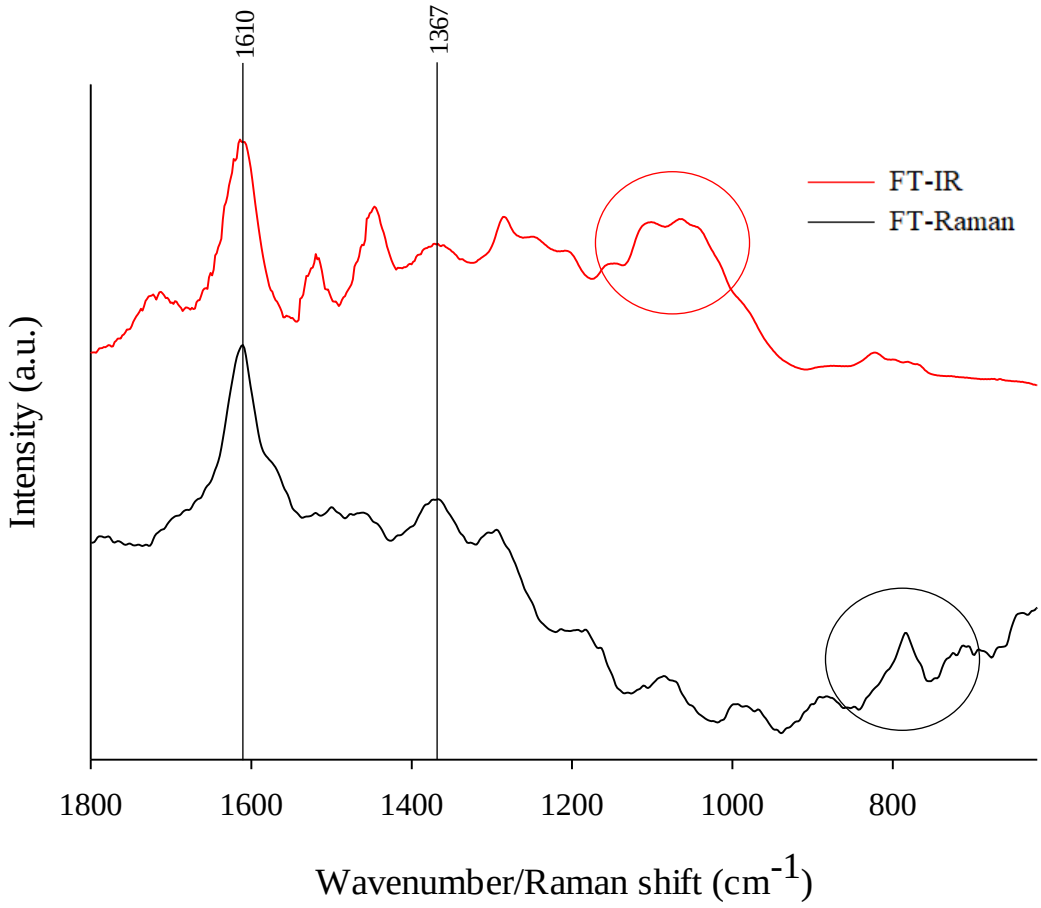

Figure 5.21. Comparison of FT-Raman and FT-MIR spectra for bulk grape marc extract kept at 250 °C for 10 minutes.

The comparison between the FT-Raman and FT-MIR spectra for bulk GME kept at 250 °C for 10 minutes (Figure 5.21) showed two similar bands in common with those seen in Figure 5.20 at 1610 and 1367 cm^{-1}. These peaks are associated with separation by heat with FT-Raman. As with Figure 5.20, the region centred around 1050 cm^{-1} is more "IR active", while the region centred around 800 cm^{-1} is more "Raman active".

5.3.3 Antioxidant activity and total phenolic content

The DPPH radical scavenging of the heated GME powders were compared with the total phenolic content, established through the Folin-Ciocalteu assay (Table 5.6 and Table 5.7). There was no significant loss in free radical scavenging as the extract was heated for a shorter period of time. The % DPPH free radicals scavenged by the extract was 78.3 ± 0.6% at room temperature, dropping to 67.6 ± 0.5% for the extract that had been heated to 150 ˚C. There was a slight increase again to 80.1 ± 1.4% for the extract that was heated to 200 ˚C. When the extracts were heated to the 100 ˚C and 150 ˚C for one hour, there was no significant loss in antioxidant activity. The % DPPH free radicals scavenged were 68.8 ± 0.3% and 75.5 ± 0.9%, respectively. In the same way, there was no significant loss in total phenolic content (TPC).

The total phenolic content is similar to that found by other researchers for such extracts. It has been reported that the TPC of grape marc and grape seed extracts ranged from 144 mg GAE g^{-1} to 444 mg GAE g^{-1}. Grape marc extract had a much lower TPC than grape seed and grape stem extracts.[238]

The small-scale extract shows an increase in TPC with rising temperature for both long and short heating times (Table 5.6 and Table 5.7). This was not seen in the bulk samples. This may be due to differences in phenolic profile, as FT-MIR and FT-Raman showed differences between the bulk and small-scale GMEs. A proportion of the TPC values for both GMEs may be derived from the reaction of other compounds with the Folin-Ciocalteu reagent. These additional compounds include sugars and tartrates.[239]

The bulk samples saw a decrease in TPC from room temperature to 150 ˚C treated samples. The TPC then increased again for the 250 ˚C treated sample. For the small-scale extract, the TPC rose from room temperature to the 200 ˚C and 250 ˚C treated samples with no significant decrease in TPC. A previous study has shown a similar effect up to 150 ˚C, though degradation was observed at 200 ˚C.[220] The reason for this discrepancy is not clear and further research may be warranted.

Table 5.6. Antioxidant activity (± SD) and total phenolic content (± SD) of bulk and small scale extracts heated to the described temperatures for 10 minutes. Values with different letters are statistically significantly different ($p < 0.05$).

	% DPPH scavenged		Total phenolic content (TPC) (mg GAE g^{-1})	
	Bulk	Small	Bulk	Small
Room temperature	65.3 ± 0.6^{a}	78.3 ± 0.6^{a}	$493 \pm 15^{a,b}$	497 ± 10^{a}
100 ˚C	70.9 ± 3.0^{b}	73.2 ± 0.3^{b}	549 ± 25^{a}	479 ± 8^{a}
150 ˚C	70.8 ± 1.3^{b}	67.6 ± 0.5^{c}	378 ± 4^{c}	$541 \pm 46^{a,b}$
200 ˚C	80.0 ± 1.8^{c}	80.1 ± 1.4^{a}	$415 \pm 25^{c,d}$	699 ± 7^{c}
250 ˚C	74.1 ± 2.6^{b}	79.4 ± 0.9^{a}	$444 \pm 26^{b,d}$	605 ± 28^{b}

Table 5.7. Antioxidant activity and total phenolic content of bulk and small scale extracts heated to the described temperatures for 60 minutes. Values with different letters are statistically significantly different ($p < 0.05$).

	% DPPH scavenged		Total phenolic content (mg GAE g^{-1})	
	Bulk	Small	Bulk	Small
100 ˚C	68.7 ± 0.2^{a}	76.8 ± 0.4^{a}	468 ± 8^{a}	477 ± 28^{a}
150 ˚C	75.5 ± 0.9^{b}	76.6 ± 0.1^{a}	435 ± 1^{b}	619 ± 22^{b}

5.3.4 Influence of heat on phenolic composition

High performance liquid chromatography was performed on the heated GME powder to determine the phenolic composition (Table 5.8 to Table 5.10). High levels of the flavan-3-ols epicatechin and catechin were observed at 79.1 ± 4.0 mg g^{-1} for the bulk GME powder at room temperature (approx. 8% by weight). The small-scale GME powder at room temperature has a similar level of catechin and epicatechin at 87.2 ± 3.3 mg g^{-1}.

Table 5.8. Mean concentrations ± SD of phenolic compounds in bulk scale extracts exposed to temperatures for 10 minutes. Units are mg g^{-1} GME powder. Concentrations in the same row with different letters are statistically significantly different ($p < 0.05$).

	RT	100 ˚C	150 ˚C	200 ˚C	250 ˚C
Gallic acid	11.7 ± 0.7[a]	10.2 ± 0.7[a]	10.4 ± 0.1[a]	9.3 ± 0.4[a]	9.7 ± 1.5[a]
(+)-catechin	21.6 ± 1.0[a]	17.8 ± 0.6[b]	16.8 ± 0.7[b,d]	12.0 ± 0.3[c]	14.7 ± 0.5[d]
Caffeic acid	7.9 ± 2.1[a]	4.1 ± 0.5[a]	2.8 ± 0.2[a]	-	-
(-)-epicatechin	57.5 ± 3.0[a]	45.2 ± 1.7[b]	40.6 ± 1.9[b]	19.4 ± 0.3[c]	29.0 ± 1.0[d]
p-coumaric acid	4.0 ± 0.2[a]	3.8 ± 0[a]	3.8 ± 0.1[a]	3.8 ± 0.3[a]	-
Quercetin glucoside[a]	12.4 ± 0.4[a]	11.4 ± 0.4[b]	10.9 ± 0.1[b]	9.09 ± 0.1[c]	9.7 ± 0.1[c]
Sum of known monomers	115 ± 7	92.4 ± 3.9	85.3 ± 3.1	53.5 ± 1.8	66.6 ± 3.8

[a] expressed in rutin equivalents

Table 5.9. Mean concentrations ± SD of phenolic compounds in small scale extracts exposed to temperatures for 10 minutes. Units are mg g^{-1} GME powder. Concentrations in the same row with different letters are statistically significantly different ($p < 0.05$).

	RT	100 ˚C	150 ˚C	200 ˚C	250 ˚C
Gallic acid	-	6.9 ± 0.4[a]	6.5 ± 0.1[a]	7.4 ± 1.1[a]	6.4 ± 0.2[a]
(+)-catechin	22.3 ± 0.8[a]	20.7 ± 1.0[a]	21.0 ± 0.7[a]	9.23 ± 0.3[b]	10.0 ± 0.3[b]
Caffeic acid	8.4 ± 0.6[a]	8.4 ± 0.6[a]	8.9 ± 1.2[a]	-	-
(-)-epicatechin	64.9 ± 2.5[a]	59.3 ± 2.1[a,b]	55.8 ± 1.9[b]	14.2 ± 0.6[c]	15.8 ± 0.3[c]
p-coumaric acid	3.8 ± 0.5[a]	3.7 ± 0.2[a]	4.4 ± 0.4[a]	3.7 ± 0.3[a]	3.6 ± 0.2[a]
Quercetin glucoside[a]	13.1 ± 2.8[a]	14.3 ± 0.6[a]	14.7 ± 0.8[a]	10.2 ± 0.2[a]	10.5 ± 0.2[a]
Sum of known monomers	113 ± 7	109 ± 8	111 ± 5	44.8 ± 2.5	44.6 ± 4

[a] expressed in rutin equivalents

Table 5.10. Mean concentrations ± SD of phenolic compounds in bulk and small-scale extracts exposed to temperatures for 60 minutes. Units are mg g^{-1} GME powder. Concentrations in the same extraction size and row with different letters are statistically significantly different ($p < 0.05$).

	Bulk		Small	
	100 ˚C	150 ˚C	100 ˚C	150 ˚C
Gallic acid	11.1 ± 0.8[a]	9.5 ± 1.7[a]	-	6.5 ± 0.2
(+)-catechin	19.5 ± 0.8[a]	11.7 ± 0.4[b]	18.4 ± 0.6[a]	10.5 ± 0.2[b]
Caffeic acid	4.9 ± 0.5	-	5.7 ± 1.5	-
(-)-epicatechin	51.2 ± 2.5[a]	20.1 ± 0.8[b]	50.8 ± 2.0[a]	20.8 ± 0.7[b]
p-coumaric acid	3.8 ± 0.01[a]	3.4 ± 0[a]	3.6 ± 0.1[a]	3.6 ± 0.1[a]
Quercetin glucoside[a]	11.8 ± 0.5[a]	9.6 ± 0.1[a]	12.1 ± 1.3[a]	12.0 ± 0.1[a]
Sum of known monomers	102 ± 5	54.5 ± 3.7	90.6 ± 5.5	53.7 ± 1.4

[a] expressed in rutin equivalents

The HPLC data (Table 5.8, Table 5.9, and Table 5.10) shows some changes with temperature for the concentrations of the observed phenolic compounds. Epicatechin and catechin significantly decreased in concentration when the samples were heated to higher temperatures. Caffeic acid was detected in the extracts kept at room temperature, 100 ˚C, and 150 ˚C, but was not detected in extracts exposed to 200 ˚C or 250 ˚C. This trend was also seen in the extracts heated for an hour, where caffeic acid was not detected at 150 ˚C (Table 5.10).

