DOCTOR OF PHILOSOPHY

Improved procedures for the transport and storage of fruit and vegetables

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Chapter 1: Introduction

1.1: Overview

Fresh fruits and vegetables are an important part of the human diet, providing many of the essential vitamins and minerals we require to remain healthy in mind and body. Heightened public awareness of these facts has led to increased consumer demand for these products. Whereas they were once grown for direct consumption or to sell at a very local level, they are now a significant component in the competitive strategies of retail outlets throughout the developed world (Eckert and Sommer 1967; Cook 1992; Korsten 2006).

The areas that produce many of these perishable commodities are often some distance from the end point of sale, thus driving the pursuit for the advancement of technology in long-distance transport and storage facilities. In turn, this has promoted greater product diversity as the market for 'exotic' fruits and vegetables has grown, as well as encouraging the demand for both seasonal and non-seasonal products throughout the year. However, the consequence of this is the increased probability of the fruits and vegetables losing the 'freshness' for which they are desired, as deterioration by physiological breakdown and/or pathogen attack is likely to be increased the greater the time and distance from harvest to consumption (Cook 1992; Moss 2002; Korsten 2006).

Postharvest losses of fresh fruits and vegetables have been estimated to be between 30-40% worldwide, although this figure may be a lot higher in developing countries due to lack of technology and adequate sanitation. In the U.K, the supermarket Tesco revealed recently that it generated almost 30,000 tonnes of food waste in the first six months of 2013, with 21% of that being fruit and vegetables (Tesco Plc 2013). With the growing worldwide concern for future food security and the fact that suitable land for crop production is already at a premium, it is vital that methods are implemented to reduce these losses in a bid to adequately feed the burgeoning human population (Wilson and Wisniewski 1989; Tripathi and Dubey 2004; Kader 2005).

This chapter shall look briefly into the physiology of fruits and vegetables and the factors influencing their breakdown, and then go on to examine some of the postharvest treatments and technologies that have been utilised to safeguard their quality. It shall then introduce the aims of and work involved in this PhD thesis.

1.2: The Physiology of Fruits & Vegetables

1.2.1: Structure & Composition

Different fruits and vegetables originate from different parts of the parent plant. Strawberries, for instance, originate from the receptacle, whilst tomatoes are derived from the septum and apples come from the carpels. Most, however, are composed of > 70% water, whilst the remaining ~ 30% is predominantly carbohydrates (with glucose and fructose found in all produce and sucrose in ~ 2/3rds), including cellulose, hemiculloses and pectin, which make up their fibrous content (Kader 1992; Wills *et al.* 1998).

Protein and lipid content are generally low (typically < 3.5% & < 1%, respectively) and although all fruits and vegetables are important sources of vitamins, minerals and organic acids, the exact composition of these vary widely. Orange and yellow vegetables, such as carrots and sweet potatoes, as well as green leafy vegetables, such as spinach and kale, are good sources of beta-carotene (the precursor to vitamin A) and, while vegetables are generally richer in mineral substances such as calcium and phosphorus, strawberries, cherries, peaches and raspberries are very important suppliers of potassium (Lee and Kader 2000). Organic acids, however, are mainly found in fruits, including malic acid in apples and citric acid in citrus fruits (hence their name), which are also excellent sources of vitamin C (ascorbic acid) along with tomatoes and cabbages (Nagy 1980; Wills *et al.* 1998).

All plants, including fruits and vegetables, have protective tissues which usually consist of specialised, compacted parenchyma cells that form a skin, peel or rind. On the outside of these structures, the epidermal cells produce a waxy covering called the plant cuticle, which is water impermeable and forms a physical barrier to pathogenic microorganisms such as bacteria and fungi (Adaskaveg 2002). The rind (flavedo) of oranges, for example, consists of cellulose and pectic substances. Plastids are also found here – chloroplasts in immature fruit which are converted to chromoplasts as they mature. The flavedo is cutinized and protected by a layer of wax platelets that consist of long-chain alkanes and alcohols, as well as long-chain aldehydes and fatty acids (Ladaniya 2008). It serves as the main barrier between the fruit and its environment, determining gas exchange rates of water vapour, oxygen and carbon dioxide. Evaporation at the flavedo creates a difference in water potential which draws water by mass flow from the epidermal cells through the

cuticular membrane to the air/water interface. The cuticle is perforated by stomatal pores that allow for gas exchange; however 12-60% of these are occluded by the natural waxes by the time they are harvested, thus blocking exchange (Ben-Yehoshua *et al.* 1995). The parenchyma of the flavedo extends through the albedo (the pale, pith below the flavedo) to the flesh, thus creating an airy network in which vascular bundles are interspersed. Large, thin-walled oil glands are found amongst the parenchyma, the oil in which consists mainly of limonene which is toxic to most of the other cells of the fruit and therefore must be contained. The albedo itself consists of deeply-lobed meristematic cells and has large air spaces (Agusti *et al.* 2001).