The sum of the known monomers for GME kept at room temperature for both extracts is around 100 mg g^{-1}, which comes to about 10% of the powder extracts. Other compounds expected to be found in GME are polymerised compounds such as procyanidin dimers, trimers, oligomers, and polymers, as well as additional compounds including sugars and tartaric acid. In section 0, the total phenolic content of the GME powder at room temperature is around 500 mg g^{-1} for both extraction methods. As the sum of the known monomers is 100 mg g^{-1}, the remaining 80% of the total phenolic content of the powders may be longer tannin molecules. These longer tannins are of more value in active packaging applications, as it is expected that these will be less likely to diffuse out of the packaging. With increasing temperature, the sum of the known monomers decreases significantly. However, the total phenolic content of the small-scale extract shows a significant increase to over 600 mg g^{-1} for the extract heated to 250 ˚C (Table 5.6). This implies that at higher temperature exposure tannins make up a greater proportion of the phenolic content. This change in composition of the extracts may lead to an increase in antioxidant capacity; the same extract heated to 200 ˚C showed a significant increase in % DPPH scavenged (Table 5.6), as longer tannin molecules have a higher antioxidant capacity compared to monomers such as catechin.[240]

5.3.5 Influence of heating on GM molecular weight distribution

Gel permeation chromatography was performed on the GME powders to ascertain the molecular weight distributions and how these change with exposure to increasing temperatures. Figure 5.22 and Figure 5.23 (small-scale extract) and Figure 5.24 and Figure 5.25 (bulk extract) show changes in distribution with both temperature and time.

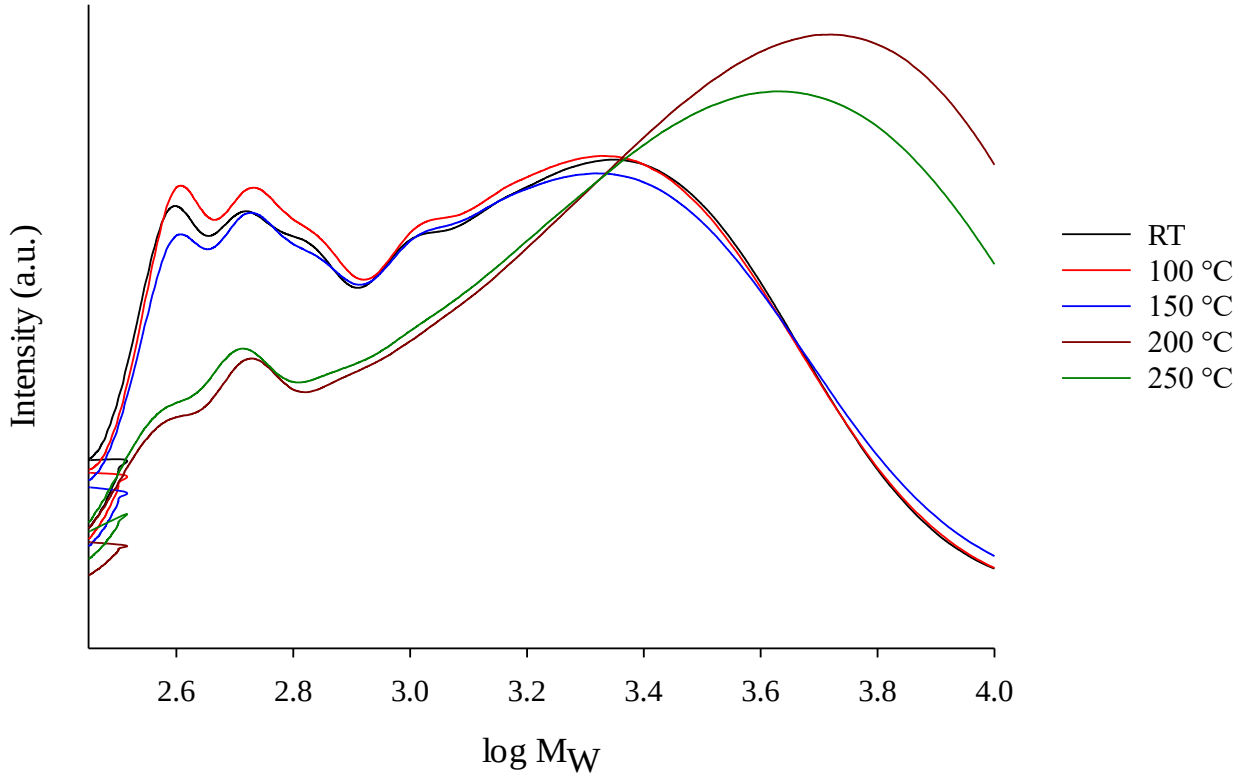

Figure 5.22. GPC results of small-scale extraction group exposed to temperatures for 10 minutes, showing the response relative to the log function of the average molecular weight of the GME powders.

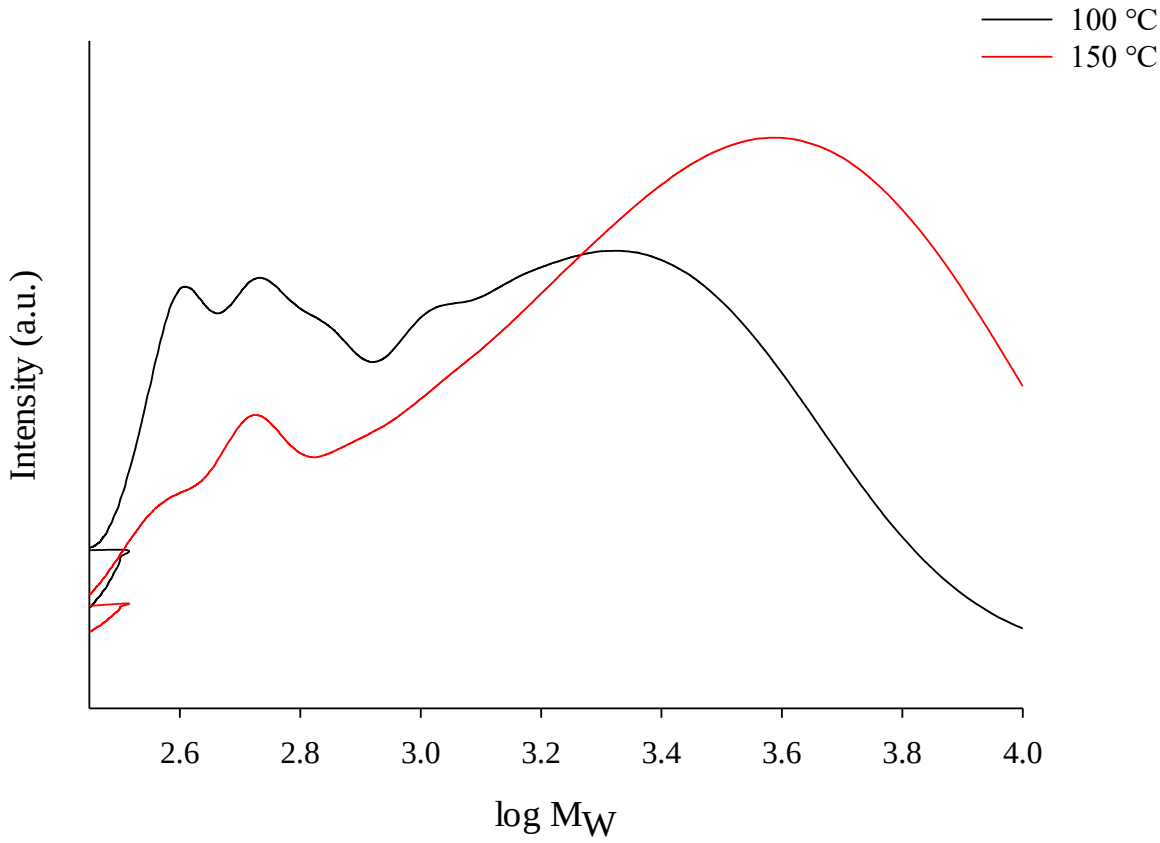

Figure 5.23. GPC results of small-scale extraction group exposed to temperatures for 60 minutes, showing the response relative to the log function of the average molecular weight of the GME powders.

The small-scale extract group showed differences in molecular weight with temperature exposure in Figure 5.22 and Figure 5.23. The GME powders exposed to 150 ˚C for one hour, 200 ˚C and 250 ˚C (both for 10 minutes) all showed a decrease in response at lower molecular weight and a shift to higher molecular weight overall. Lower temperature powders (RT, 100 ˚C for both 10 minutes and one hour, and 150 ˚C for 10 minutes) showed peaks at 2.61 and 2.73, which correspond to 410 g mol^{-1} and 540 g mol^{-1}, respectively. There is a shoulder at 3.06 (1150 g mol^{-1}) and a broad peak at 3.3 (2000 g mol^{-1}). The peak at the lowest molecular weight corresponds to catechin or epicatechin monomers at 290 g mol^{-1}, as this is within the uncertainty from the pullulan saccharide standards used. The peak at 540 g mol^{-1} may be

attributed to trisaccharides containing glucose and/or fructose, which each have a molecular weight of 180 g mol^{-1}. The 1150 g mol^{-1} shoulder can be attributed to a procyanidin tetramer, and the 2000 g mol^{-1} peak to a pentamer.

In the remaining extracts, the response for the peaks at lower molecular weight has decreased and for higher molecular weight has increased. In each case, there was a peak at 2.60 and 2.72 (400 and 520 g mol^{-1}) and a broad peak at higher molecular weight. Relative to the first group of GME powders, this broad peak has shifted to higher molecular weights. The extract exposed to 150 ˚C for one hour has shifted the least, followed by that exposed to 250 ˚C for 5 minutes, 200 ˚C for 10 minutes and finally 200 ˚C for 15 minutes. This implies that at higher temperatures, both the temperature and the time of exposure are factors that influence the resulting molecular weight of the powders. The increased molecular weight with higher treatment temperature may be due to agglomeration or crosslinking of smaller compounds together. This is further supported by the decreased concentration of the monomeric compounds of catechin and epicatechin with increased temperature treatment while the total phenolic content remained the same or increased (Table 5.6, Table 5.8, and Table 5.9).

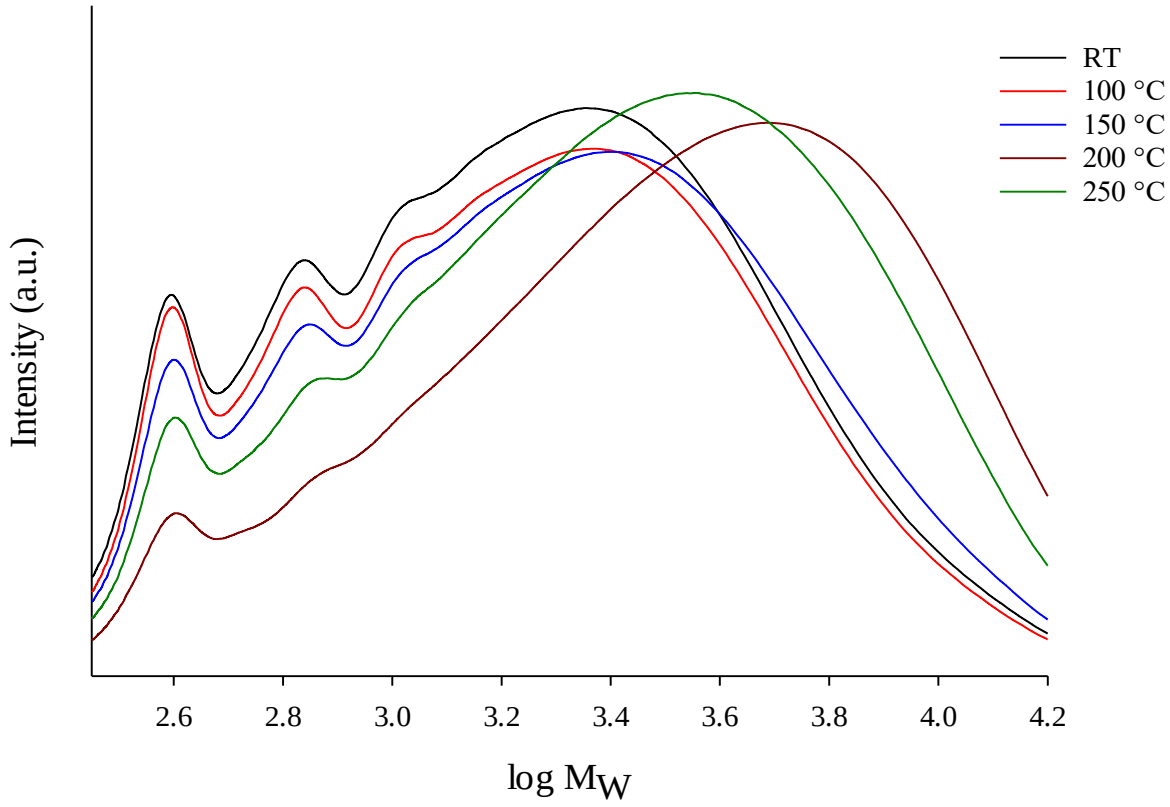

Figure 5.24. GPC results of bulk extraction group exposed to temperatures for 10 minutes, showing the response relative to the log function of the average molecular weight of the GME powders.

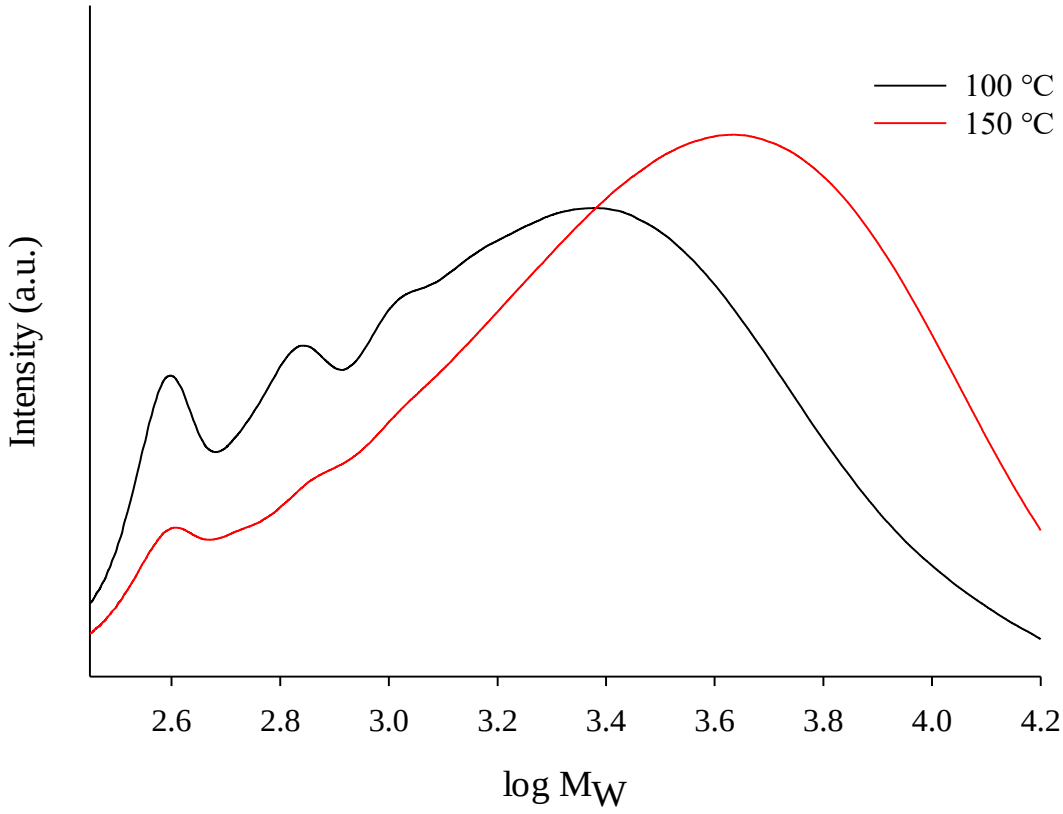

Figure 5.25. GPC results of bulk extraction group exposed to temperatures for 60 minutes, showing the response relative to the log function of the average molecular weight of the extracts.

The bulk extraction showed similar results to the small-scale extraction. There was a peak at 2.60 (400 g mol^{-1}), 2.85 (710 g mol^{-1}), 3.06 (1150 g mol^{-1}), and a broad peak at 3.35 (2240 g mol^{-1}) for the lower temperature GME powders (Figure 5.24 and Figure 5.25). This broad peak shifted to higher molecular weight for the extracts subjected to 150 ˚C for one hour (Figure 5.25), and 200 ˚C and 250 ˚C for 10 minutes (Figure 5.24). The broad peak shifted the least for the 250 ˚C for 5 minutes, then for 150 ˚C for one hour, 200 ˚C for 10 minutes, and 200 ˚C for 15 minutes. This again follows the trend seen for other samples, that exposure to increased temperature and time increased the average molecular weight of the longer tannins. This implies there may be some aggregation or crosslinking of tannin molecules at higher temperatures.

5.3.6 Antimicrobial activity

The minimum bactericidal concentration (MBC) of the extracts were established against *E. coli* and *S. aureus* as representative gram negative and gram positive bacteria, respectively.

Table 5.11. MBC (%) for the two extracts at different temperature treatments against *E. coli*.

MBC (%) / Sample	Bulk			Small		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
RT	4	4	4	4	4	4
100 ˚C	2	2	2	2	4	4
150 ˚C	2	2	2	1	1	2
200 ˚C	2	2	2	1	1	1
250 ˚C	2	2	2	1	1	2

Table 5.12. MBC (%) for the two extracts at different temperature treatments against *S. aureus*.

MBC (%) / Sample	Bulk			Small		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
RT	0.25	0.25	0.25	0.25	0.25	0.5
100 ˚C	0.25	0.25	0.25	0.25	0.25	0.25
150 ˚C	0.25	0.25	0.25	0.25	0.25	0.25
200 ˚C	0.5	0.5	0.5	0.5	0.5	0.5
250 ˚C	0.5	0.5	0.25	0.25	0.25	0.25

The MBC for the room temperature extract was 4.0% against *E. coli* (Table 5.11) and 0.25% against *S. aureus* (Table 5.12). The extracts were more effective against *S. aureus* than *E. coli*, which confirms results from previous literature suggesting that plant polyphenols are more effective against gram positive than gram negative bacteria.[241] However, other studies have

shown little difference between the antimicrobial properties of both red and white grapes on gram negative and gram positive bacteria.[242] Gram negative bacteria like *E. coli* have a two layer cell membrane and strong hydrophilicity of the outer membranes, which could explain the difference in activity against the two bacteria types.[243]

The extracts were not effective against *C. albicans* under the concentrations tested of 4.0% and below. MBC was recorded instead of minimum inhibitory concentration (MIC) as the colour of the extract made establishing the MIC inconsistent. Upon heating, the extracts retained antimicrobial activity against both *S. aureus* and *E. coli* at all temperatures. Against *S. aureus*, the MBC of the extract heated to 250 ˚C declined by 24% compared to the original value. This is consistent with the MBC values recorded for the extracts heated to 100 ˚C and 150 ˚C. However, the extracts heated to 200 ˚C showed an increase in MBC to 0.5%. Against *E. coli*, there was a reduction in MBC of 50% in the extract heated to 250 ˚C, which is the same reduction as the extract heated to 150 ˚C. However, there was no change in MBC for the extract heated to 100 ˚C, and a 33% decrease in MBC for the extract heated to 200 ˚C. There was no total loss in antimicrobial activity against either *S. aureus* or *E. coli*.

Interestingly, despite the changes in the chemistry of the extracts due to heating seen in the FT-MIR and FT-Raman spectra, antimicrobial activity was retained against *E. coli*, but was lessened against *S. aureus*.

The change in MBC may be due to a change in the structure of the extracts upon heating. There are some indications that antimicrobial activity can be dependent on the structure of the polyphenols. Oligomers of catechin and epicatechin have been shown to be active against *S. aureus* and *E. coli* strains, while monomeric fractions were not active against these.[52] Oligomeric proanthocyanidins are thought to have the most effective antimicrobial activity, as they are small enough to reach and interact with microbial proteins, but also large enough to

effectively cross-link. Condensed tannins can bind with proteins via hydrogen bonds between the phenol group of the tannin and the carbonyl group of the peptide in the microbial protein. This may inhibit key enzymes.[9]

This concurs with the observations made using the gel permeation chromatography in section 5.3.5, where increased average molecular weight for tannins was found with increased temperature. In HPLC for monomers (section 0), the concentration of catechin and epicatechin decreased with increased temperature. The tannin molecules may have aggregated together to form oligomers from dimers or trimers, thus increasing their antimicrobial activity against *E. coli*.

5.4 Conclusion

Grape marc extract was exposed to temperatures comparable to that experienced in the melt blending process. Antioxidant testing showed that there was no loss with increasing temperature exposure, while the antimicrobial activity of the GME did show a modest loss in activity against *S. aureus*. Using spectroscopic analysis through FT-MIR and FT-Raman, it was determined that there was no significant degradation of the phenolic compounds when exposed to increased temperatures, although some changes in hydrogen bonding were observed. Gel permeation chromatography suggested that the smaller procyanidin molecules may aggregate together at higher temperatures to form oligomeric molecules. This rearrangement may have helped to maintain, and even improve, the antimicrobial activity against gram positive bacteria. In a powder form, GME demonstrated tolerance to heat as high as 250 ˚C for 10 minutes. However, the tolerance of GME when distributed in a polymer film needs further investigation.

6 Development of antifungal LLDPE films

6.1 Introduction

Food spoilage is a global concern, with around one third of all produced food worldwide being spoiled or otherwise disposed of before it can be consumed.[78] One factor in food spoilage is microbial spoilage, where bacteria or other microbial organisms grow on food to make it inedible. With bread, major microbes of interest include the moulds *Aspergillus* and *Penicillium*. A common way to preserve bread is by the addition of weak organic acids like benzoic, sorbic, and propionic acids.[244] Consumers are increasingly interested in minimally processed products without synthetic preservatives, so antimicrobial compounds from natural sources are increasingly being used.[245] While these compounds can be directly incorporated into the food product, to avoid sensory issues companies are increasingly turning to active packaging.[246] Active packaging involves interactions between the packaging and the product to improve the shelf-life or consumer experience. This can be achieved through migration of active, volatile compounds into the headspace between the product and the packaging, or by direct physical contact between the product and the packaging.[247]

The series of experiments reported here were conducted in association with an industry partner. Their specifications were to find an antifungal compound which, if possible, would:

- be of New Zealand origin, or could be sourced in New Zealand
- be incorporated into their existing manufacturing systems
- be fungicidal against *Aspergillus brasiliensis*, *Penicillium citrinum*, and *Pichia burtonii* when incorporated into a polyethylene matrix
- have no negative sensory effects on their products
- have positive marketing possibilities around its ‘story’ or origin, i.e. no synthesised chemicals

A list of around thirty compounds was assembled, based on a combination of literature evidence of their efficacy against the selected organisms and the marketing potential of each extract, specifically in New Zealand. Emphasis was given to New Zealand based sources, as well as those that are already easily extracted and are available in food grade. Essential oils were included alongside plant extracts, although these needed to have a much lower minimum fungicidal concentration (MFC) for consideration to avoid potential adverse sensory effects and migration issues. In addition, essential oils are volatile compounds that have sensitivity to heat, which is a limitation for many packaging applications under current manufacturing systems.[248]

In consideration of the sources of natural extracts and essential oils with potential as an antimicrobial agent for use in food packaging, a variety of New Zealand plants were identified that have been used by the indigenous Māori for medicinal purposes.[140,249] Among these were manuka (*Leptospermum scoparium*), akeake (*Dodonaea viscosa*), kawakawa (*Piper excelsum*), harakeke (*Phormium tenax*), totara (*Podocarpus totara*), and horopito (*Pseudowintera colorata*). A selection of non-NZ native plant extracts was also identified as alternative candidates, including hops extract, *Galenia africana* extracts, and grapefruit seed extract (GFSE).

As the final group of candidate antimicrobial agents, a number of essential oils were considered for analysis in this study, extracted from: thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum* sp.) and its major component cinnamaldehye, clove (*Syzygium aromaticum*), tea tree (*Melaleuca alternifolia*), and sugi (*Cryptomeria japonica*).

In this chapter I follow a strategy of *in vitro* testing of natural compounds and essential oils for antimicrobial activity against key species, selecting the best candidates based on a series of

qualifying factors. These are antimicrobial activity against the aforementioned species, tolerance to elevated temperatures, a connection to New Zealand, and consumer acceptance. Consumer acceptance was defined as being from a natural source that most people were familiar with, and not adding any unwanted sensory effects to the consumer experience.