1.2.2: Life after Harvest

An essential aspect to keep in mind when thinking about maintaining the quality of fruits and vegetables after they are harvested is the fact that they are still alive. Harvesting cuts them off from their water supply, but continued respiration, which produces carbon dioxide and heat, as well as transpiration, which adds to evolved moisture, are important biological factors influencing their postharvest condition (Wills *et al.* 1998). Water transport in all harvested fruit occurs primarily through transpiration and evaporation as respiration rates are generally low (Ben-Yehoshua *et al.* 1985; Saltveit 1991). The production of ethylene, which is involved in the processes of ripening and senescence, as well as compositional changes such as loss of chlorophyll, changes in phenolic compounds and organic acids, and carbohydrate conversion (sugars to starch or vice versa) also contribute to their taste, texture and overall quality characteristics (Kader 1992; Suslow 2000).

Environmental factors, particularly temperature and relative humidity, have a huge influence on these internal processes and conditions, and methods that control and maintain their external surroundings to an optimum can extend their postharvest life-span. Moreover, careful handling of the products at all stages of the postharvest chain is essential, as physical damage of any sort not only makes them look less attractive to the consumer, but also allows easy access to pathogenic microorganisms, which escalate their physiological breakdown (Eckert and Sommer 1967; Kader 1992).

1.3: Physical Means to Prevent Deterioration

1.3.1: Controlling Temperature and Relative Humidity

Temperature is probably the most important factor in determining the rate of deterioration of fruits and vegetables once harvested (Paull 1999). It is the major determinant of product respiration rate (Burton 1977). Between 0-30°C, each 10°C rise in temperature increases respiration by a factor of 2-4. It also greatly influences transpiration and evaporation and, therefore, water loss (Mitchell 1992).

Low temperature storage is the first option in any postharvest management system and has been used as a tool by mankind since antiquity, with the utilization of caves, cellars and ice houses to keep commodities cool and extend their life-span (Suslow 2000). Modern trading practices have meant that cooling techniques have had to change dramatically - with refrigerated transport switching from ice in bunkers to mechanical systems – but these contemporary methods are not necessarily improvements. Previous practices, when utilised properly, provided adequate air circulation around the products, which allowed them to be get sufficiently cooled. However, implementing this facet into current refrigeration systems has been problematic; a negative impact exacerbated by the tendency to minimize transport costs by using high-density loads (Mitchell 1992).

To combat this problem, produce has to be rapidly cooled prior to being put into cold storage (King *et al.* 1988; Yang and Irudayaraj 2003). These two systems have to be separate, as sufficient cooling can only be obtained if each individual product has space around it for air to flow. This may mean that up to 100 times more capacity may be required to cool the same volume of produce as to store it (Mitchell 1992).

Once the commodity is at its optimum temperature it can then be placed in storage, where air speed must be kept at a minimum to prevent the effects of dehydration caused by evaporation (Mitchell 1992). This is when relative humidity (RH) also becomes an especially important factor, as it has a direct relationship with temperature and will influence the rate of water loss via transpiration (Harvey 1978; Sharkey and Peggie 1984; Porat *et al.* 2004). The RH of the produce is essentially 100% because of its high water content, so the greater the difference between that and the RH of the ambient air the more the produce will transpire (Suslow 2000).

If all produce had the same optimum storage temperature and RH requirements, it would be a much simpler task to maintain them all in good condition between harvest and consumption. However, different products respond in various ways to low temperatures, with some crops (particularly fruits from tropical and subtropical climates) being susceptible to a condition known as chilling injury (CI) when stored at temperatures below 10°C (Purvis 1984; Schirra 1992; Marangoni *et al.* 1996; Lurie and Sabehat 1997; Henriod 2006). The symptoms of CI, such as pitting of the skin or peel and the development of discoloured areas, often do not become apparent until after the fruit has been removed from cold-storage, such as the time when it gets transferred to ambient temperatures at its retail destination. Much research has, therefore, been conducted into finding the temperature tolerances of different fruits and vegetables, so that they can be stored at the lowest temperatures they can withstand without developing CI (Suslow 2000).