6.2 Experimental Methods

6.2.1 Antimicrobial Susceptibility Test (AST)

The minimum fungicidal concentration of the extracts/essential oils was attained using the standardised broth microdilution protocol described in 2.3.2.2. The extracts were prepared as described in Table 2.1.

6.2.2 Heated extract testing

Results from the AST were used to narrow down the extracts to six candidates for the heated extract stage. The extracts were then tested as described in 2.3.2.3.

6.2.3 Thermogravimetric analysis (TGA)

TGA was carried out as described in 2.3.12.

6.2.4 Film Production

Linear low-density polyethylene (LLDPE) was processed using a Brabender DSE25 twin-screw extruder (Duisburg, Germany). The LLDPE pellets were mixed for 1 minute at 200 ˚C, before the plant extract was added, and mixed for a further 2 minutes at 200 ˚C. After this, the mix was allowed to cool slightly and cut into small pellets. Extracts were incorporated into the films at concentrations of 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, and 5% (*w/w*). Although a range of extracts were tested, only three compounds were selected for further testing based on their screening for thermal stability and antifungal activity. LLDPE only mixtures were also

prepared as controls. The total mass of all components within each batch was 37 g. The measured torque range was 100 Nm.

Thin films were prepared with a 20-ton LabTech LP-S-20 (Samutprakarn, Thailand) hydraulic press with heated platens. Pressing of thin films was undertaken at 200 ˚C, with a 3.5-minute pre-heating cycle, a 30 second pressing cycle at 1000 psi, and a 4-minute cooling cycle.

6.2.5 Antifungal activity of films

The antifungal activity of the films was established using a modified JIS method, described in 2.3.2.5.

6.2.6 Antioxidant testing

Antioxidant activity was established using the DPPH radical scavenging assay described in 2.3.1.1.

6.2.7 Leachable solids

Leaching of the plant extracts from polyethylene films were measured as described in 2.3.10.

6.2.8 Mechanical strength testing

This mechanical testing was performed as described in 2.3.13. Films containing extracts at concentrations of 1%, 2%, 3%, and 5% were tested. Combination films containing two extracts at concentrations of 2.5% each were also tested. A control film of polyethylene was used as a comparison. Each film was tested with five technical replicates.

6.2.9 Statistical analysis

Statistical analysis of data using single factor ANOVA and post hoc test Tukey-Kramer was performed with Excel 2016 for Windows.

6.2.10 Naming of extracts

The extracts analysed in this chapter are coded to maintain confidentiality requirements. Further information on some of the extracts is included here.

A1, A2, and A3 extracts are separated by 1, 2, and 3 to show the different extracts supplied by a confidential company. Each was extracted in a slightly different solvent, which were included in tests as a control to make sure that any antifungal activity was due entirely to the extract. The extract H1 is from the same plant source but is a raw extract.

B1, B2, B3, B4, and B5 extracts are essential oils from related sources. E1, E2, E3, E4, E5 are common essential oils.

Extracts with a New Zealand origin or source include D1, F1, K1, L1, M1, and N1.

6.3 Results and Discussion

6.3.1 Thermal stability testing of extracts

Thermogravimetric analysis (TGA) was performed on all extracts to gain a sense of their thermal tolerance to elevated temperatures. Of particular interest was the stability of the extracts in the temperature range 200 to 250 ˚C, the temperature range that the extracts would be subjected to during film processing.

As expected, due to their volatile properties, most of the essential oils tested did not have stable thermal properties at high temperatures (Table 6.1). B5, E1, E2, E3 (Figure 6.1), E4, and E5 essential oils showed a total or near total loss of mass by 250 ˚C. Interestingly, E3 and E4 did show some thermal stability at low temperatures, retaining 37.0% and 45.0% mass, respectively, at 150 ˚C. G1, the main component in E3, had some thermal stability at 150 ˚C, retaining 57.9% of its original mass. However, once exposed to temperatures of 200 ˚C and higher, G1 showed a near total loss of mass. B2, B3, and B4 showed some variation, retaining between 18.5% and 35.2% of total mass at 250 ˚C. While B1 showed only 0.3% mass remaining at 250 ˚C, it had fair stability at 150 ˚C, with 65.7% mass remaining.

The ethanolic extracts of K1, L1, M1, and N1 showed low thermal stability, even at 150 ˚C. This may be due to the high content of ethanol in the extracts (between 45 to 65%). They are unsuitable for incorporation into melt blended films, retaining less than 20% of their mass at 150 ˚C.

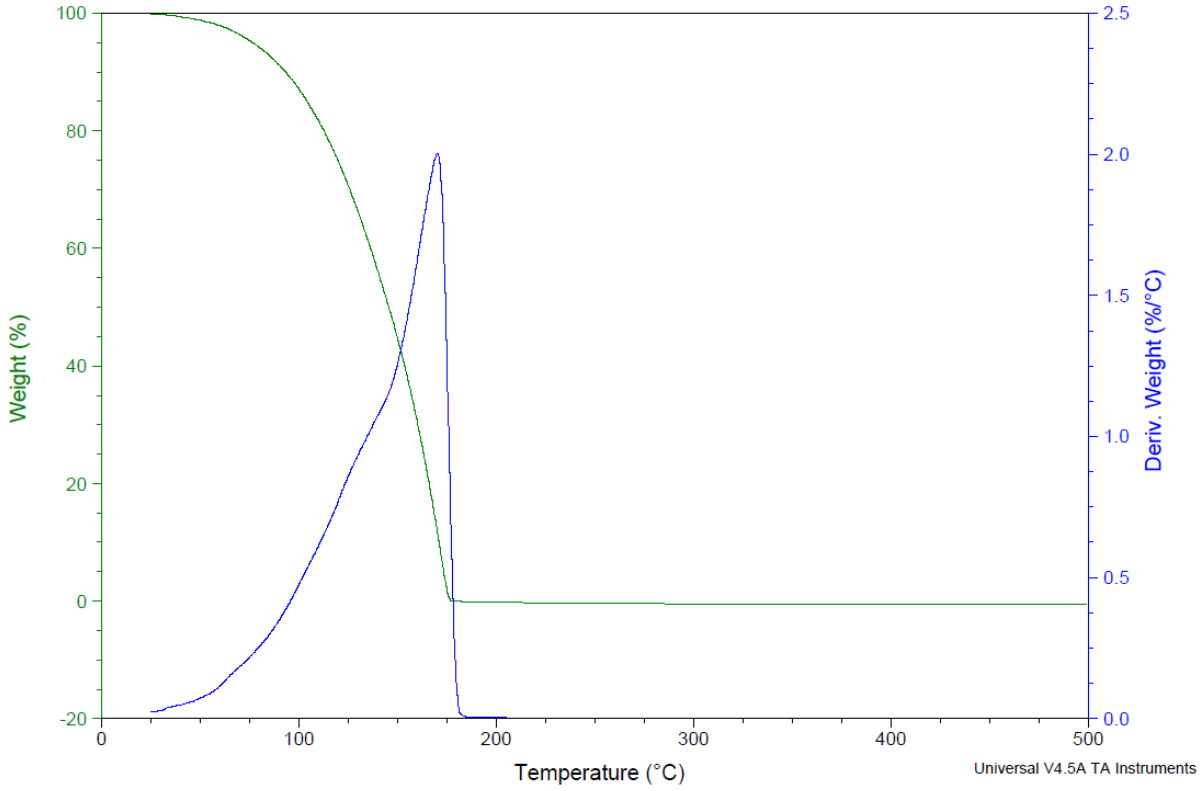

Figure 6.1. TGA plot of E3.

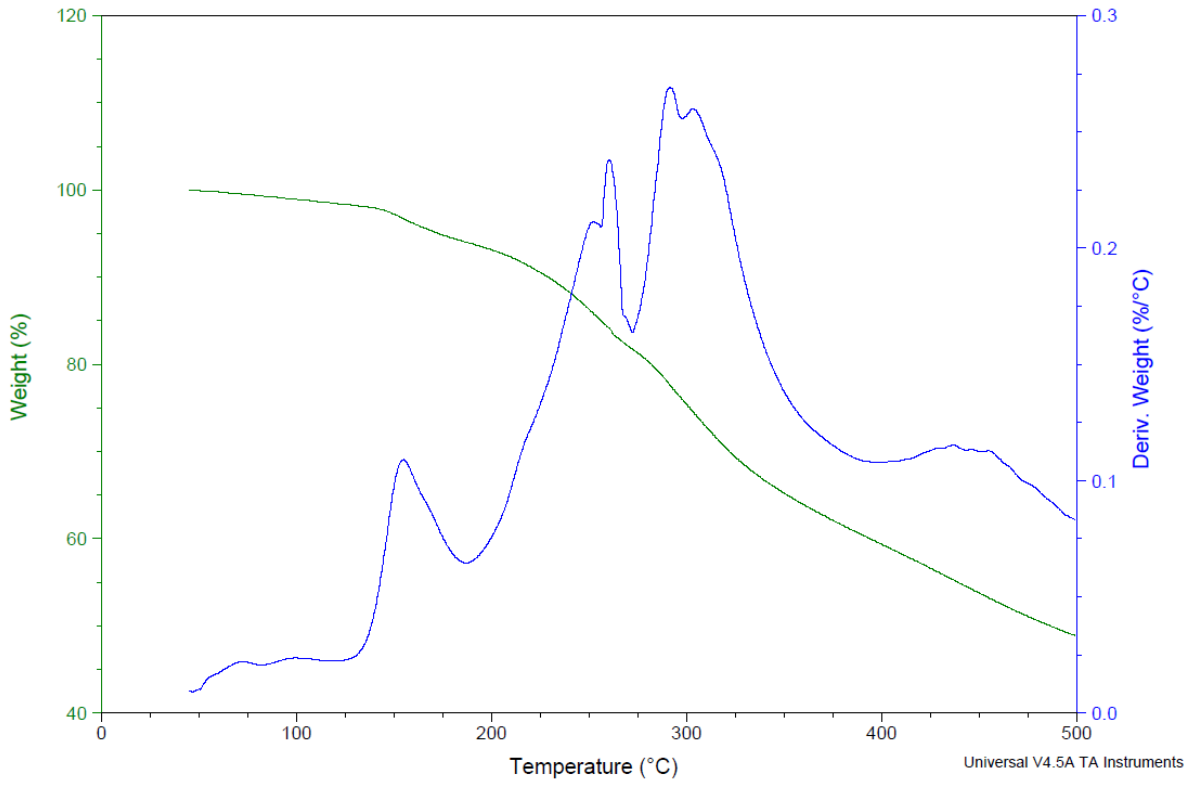

Figure 6.2. TGA plot of H1.

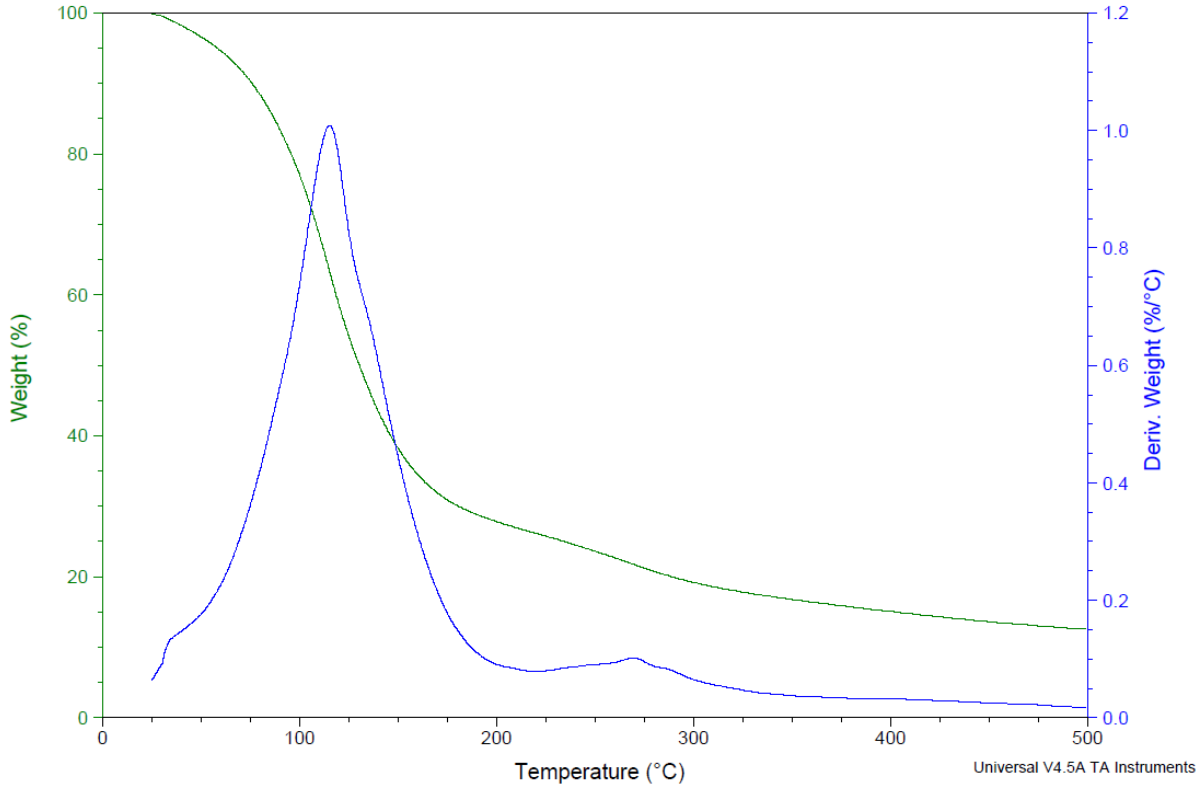

Figure 6.3. TGA plot of A3.

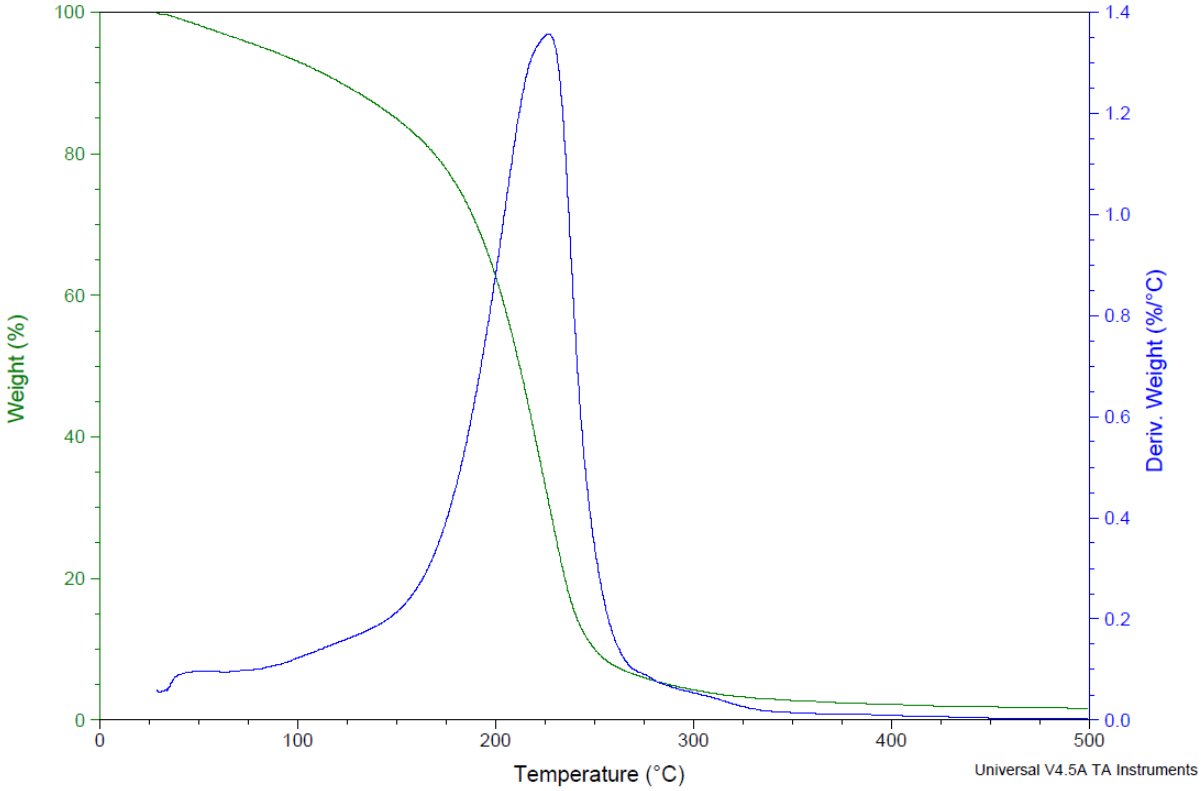

Figure 6.4. TGA plot of J1.

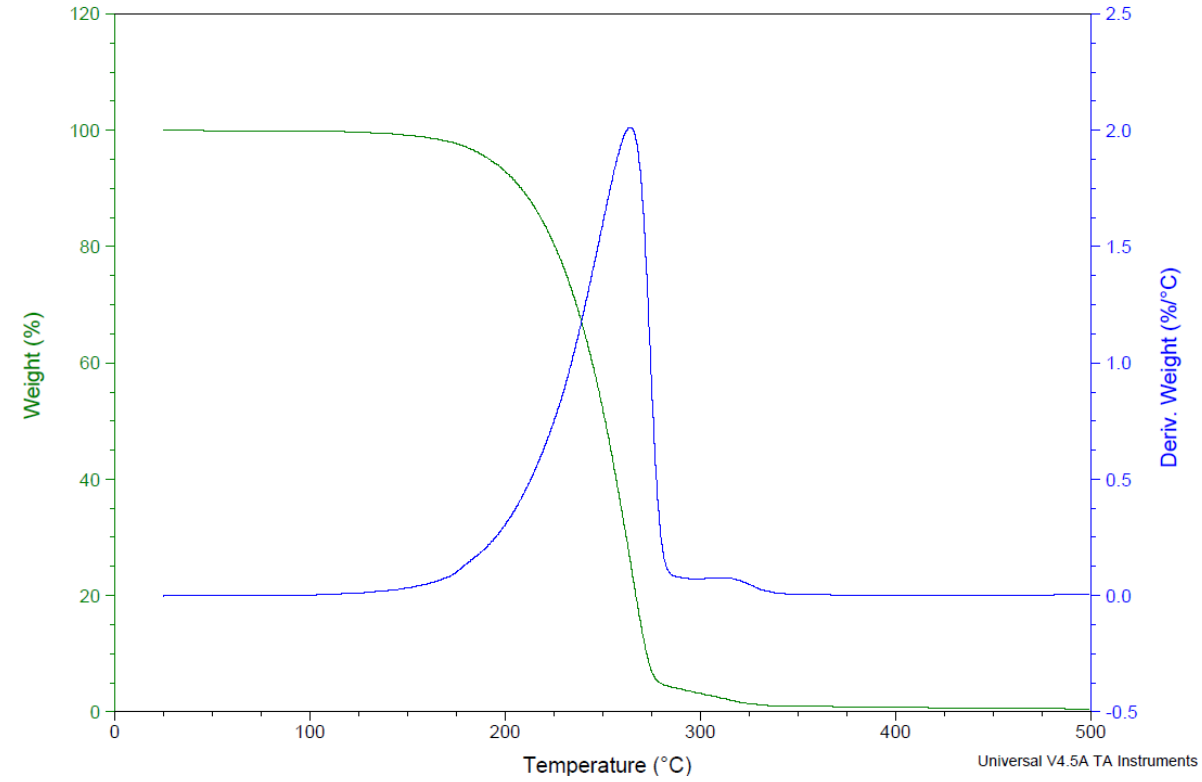

Figure 6.5. TGA plot of D1.

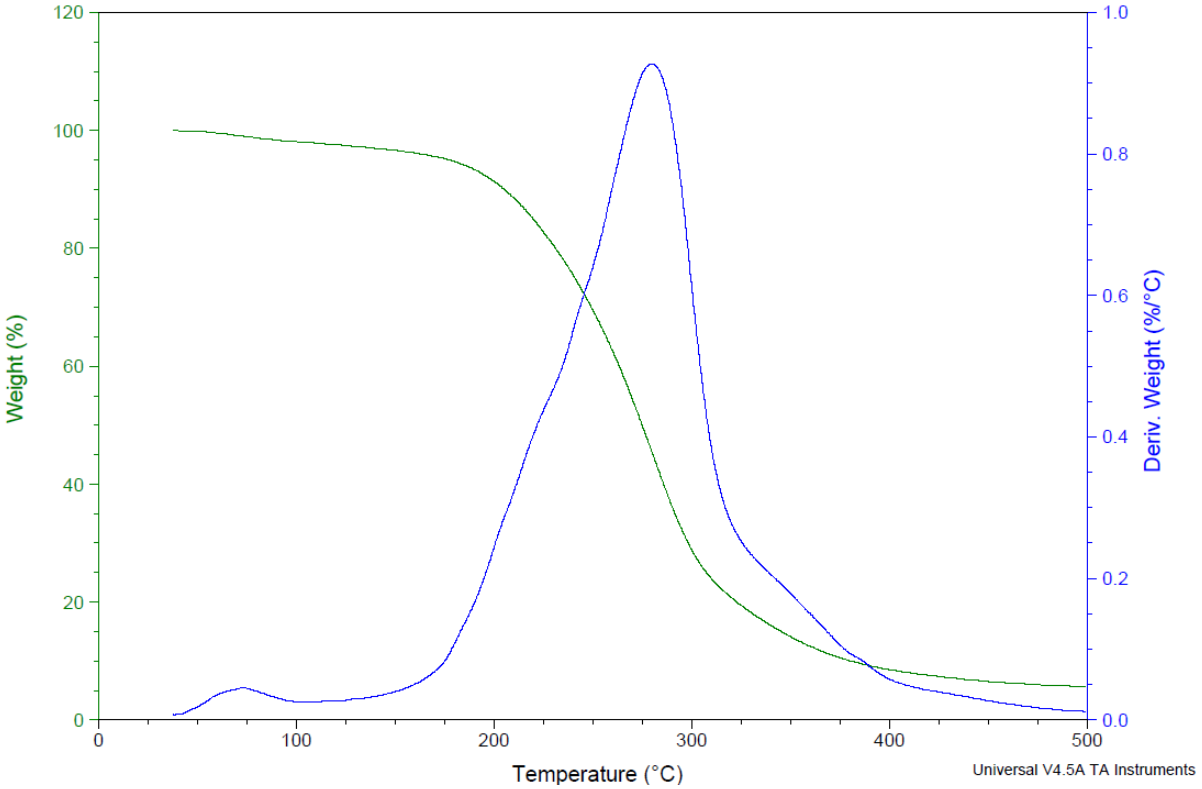

Figure 6.6. TGA plot of F1.

Table 6.1. TGA results from samples showing extrapolated onset temperature, mass of sample remaining at 150, 200, and 250 ˚C, and the temperature where the sample is fully decomposed.

	T_{Onset} (˚C)	Mass at 150 ˚C (%)	Mass at 200 ˚C (%)	Mass at 250 ˚C (%)
A1	85.9	38.2	27.8	23.6
A2	70.2	39.2	29.0	24.4
A3	41.9	41.9	30.3	24.2
H1	217.9	97.3	93.1	86.4
B1	55.9	53.3	40.4	35.2
B2	81.5	32.3	20.3	18.5
B3	99.3	44.2	29.3	27.4
B4	143.5	65.7	0.8	0.3
B5	67.0	0.2	0.0	0.0
D1	227.6	99.2	92.8	50.6
E1	76.6	2.6	0.0	0.0
E2	59.6	2.2	1.9	1.8
E3	128.4	45.0	0.0	0.0
E4	120.7	37.0	0.5	0.0
E5	63.5	0.6	0.5	0.4
F1	135.8	57.9	1.9	1.7
G1	217.9	97.3	93.1	86.4
J1	185.6	85.0	61.9	9.7
K1	38.8	16.5	14.4	11.1
L1	47.0	8.7	6.8	5.3
M1	42.7	14.2	11.8	9.5
N1	44.8	12.6	10.5	7.8

The A1, A2, and A3 extracts also did not show great thermal stability, although all three extracts were similar. By 150 ˚C they had lost around 60% total mass, which increased to a loss of over 75% total mass by 250 ˚C. However, this is probably due to the loss of solvent, which showed total loss by 250 ˚C. The H1 extract showed very different properties due to the lack of solvent (Figure 6.2). At 150 ˚C, it retained 97.3% total mass, and by 250 ˚C had only lost 13.6%. This indicates that these extracts may be suitable for inclusion in melt blended films.