Temperature and RH also affect the microorganisms that may be living on any particular fruit or vegetable. As a general rule, bacteria are more prevalent on vegetables and fungi are more frequent on fruit, due to their having a lower surface pH, but the growth of both are heavily influenced by environmental conditions (De Roever 1998; Teixido *et al.* 1999b). Throughout a crop's life cycle, there may be large numbers of different microbial species associated with it, most of which will be natural epiphytes rather than pathogens. However, once they have been harvested, the microbial population will start to change, latent infections will take hold and more opportunistic pathogenic species will invade (Korsten 2006). Growth rate is highly dependent on temperature, as is spore germination in fungi, and the optimum temperature for each will vary widely amongst different species. However, even though the growth of most fungi is completely halted at temperatures approaching 0°C they are still alive and will continue growth and spore production immediately upon transference into more ambient conditions (Eckert and Sommer 1967).

1.3.2: High Temperature Treatments

Brief treatments at high temperatures (like pasteurisation) have been used to reduce infections by pathogens. For example, Fallik and colleagues (1996) showed that treatments with hot air (38°C) for 4 days prior to storage reduced or eliminated decay caused by the green mould fungi *Penicillium expansum* on apples (Fallik *et al.* 1996).

However, as heat is transferred more freely in water than air (Fallik *et al.* 2000; Schirra *et al.* 2004), hot water dips with temperatures of 45-56°C are used in many packinghouses in South Africa to treat citrus and mango fruit (Janisiewicz and Korsten 2002).

These treatments have worked reasonably well, but precise temperature control is paramount, as the heat required to kill pathogens is usually very near to that which would also seriously injure the plant tissue of the fruit or vegetable. Besides this, there is the issue of cultivar effect and the need for uniform treatment of each individual product, so the practicalities of utilising this technology are somewhat unfavourable to its widespread implementation (Eckert and Sommer 1967; Wills *et al.* 1998).

1.3.3: Modified Atmosphere Treatments

Techniques that modify the composition of atmospheric gases in rooms or storage boxes where fruits and vegetables are kept have been utilised with varying degrees of success. Increasing CO_2 and decreasing O_2 concentrations, for instance, slows the respiration rate of the produce, thus extending its life (Kader 2005). However, although this will also slow the growth rate of most fungi, some are at least partially anaerobic and will thrive unless the O_2 levels are > 1%. Unfortunately, this will sometimes induce adverse effects in the commodity one is trying to protect, with problems such as off-flavours often being reported (Li and Kader 1989). Technological advancements have meant that this technique has become more successful in recent years and is now standard industry practice for many fruits, including apples, pears, strawberries, as well some root vegetables. The concept of modifying the storage atmosphere of a product has been extended to the use of perforated films known as modified atmosphere packaging (MAP), and many different types for use with different produce are now available (Sandhya 2010). The technology is, however, very cultivar dependent and has to be implemented and used with caution (Eckert and Sommer 1967).

1.3.4: Ultraviolet Light

Ultraviolet light C (UV-C) has been reported to induce natural plant defence responses by a number of authors (Chalutz *et al.* 1992; Stevens *et al.* 1996; Shama and Alderson 2005). These include reduced latent infection caused by *Botrytis cinerea* in tomatoes treated with UV-C (Charles *et al.* 2008; Charles *et al.* 2009), reduced decay by *Penicillium* species in grapefruit (Lers *et al.* 1998; D'Hallewin *et al.* 2000), and also increased resistance against

Colletotrichum gloeosporioides, *Monilinia fructicola* and *Penicillium digitatum* in apples, peaches, and tangerines, respectively (Stevens *et al.* 2005).

To summarize, storage and transport of fresh produce at the lowest temperature it can withstand, plus high RH conditions, are the primary methods of preservation of quality. Treatments such as irradiation (UV-C) and modified atmosphere packaging (MAP) have been implemented, but are cultivar dependent, quite costly and ultimately rely on tight temperature controls. Furthermore, as the problem of bacterial and fungal growth is only slowed at low temperatures, other means to eliminate the threat of infection are often required in combination.