J1 shows good thermal stability up to 200 ˚C, retaining 85% and 61.9% of its total mass at 150 ˚C and 200 ˚C, respectively (Figure 6.4). However, it showed a significant loss in mass between 200 ˚C and 250 ˚C. Its extrapolated onset temperature is 185.6 ˚C. It is still a potential candidate for melt blended films. F1 and D1 both showed good thermal stability compared to most other extracts (Figure 6.5 and Figure 6.6). At 150 ˚C and 200 ˚C, both retained more than 90% of the total mass. At 250 ˚C, they retained 69.2% and 50.6% mass, respectively. The extrapolated onset times for both were around 227 ˚C.

6.3.2 Antimicrobial Susceptibility Test

To determine which of the candidates identified from literature searches were active against fungi MIC and MFC were determined for *A. brasiliensis* (Table 6.2) and *P. citrinum* (Table 6.3). The best six candidates, identified in Table 6.4, were chosen for further study.

Table 6.2. AST results of all extracts against *A. brasiliensis*. The median value of the three biological repeats is recorded, of which each contains three technical replicates. 'I' indicates inhibition where no clear value was able to be discerned. 'NI' indicates no inhibition at the highest tested concentration.

Test Sample	MIC (%)			MFC (%)		
Technical replicates	1	2	3	1	2	3
A1	0.0625	0.0625	0.0625	0.25	1	0.25
A2	I	I	0.03125	0.5	0.5	0.5
A3	0.125	0.25	0.25	1	1	1
H1	0.1	0.05	0.1	0.1	0.1	0.4
B1	I	I	I	NI	NI	NI
B2	0.4	0.4	NI	NI	NI	NI
B3	0.4	0.4	NI	NI	NI	NI
B4	0.4	0.4	NI	NI	NI	NI
B5	NI	NI	NI	NI	NI	NI
D1	NI	NI	0.4	0.4	0.1	NI
E1	0.8	0.8	0.4	0.8	0.8	0.8
E2	NI	NI	NI	NI	NI	NI
E3	0.05	0.05	0.05	0.1	0.1	0.1
E4	0.1	0.1	0.1	0.1	0.1	0.1
E5	NI	NI	NI	NI	NI	NI
F1	0.2	0.1	0.1	0.1	0.2	0.2
G1	0.025	0.025	0.025	0.05	0.05	0.1
J1	0.00625	0.00313	0.00313	0.00625	0.00625	0.00625
K1	2.2	NI	NI	NI	NI	0.275
L1	I	I	NI	NI	NI	2.2
M1	0.55	0.275	NI	NI	0.1375	1.1
N1	NI	2.2	NI	2.2	NI	2.2

Table 6.3. AST results of all extracts against *P. citrinum*. The median value of the three biological repeats is recorded, of which each contains three technical replicates. ‘I’ indicates inhibition where no clear value was able to be discerned. ‘NI’ indicates no inhibition at the highest tested concentration.

Test Sample	MIC (%)			MFC (%)		
Technical replicates	1	2	3	1	2	3
A1	I	I	I	0.125	0.0625	0.125
A2	I	I	I	0.125	0.0625	0.125
A3	I	I	I	0.125	0.125	0.25
H1	I	I	I	0.05	0.025	0.5
B1	I	I	I	NI	NI	NI
B2	I	I	I	0.4	NI	0.2
B3	I	I	I	0.2	0.4	0.2
B4	I	I	I	0.4	NI	NI
B5	I	I	I	0.5	2	1
D1	I	I	I	NI	0.4	0.1
E1	I	I	I	0.2	0.4	0.1
E2	I	I	I	0.05	NI	0.0125
E3	I	I	I	0.00625	0.00313	0.00625
E4	I	I	I	0.05	0.2	0.1
E5	I	I	I	0.025	NI	0.05
F1	I	I	I	0.4	0.4	0.00625
G1	I	I	0.00625	0.00625	0.00313	0.0125
J1	I	I	I	0.00625	0.00156	0.00156
K1	I	I	0.275	NI	2.2	NI
L1	I	I	NI	NI	NI	NI
M1	I	I	0.55	NI	1.1	NI
N1	I	I	2.2	2.2	1.1	NI

The MICs and MFCs of the plant extracts against *A. brasiliensis* (Table 6.2) show that E2, E5, and B1-5 essential oils have no fungicidal activity against *A. brasiliensis* at the highest concentrations tested. However, A1-3, H1, F1, and E1 showed MFCs ≤ 1%. E3 showed an MFC of 0.1%, while G1 had MFCs of 0.1% and 0.05%. The best performing extract was J1, which had an MFC of 0.00625%. The four extracts tested that were pre-dissolved in ethanol (K1, L1, M1, N1) all gave inconsistent MFC values. This may be due to inconsistency within the extracts, or because the tested concentrations were not high enough to accurately find the MFCs for these extracts.

The MICs and MFCs of the extracts against *P. citrinum* (Table 6.3) show that more extracts were active against *P. citrinum* than against *A. brasiliensis*, albeit with more variation between biological replicates. L1 and B1 showed no activity against *P. citrinum* at the highest concentration tested, while the extracts of B2-4, D1, E2, E5, M1, and N1 gave inconsistent results between levels of fungicide and no inhibition. The best performing extracts were J1, with a median MFC of 0.00156%, and E3 and G1, both with a median MFC of 0.00625%. E1 gave a median MFC of 0.2%, while E4 had a median MFC of 0.1%. F1 gave a median MFC of 0.4%, although one biological replicate showed an MFC of 0.00625%, a number comparable to the most active extracts tested. The extracts A1-3 and H1 all showed fungicidal activity against *P. citrinum*, with MFCs between 0.00625 to 0.5%. The more processed extracts (A1-3) performed better than the raw extract (H1). B5 essential oil was active against *P. citrinum*, while it showed no activity against *A. brasiliensis*.

From the MIC and MFC results, six extracts were shortlisted that showed the best fungicidal activity against both *A. brasiliensis* and *P. citrinum*. Both the A1 extract and H1 were chosen as they showed interesting antifungal activity that differed between organisms. D1 and F1 both showed MFCs ≤ 0.4%, albeit with some inconsistencies. However, D1 did show some potential, and has a strong New Zealand connection which was very appealing to

the commercial partner. E3 and J1 were the two strongest plant-sourced extracts that were tested, with MFCs ≤ 0.00625% for E3 against *P. citrinum* and for J1 against both organisms. G1 also showed very good activity but did not meet the marketing requirements for the commercial partner, as it was purchased as a synthetic chemical. These six extracts were subsequently used in the following parts of the experiment (Table 6.4).

Table 6.4. Criteria for choice for compounds moving on to next step.

	Activity against A. brasiliensis (MFC < 0.2%)	Activity against P. citrinum (MFC < 0.2%)	NZ connection	Heat tolerance (from TGA results)
A1	X	✓	X	X
D1	X	X	✓	✓
E3	✓	✓	X	X
F1	X	X	X	X
H1	X	✓	X	✓
J1	✓	✓	X	✓

6.3.3 Antimicrobial Susceptibility Testing of heated samples

The ultimate aim of this project was to incorporate the antifungal agent into a melt blended film for food packaging. In this process the material needs to be heated to temperatures around 200 ˚C and so it is important that the antifungal additive is able to retain activity after heating to these temperatures. To determine this, the six candidate antifungal extracts were heated to 200 ˚C for 10 minutes and then tested for MIC and MFC against *A. brasiliensis* (Table 6.5), *P. citrinum* (Table 6.6) and *Pichia burtonii* (Table 6.7) in comparison with unheated extracts. *P. burtonii* was included as a yeast. Only extracts with some heat stability were considered for further testing.

Table 6.5. AST results of heated extracts against *A. brasiliensis*. The median value of the three biological repeats is recorded, of which each contains three technical replicates. 'I' indicates inhibition where no clear value was able to be discerned. 'NI' indicates no inhibition at the highest tested concentration.

	MFC of heated extract (%)			MFC of non-heated extract (%)		
	1	2	3	1	2	3
A1	2	2	0.4	0.25	1	0.25
E3	0.1	0.1	0.05	0.1	0.1	0.1
F1	NI	0.4	NI	0.4	0.1	NI
H1	NI	NI	NI	0.1	0.1	0.4
J1	0.00625	0.00625	0.00625	0.00625	0.00625	0.00625

H1 did not show any antifungal activity against *A. brasiliensis* at the highest tested concentration (Table 6.5). The extract F1 gave inconsistent results, with only one biological replicate giving a valid MFC of 0.4%. This indicates that the true MFC for this extract is at a higher concentration than those tested. The extract A1 showed a lower antifungal activity when heated compared to the non-heated extract. However, the extract was still active against *A. brasiliensis* with a median MFC of 2%. E3 and J1 were the two extracts that did not show a reduction in antifungal activity when heated. One biological replicate for E3 showed a decrease in MFC to 0.05%. The MFC for J1 stayed constant at 0.00625%, the same value as for the non-heated extract.

Table 6.6. AST results of all extracts against *P. citrinum*. The median value of the three biological repeats is recorded, of which each contains three technical replicates. 'I' indicates inhibition where no clear value was able to be discerned. 'NI' indicates no inhibition at the highest tested concentration.

	MFC of heated extract (%)			MFC of non-heated extract (%)		
	1	2	3	1	2	3
A1	0.05	0.05	0.05	0.125	0.0625	0.125
E3	0.05	0.00313	0.00313	0.00625	0.00313	0.00625
F1	0.05	0.1	0.025	0.4	0.4	0.00625
H1	0.05	0.1	0.05	0.05	0.025	0.5
J1	0.00156	0.00156	0.000781	0.00625	0.00156	0.00156

All of the heated extracts were active against *P. citrinum* (Table 6.6). The extract H1 showed lower antifungal activity in the heated extract compared to the non-heated extract; the heated extract had the same median MFC, but a higher mean than the non-heated extract. However, A1 showed a decrease in median MFC from 0.125% to 0.05% when heated. This may be due to interactions between the extract and the solvent. The other heated extracts also showed a general increase in antifungal properties as seen by the median MFC values. The extract F1 showed a decrease in MFC to 0.05% from 0.4%, and E3 a decrease from 0.00625% to 0.003125%. However, E3 did show some inconsistency, with one biological replicate giving an MFC of 0.05%. The extract J1 showed the same median MFC values when heated, at 0.00156%. However, one biological replicate showed an MFC of 0.000781%, the lowest MFC value recorded in this series of experiments.

The heated extracts were also tested against *P. burtonii* as the yeast is another organism that is often found to cause bread spoilage (Table 6.7). All the extracts were found to have antifungal properties against the organism, although F1 did show some inconsistencies. The

MFC values were higher than those observed for the other two organisms. This may be due to a different method of action, as *P. burtonii* is a yeast and the other organisms are filamentous fungi. J1 again performed the best, however, with an MFC of 0.0125%. The extracts A1, H1, and E3 all had the same MFC of 0.05%. The extract F1 performed the worst, with a median MFC of 0.2%. The candidate compounds retaining sufficient activity for further testing were identified as J1, A1, and E3.

Table 6.7. AST results of all extracts against *P. burtonii*. The median value of the three biological repeats is recorded, of which each contains three technical replicates. 'I' indicates inhibition where no clear value was able to be discerned. 'NI' indicates no inhibition at the highest tested concentration.

	MIC of heated extract (%)			MFC of heated extract (%)		
Technical replicates	1	2	3	1	2	3
F1	0.1	0.025	0.0125	NI	0.1	0.2
H1	0.05	0.025	0.025	0.05	0.05	0.05
A1	0.125	0.0125	0.0625	0.05	0.05	0.05
E3	0.05	0.025	0.025	0.05	0.05	0.05
J1	0.00625	0.00625	0.00625	0.0125	0.0125	0.0125

6.3.4 Antifungal properties of films

The best candidate antifungals retaining activity after heating were incorporated into LLDPE films and tested for antifungal activity using an adaptation of JIS Z 2801 (see section 6.2.5). Activity against *A. brasiliensis*, *P. citrinum* and *P. burtonii* (Table 6.8) were recorded. The median value of the three biological repeats is recorded, of which each contains three technical replicates.

Table 6.8. JIS results of films containing G. africana extract, J1, and a combination film containing both extracts at a percentage of 2.5% each (w/w). 'I' indicates inhibition where less than full growth was observed. 'NI' indicates no inhibition at the highest tested concentration.

Sample	Microorganism								
	A. brasiliensis			*P. citrinum*			*P. burtonii*		
Technical replicates	1	2	3	1	2	3	1	2	3
A1 (5%)	I	NI	NI	I	NI	NI	NI	NI	NI
J1 (5%)	I	NI	NI	I	NI	NI	NI	NI	NI
Combination	NI	NI	NI	NI	NI	NI	NI	NI	NI

There was no consistent inhibitory effect from films containing J1, A1, or a combination of the two against *A. brasiliensis*, *P. citrinum*, or *P. burtonii*. The combination films showed no inhibitory activity against any of the three organisms at the tested concentrations. None of the films showed any inhibition against *P. burtonii*. However, the films containing A1 and J1 did show inhibitory activity against *A. brasiliensis* and *P. citrinum* for one biological repeat each. This implies that there is still some interaction happening between the extracts and the organisms while the extracts are within the polymer matrix. However, the effects were not consistent, even at a concentration of 5% (*w/w*). This may be due to there being a lack of extract material present at the surface, or because the mechanism of action of the extracts requires more mobility. Active sites may be prevented from reaching the organisms when held in the film.

Further film production involving these extracts may be undertaken by forming emulsions or other stabilising methods to ensure the extracts are stable and available for their intended use. This has precedence in literature, with cinnamon essential oil undergoing the Pickering stabilisation method to create antifungal films.[244] The antimicrobial activity of the films may also be dependent on the polymer type. Previous studies have shown that polylactic acid films containing plasticiser and grapefruit seed extract showed strong antibacterial activity against *E. coli* and *Listeria monocytogenes*, while LLDPE films showed low antimicrobial activity.[250]

6.3.5 Antioxidant testing of films

There was a significant difference in antioxidant activity for LLDPE films containing one or both A1 and J1 compared to the control ($P < 0.05$). The control film, containing no additional extract, scavenged just 7.6 ± 0.7 % of the DPPH free radicals present. By comparison, J1 and A1 containing films scavenged 78.3 ± 4.5 % and 66.3 ± 0.1 %, respectively. The addition of the extracts at such a low loading significantly increased the antioxidant activity of the films. This indicates that active compounds from the extracts remain active within the films after the heat treatment necessary for production.

Although the films show good antioxidant activity, they did not retain the antimicrobial activity seen with the pure extracts. This may be due to the availability of the extracts at the surface of the film and the mechanism of antimicrobial action. The assay used to determine antioxidant activity involves submerging the film within the solvent containing DPPH free radicals. The protocol for testing the films for antimicrobial activity involves 100 µL of inoculum in contact with the surface of the film. This means that the extracts or their active antimicrobial sites may not be present or accessible at the surface of the films.

6.3.6 Leaching of solids from films

To measure the stability of the extracts in films, the level of leaching was measured in both water and ethanol.

Table 6.9. Leaching results of films containing A1, E3, and J1 in water. Results shown are mean with standard deviation. Values in the same column not connected by the same letter are significantly different ($p < 0.05$).

Film leaching (mg) / Samples	1%	5%
Control	1.45 ± 0.07^{a}	1.45 ± 0.07^{a}
A1	4.53 ± 1.80^{a}	12.53 ± 1.53^{a}
E3	2.40 ± 0.28^{a}	15.00 ± 2.43^{b}
J1	4.27 ± 0.93^{a}	9.05 ± 7.71^{a}

Table 6.10. Leaching results films containing A1, E3, and J1 in ethanol. Values not connected by the same letter are significantly different ($p < 0.05$).

Film leaching (mg) / Samples	1%
Control	3.10 ± 0.28^{a}
A1	3.47 ± 1.02^{a}
E3	3.20 ± 0.10^{a}
J1	5.73 ± 2.03^{a}

A1, E3 and J1 in the 1% film showed no significant level of leaching in water compared to the control ($p > 0.05$). E3 had the highest levels of leaching at 15.0 ± 2.4 mg for the 5% film, which was significantly higher than the control ($p < 0.05$). It is not surprising that the E3 showed significant leaching at this level of incorporation into the films. As an essential oil, it

has well documented volatile properties that make leaching very likely. While J1 showed the lowest average level of leaching in water at a film loading of 5%, it also showed the most variation between samples. In ethanol, films containing J1, A1 and E3 showed no significant change from the control film ($p > 0.05$).

The acceptable limit for additives in food packaging, set out by the European Food Safety Authority (EFSA) is 10 mg dm^{-2} of the material.[251] At 1% loading of all extracts, the leaching fell below this acceptable limit. However, in the 5% films, the leaching of E3 and A1 were above this limit. This means that at higher extract concentrations, the leaching of these may not be appropriate for food packaging applications.

6.3.7 Mechanical strength testing of films

LLDPE films containing A1 and a combination of both A1 and J1 were tested against a LLDPE control film for their mechanical properties, which are shown in Table 6.11.

Table 6.11. Tensile strength results of films containing A1 and the combination film containing both A1 and J1 at a loading of 2.5% (w/w) each. Films containing J1 gave highly variable results and are not shown. Values in the same column not connected by the same letter are significantly different ($p < 0.05$).

	Ultimate tensile strength (MPa)	Tensile strain at break (%)	Yield strength (MPa)	Thickness (mm)
Control	22.4 ± 0.2^{a}	721 ± 9^{a}	10.0 ± 0.3^{a}	0.223 ± 0.011
1% A1	21.9 ± 1.3^{a}	736 ± 50^{a}	9.65 ± 0.83^{a}	0.215 ± 0.057
2% A1	21.8 ± 1.3^{a}	688 ± 83^{a}	9.03 ± 0.33^{a}	0.202 ± 0.019
3% A1	22.9 ± 1.8^{a}	752 ± 38^{a}	9.01 ± 0.21^{a}	0.188 ± 0.034
5% A1	23.1 ± 1.7^{a}	756 ± 35^{a}	9.20 ± 0.05^{a}	0.198 ± 0.007
A1 and J1	42.8 ± 2.0^{b}	749 ± 56^{a}	27.8 ± 1.8^{b}	0.158 ± 0.016

The tensile strength at break and ultimate tensile strength were not significantly different for films containing A1 ($p > 0.05$). The addition of A1 at concentrations up to 5% did not adversely affect the mechanical properties of the LLDPE films. The combination film

containing both extracts showed a significantly higher ultimate tensile strength compared to the control ($p < 0.05$). This indicates that the addition of the mixture A1 and J1 at a total concentration of 5% did affect the tensile properties of the film compared to the control.

6.4 Conclusions

Natural plant extracts are potential candidates for active packaging, specifically for the preservation of bread. A series of plant extracts and essential oils, including those of New Zealand origin, showed various levels of antifungal activity against *A. brasiliensis*, *P. citrinum*, and *P. burtonii*. A1 and J1 showed the most promise due to their superior antifungal properties in conjunction with evidence of thermal stability and a consideration of low adverse sensory effects. Films showed significantly improved antioxidant properties, as shown by the DPPH radical scavenging assay. Due to the results in Table 6.8, no direct testing with bread was undertaken within the PhD thesis. Further work may identify appropriate strategies such as encapsulation, to improve the antifungal activity of the films containing extracts. The incorporation of A1 did not adversely affect the mechanical properties of the LLDPE films, while combining A1 and J1 showed significantly improved mechanical properties. Overall, natural extracts like A1 and J1 are an exciting option for the food packaging industry.

7 Conclusions and Future Work

7.1 Conclusions

In this research project, the aim was to explore the properties of natural extracts and their potential for use in active food packaging. This can be split into two different parts: the extraction of phenolic compounds from grape marc and the incorporation of the extracts into packaging, and the use of a diverse range of natural extracts and essential oils to produce antifungal packaging that could potentially be used for bread preservation.

Grape marc was identified as a waste product that could be valorised, as it contains high levels of phenolic compounds. Conventional solvent extraction using water was used in the proceeding work with grape marc, as it allowed easy purification of the extract using Amberlite resin, removing sugars and other compounds such as tartaric acid. Extraction methods and solvents were compared using microwave-assisted extraction, ultrasound assisted extraction, and conventional solvent extraction. Additionally, in order to keep the potential applications for the extract as wide as possible, it was prudent to minimise solvents that may not be regarded as safe around food. The solvents used were water, 20% ethanol, 80% ethanol, and 80% ethanol with 1% acetic acid.

Once the extraction process was finalised, the suitability of the grape marc extract (GME) in biodegradable and bio-sourced films was investigated. Incorporation of GME into soy protein, fish gelatin, and ethyl cellulose films showed that the antioxidant capacity of the GME could be maintained in films. In addition, the ethyl cellulose films incorporated with GME showed a significant increase in antibacterial activity against *S. aureus*. The mechanical properties of the soy protein isolate films decreased with GME concentration, which could have an impact on the application of the films as packaging materials. While bio-sourced

polymers are attractive in theory, the plastic packaging industry currently uses polyolefins and melt blending for packaging. As the bio-sourced polymers were processed at temperatures under 150 ˚C, it was necessary to investigate the properties of GME when exposed to heat, to test their suitability for use in current polyolefin plastics.

In powder form, GME showed tolerance to temperatures as high as 250 ˚C for 10 minutes. The antioxidant capacity of the powders showed no loss with increasing temperature, which was an unexpected result. While there was a small loss in antimicrobial activity against *S. aureus*, heating the GME to higher temperatures showed an improvement in activity against *E. coli*. Structurally, no major degradation was seen in the powders, although some changes in hydrogen bonding was observed at higher temperatures. Smaller molecules such as catechin and epicatechin may aggregate together at higher temperatures to form longer tannin structures. This may make GME more attractive as an additive in melt blended packaging, as longer tannin structures are less likely to leach from the film. This work demonstrates the potential for GME to be applied in films produced via melt blending at temperatures up to 250 ˚C, providing the antioxidant capacity and antimicrobial properties carry through to its incorporation in the films.

Work was then undertaken from a different angle. While the GME work started with a natural product and applications were explored, the next phase of work started with an application and natural products were investigated to find a solution. Bread spoilage is a significant problem worldwide and a solution could have wide-ranging economic and social effects. A1 and J1 were both identified as promising additives for active packaging of bread due to their activity against *Aspergillus brasiliensis*, *Penicillum citrinum*, and *Pichia burtonii*. The mechanical properties of the films were not affected by the inclusion of A1, and the combination of both A1 and J1 improved the mechanical properties. Incorporation into films significantly improved the antioxidant capacity, however the antifungal activity of the extracts did not carry over into the films.

The research in this thesis presents options for active packaging additives originating from natural sources. Grape marc extract is an exciting option for wineries to further valorise a waste product. For the packaging industry, it is a source material with high volume and a low-cost extraction process. Blending with ethyl cellulose, soy protein isolate, and fish gelatin produced films with high antioxidant capacity, while its heat tolerance was also demonstrated for further use in melt blended polymers. Antifungal active packaging was also targeted, although more work must be done to transition the antifungal activity of the raw extracts to films.

7.2 Future Work

Further work on SPI, fish gelatin, and ethyl cellulose films needs to be undertaken to expand knowledge on film properties. Ethyl cellulose films showed some evidence of antimicrobial capacity and this should be investigated in more depth.

More research needs to be undertaken on grape marc extract and its incorporation into melt blended polymers. While this research project ascertained the heat tolerance of the extract for this purpose, it is vital that the extract be incorporated into melt blended films and the film characteristics be fully established. It is especially important that the antimicrobial activity of the films against gram positive and gram negative bacteria be recorded to see whether the changes in activity seen in the powder extract with heat exposure are consistent. Additional work on the structural changes seen in GME when exposed to heat are also important, to confirm how the changes in molecular weight are affecting the phenolic compounds. An important follow-up to this work is examining the real-world application of the melt blended films through testing its effect on the accelerated ageing of oil.

As both A1 and J1 showed promise as antifungal additives in bread packaging, it is important that further work be done in the space to produce antifungal films. This may include using additional techniques such as encapsulation to protect the extracts during the melt

blending process. The films can also be used to package bread to quantify any positive effect on the shelf life of the product. Another line of investigation is the incorporation of these additives in compostable or bio-based packaging to form an environmentally friendly antifungal alternative to current bread packaging. Other extracts, especially E3 essential oil, showed promise. Further work needs to be started to investigate how to mitigate sensory issues in this space for it to be a viable candidate.

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9 Appendix

9.1 Linear calibration curves

9.1.1 Phenolic determination

Linear calibration curves were established using standards of malvidin-3-O-glucoside (oenin) for the PDA detector (Figure 9.1) and catechin for the RF detector (Figure 9.2).