1.4: Chemical Means to Prevent Deterioration

The practice of using chemical fungicides to control pathogen infections in fruits and vegetables has been around for over a hundred years, probably since the accidental discovery of Bordeaux mixture (made with copper (II) sulphate ($CuSO_4$) and slaked lime ($Ca(OH)_2$) in the late nineteenth century (Somerville 1986). As early as the 1930s, sodium salicylanilide was shown to be effective against *Penicillium, Phomopsis* and *Diplodia* fungal species and was used commercially as a fungicidal treatment of bananas (Meredith 1961; Eckert and Sommer 1967). Ammonia gas and volatile aliphatic amines were also found to reduce fungal decay by increasing the pH of the surface of the fruit to levels above which the pathogens could tolerate. However, the evolution of ammonia gas was too difficult to control in a commercial environment and only the amine, 2-aminobutane (sec-butylamine), was found to be sufficiently fungistatic at concentrations that were tolerated by the fruit (Eckert and Kolbezen 1962; Eckert and Sommer 1967). This compound has now also been withdrawn from commercial use, primarily due to fungal resistance (Ogawa *et al.* 1975).

Dipping the cut stems of bananas in Maneb (manganese ethylene bisdithiocarbamate) was introduced as a commercial practice in the 1960s to combat the rot caused by several pathogenic microorganisms (Eckert and Sommer 1967). This chemical is still in use. However, there have been some scientific studies in recent years that provide possible evidence for a link between exposure to Maneb and Parkinson's disease (Thiruchelvam *et al.* 2002; Costello *et al.* 2009). Chlorine-based solutions have been used in the post-harvest industry for many years, particularly hypochlorous acid and its salts (hypochlorites). These are primarily used to sanitize baths and hydrocoolers, where fruits and vegetables are washed in packing houses prior to storage and transport (Eckert and Sommer 1967). However, compounds containing 'positive' chlorine bonded to nitrogen (chloramines), such as nitrogen trichloride (NCl_3), have also been used with some success as fumigants to treat citrus fruits, melons and tomatoes (Ogawa *et al.* 1975; Wills *et al.* 1998).

Regarding citrus fruit and the control of *Penicillium* pathogens, treatment with sodium *o*-phenylphenate (SOPP) and thiabendazole (TBZ) had been used routinely for many years after their introduction in the 1960s. However, by the late 1970s a serious problem of

pathogen-resistance had already been discovered and imazalil (IMZ) was adopted by the citrus industry to complement or replace them (Siegel and Ragsdale 1978; Holmes and Eckert 1999; Kinay *et al.* 2007). Unfortunately, less than ten years after its introduction resistant strains of fungi were reported (Bus *et al.* 1991; Eckert *et al.* 1994) and, although still used in combination with the other chemicals, other fungicides had to be sought. Pyrimethanil (PYR) and fludioxonil (FLU) were eventually brought onto the market (Smilanick *et al.* 2006), with FLU being used to control the fungal pathogens of other fruits as well as those of citrus (Leroux 1996; Rosslenbroich and Stuebler 2000; Errampalli *et al.* 2005; Schirra *et al.* 2005).

The major problem with the use of these synthetic chemicals is that the manner in which they are utilised is highly conducive to the creation and propagation of resistant biotypes of fungal pathogens. Fungicide resistance is a result of chromosomal changes in the fungus, often brought about by a mutation in a single gene. The over-use of one type of fungicide means that selection pressure on the fungal population will allow mutations that provide resistance to it to build-up (Eckert 1977). Once resistance has propagated in a population it can spread very easily, often as a result of the post-harvest practices used to try to eliminate them. For example, the practice of re-packing after storage to remove any diseased fruit results in the aerial dispersal of fungicide-resistant spores to recently harvested fruit, thus compounding the problem (Holmes and Eckert 1995). Furthermore, many packing-houses process fruit all year around, which gives rise to continuous selection pressure on the pathogen population (Kinay *et al.* 2007; Nunes 2012). Chemical residues on produce is also a concern, as whether it be via dips, sprays or impregnation into commercial fruit waxes, the surface of the fruit is completely covered by the fungicides, and any residues will remain for the life of the product (Holmes and Eckert 1999). In the U.K, the practice of dipping or drenching apples was banned a long time ago (Pennell 2006). All these factors, plus public concern over environmental issues surrounding the extensive use of synthetic chemicals in food production, means that alternative measures have to be sought as a matter of great importance (Tripathi and Dubey 2004).

1.5: Biological Means to Prevent Deterioration

Biological control methods to prevent decay have been the focus of scientific research for over twenty years (Droby *et al.* 2009). The problem of pathogen-resistance to chemical fungicides originally just made those in the industry turn to different or more potent substances (Spotts and Cervantes 1986; Wilson and Wisniewski 1989). However, as their potency has been enhanced so has the realisation as to their negative effects on the environment and public health, such as pollution due to their high and acute residual toxicity and long degradation periods, as well as their carcinogenicity and other possible dangers to people who inadvertently consume them (United States National Research Council 1993). Discovering more natural methods of controlling decay has, therefore, been pushed high up the agenda of agricultural scientific research and has made it one of the major concerns in the subject of future food security (Kader 2005).

The postharvest environment is more favourable for the use of biological control methods as opposed to the pre-harvest one. Many factors can be controlled once produce has been harvested and is removed from the effects of natural microbial ecosystems, thus creating an almost artificial ecological island where the introduction of alternative biological processes can be effective (Wilson *et al.* 1991).

There are three main categories for biological control in agriculture: One is the utilization of microbial antagonistic organisms, such as bacteria, yeast or fungi, which either kill or compete with and reduce the ability of pathogenic fungi to rot the produce; another is the use of naturally-occurring compounds with antimicrobial action; whilst a third is the inducement of innate plant defence mechanisms to repel pathogenic attack (Baker 1987; Wilson and Wisniewski 1989; Wilson *et al.* 1991; Droby *et al.* 2009).

1.5.1: Antagonistic Microorganisms

Research has shown that unwashed fruit and vegetables often have more resistance to pathogens than washed produce, due to natural antagonistic organisms remaining on their surfaces that would not be there otherwise (Wilson and Wisniewski 1989). As long ago as the 1970s, people began looking into the possibility that naturally occurring antagonists to plant pathogens may have uses in controlling postharvest rots (Tronsmo and Dennis 1977). However, identifying these organisms has proved somewhat difficult and finding ways to promote and manage them even more so. Greater understanding of

host/pathogen/antagonist interactions is essential before methods can be found to either encourage those that already exist on particular fruits and vegetables or artificially introduce those that may have the desired effect (Andrews 1992).

A lot of research has been conducted into discovering antagonistic microorganisms that prevent or reduce pathogen infection in fruits and vegetables, resulting in some promising candidates. For instance, *Bacillus* species of bacteria have been shown to have antifungal activity against the pathogens of mango (Koomen and Jeffries 1993; de Jager *et al.* 2001), citrus fruits (Huang *et al.* 1992) and litchi fruit (Jiang *et al.* 2001); whilst *Trichoderma* species of fungi appear to be effective biocontrol agents for citrus (Borras and Aguilar 1990) and strawberries (Tronsmo and Dennis 1977).

Yeasts, however, seem to have the most potential and there are many examples that demonstrate this. These include *Candida* species on apples (Teixido *et al.* 1999b; Usall *et al.* 2000), citrus fruits (El-Ghaouth *et al.* 2001) and tomatoes (Saligkarias *et al.* 2002); *Debraryomyces hansenii* and *Pichia guilliermondii*, on citrus fruits (Droby *et al.* 1993); *Rhodotorula* species on oranges (Zheng *et al.* 2005; Zhang *et al.* 2008) and *Cryptococcus* species on sweet cherries (Chand-Goyal and Spotts 1996b), as well as both yeasts on apples and pears (Chand-Goyal and Spotts 1996a; Castoria *et al.* 1997; Chand-Goyal and Spotts 1997). Some of these yeast treatments have been developed into commercial products, such as Aspire™, containing *Candida oleophila*as as the active ingredient (Droby *et al.* 1998), BioCoat™, containing *Candida saitoana* (El Ghaouth *et al.* 2003) and Pro Yeast-ST which contains *Metschnikowia fructicola* (Karabulut *et al.* 2004).