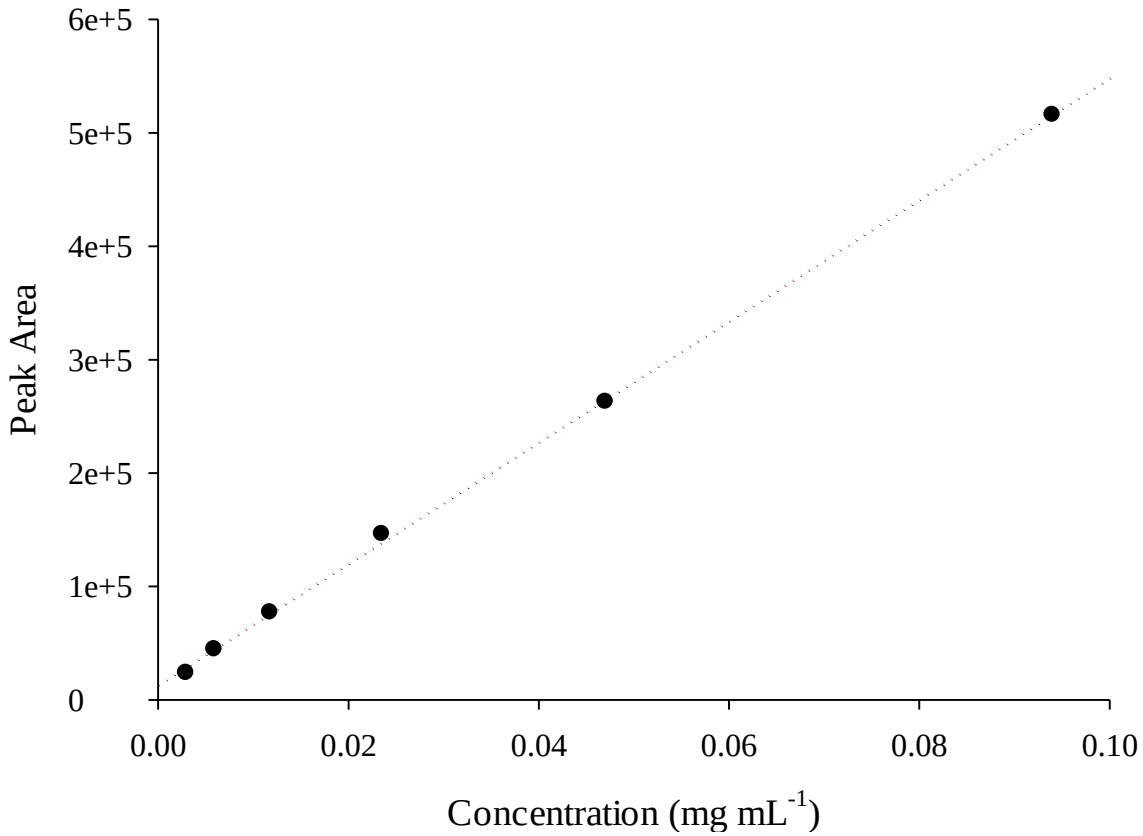

Figure 9.1. Linear calibration curve of malvidin-3-O-glucoside with linear regression $y = 5.35 \times 10^6 x + 13172$, $r^2 = 0.9995$.

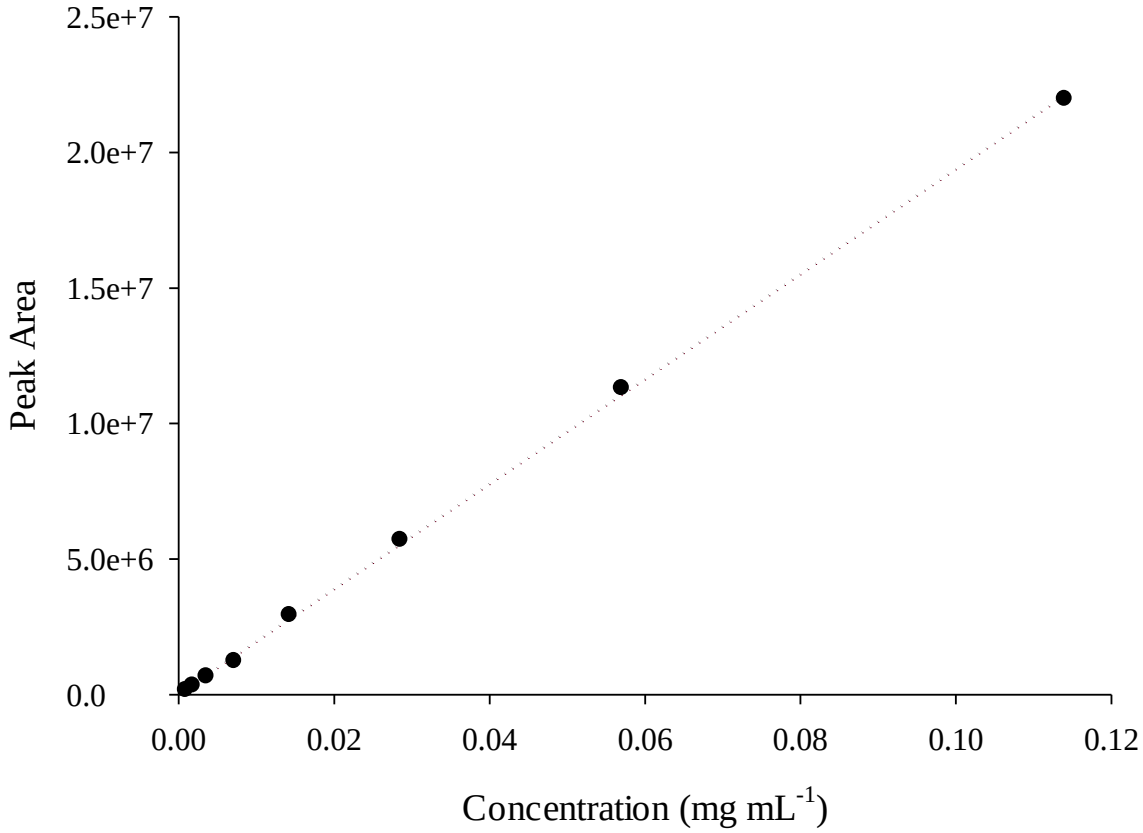

Figure 9.2. Linear calibration curve of catechin with linear regression $y = 1.93 \times 10^8 x + 42778$, $r^2 = 0.9996$.

9.1.2 Sugar determination

Glucose and fructose standards at concentrations of 0.25, 0.5, 1.0, 5.0, and 10 g L^{-1} were used to establish a linear calibration curve.

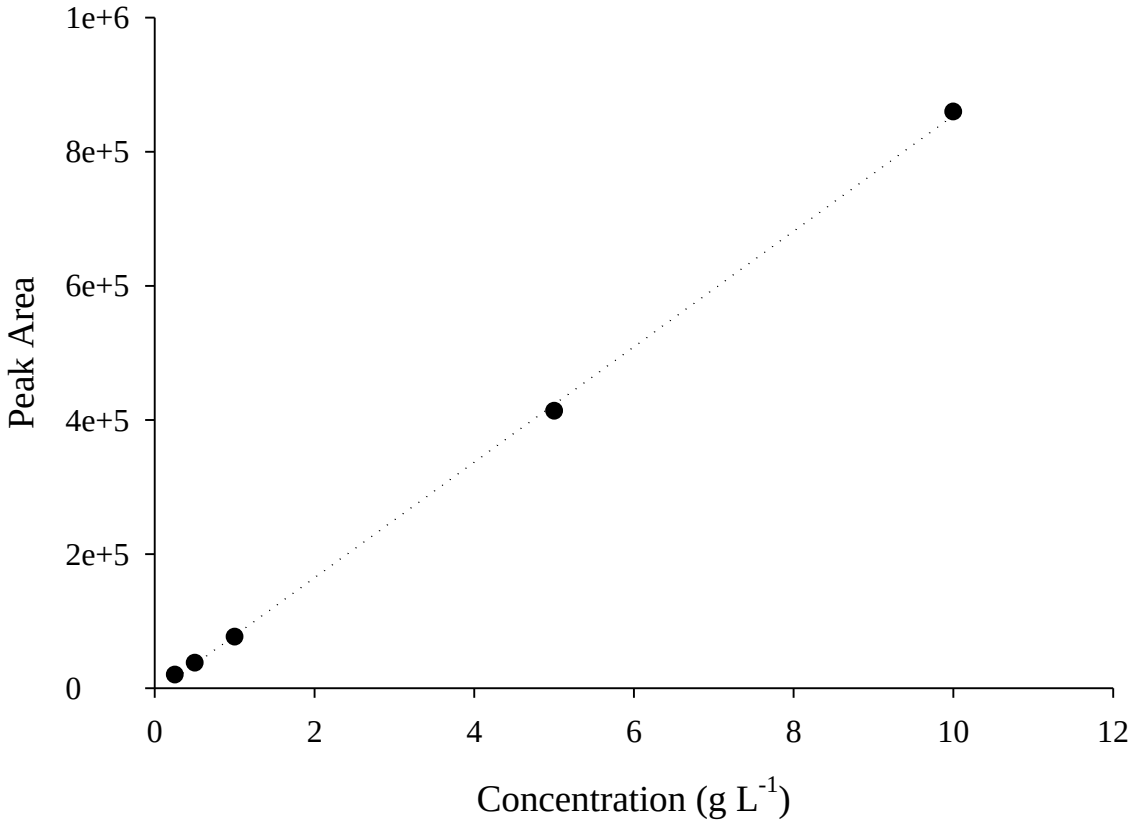

Figure 9.3. Linear calibration curve of glucose with linear regression $y = 8.62 \times 10^4 x - 7414$, $r^2 = 0.9997$.

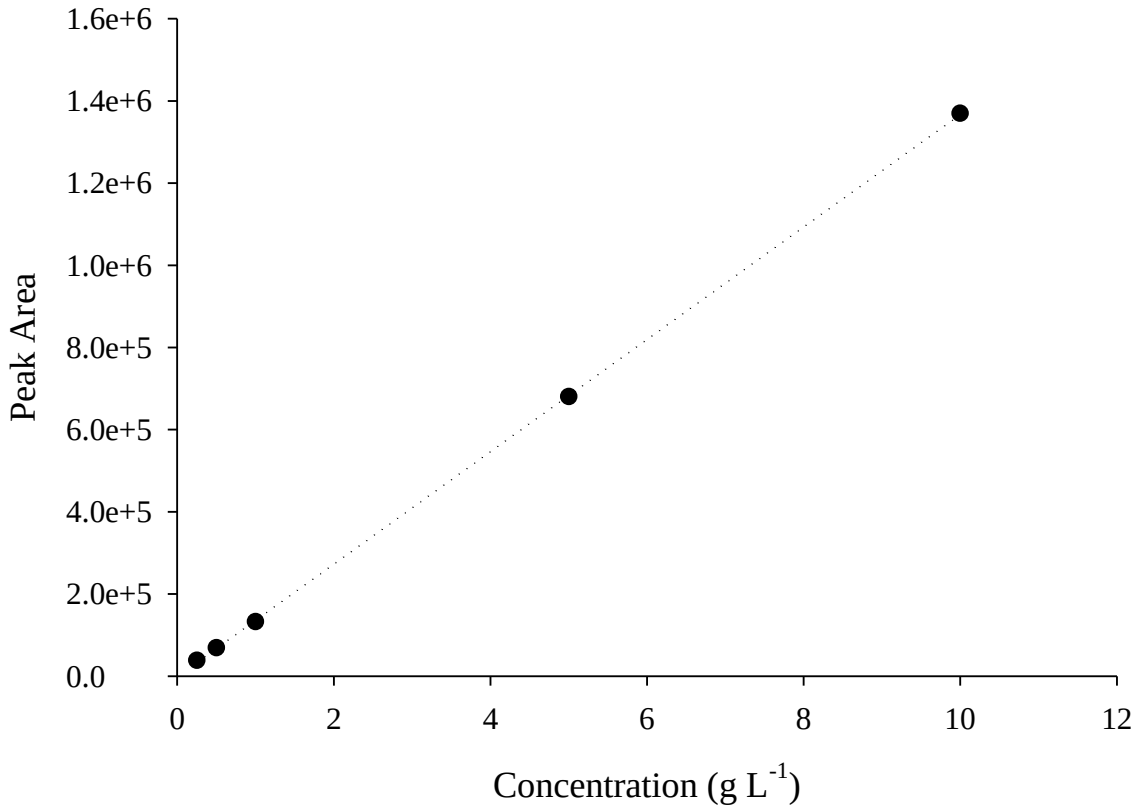

Figure 9.4. Linear calibration curve of fructose with linear regression $y = 1.37 \times 10^5 x - 697$, $r^2 = 0.9999$.