Some public concern has been raised about the use of microbial antagonists, but most of those looked at so far were originally isolated from fruits and vegetables and, although introduced in greater numbers than would be found in a natural setting, do not present any threat to human health (Droby *et al.* 2009). The recent popularity of probiotic yogurts, for instance, has shown that an educated general public will embrace the introduction of ‘good’ microorganisms into their diet, so this is only a minor hurdle that researchers and developers need to overcome.

1.5.2: Natural Plant Products

Plants produce an array of secondary metabolites, many of which are known to have a function in either attracting or repelling other organisms, as well as medicinal properties (Tyler 1999; Louw *et al.* 2002). A great number of traditional treatments and modern commercial products have, therefore, been developed in which the main active ingredient has been derived from plants (Wilson *et al.* 1991) and much research into their possible role as antimicrobials has also been conducted (Droby *et al.* 2009).

Acetic acid, for instance, occurs naturally in many fruits and its vapour has been shown to be effective as a fumigant on sweet cherries (Sholberg 1998; Chu *et al.* 1999), whilst acetaldehyde vapours have been found to be active against *Botrytis cinerea* and *Rhizopus stolonifer* on strawberries (Avissar and Pesis 1991). *In vitro* trials with acetaldehyde, plus other plant volatiles such as benzaldehyde, cinnamaldehyde, ethanol and trans-2-hexenal ((E)-2-hexenal), have also indicated that these compounds have antifungal activity against *R. stolonifer, B. cinerea,* as well as *Penicillium digitatum* (Hamilton-Kemp *et al.* 1992; Hatanaka 1993; Utama *et al.* 2002).

Volatiles are small-molecular-weight organic compounds that have significant vapour pressure at ambient temperature. Plants emit a wide range of these compounds, many of which are important flavour and aroma factors in fruits, vegetables, herbs and spices (Wills *et al.* 1998). A number of them have been shown to inhibit the growth of microorganisms (Linton and Wright 1993) and they could therefore be utilised as a biological approach for controlling post-harvest decay of fruits and vegetables (Wilson and Wisniewski 1989). The essential oils of plants contain these volatile compounds, many of which are terpenes with the general structure of $C_{10}H_{16}$. When terpenes contain additional elements such as oxygen they are called terpenoids (Cowan 1999) and many of these have been reported to be active against pathogenic fungi (Harrigan *et al.* 1993; Kubo *et al.* 1993; Ayafor *et al.* 1994; Rana *et al.* 1997; Suresh *et al.* 1997).

Jasmonates (methyl jasmonates and jasmonic acid) are oxylipins and are known to regulate plant development as well as be involved in responses to environmental stresses (Sembdner and Parthier 1993; Creelman and Mullet 1995; Creelman and Mullet 1997). There is also evidence that they are involved in plant defence mechanisms (Gundlach *et al.* 1992; Nojiri *et al.* 1996) and some have been shown to activate genes which encode

antifungal proteins and/or phytoalexins (Andresen *et al.* 1992; Creelman *et al.* 1992; Xu *et al.* 1994).

Brassicas, such as cabbages, broccoli and turnips, contain glucosinolates and the hydrolysis of these produce compounds with possible antifungal activity (Tripathi and Dubey 2004). One of them, isothiocyanate (ITC) has been shown to be particularly effective, both as glucoraphenine ITC against *Monilinia laxa* (Mari *et al.* 1993; Mari *et al.* 1996) and as allyl-isothiocyanate (AITC) against *P. expansum* (Mari *et al.* 2002).

There has also been some research into plant essential oils, which, in their vapour phase, could provide possible fumigants against pathogen attack as they are thought to play a role in plant defence mechanisms (Mihaliak *et al.* 1991; Dixit *et al.* 1995; Meepagala *et al.* 2002). For example, thymol (from thyme) used as a fumigant for sweet cherries was found to be effective against the grey mould *B. cinerea* (Chu *et al.* 1999), whilst eugenol oil has been shown to inhibit the fungal pathogens of apples (Amiri *et al.* 2008). Basil oil, which is already commercially available, has also been reported to be as effective as 125 ppm chlorine in disinfecting lettuce leaves (Wan *et al.* 1998).

1.5.3: Inducement of Plant Defence Mechanisms

Besides the direct anti-fungal activity of natural compounds or microorganisms, the possibility that some could induce plant defence mechanisms inherent in harvested fruits and vegetables has also been examined. For example, volatile alkenals and alkanals were shown to elicit defence responses in artificially wounded cotton bolls (Zeringue 1992), and antagonistic yeasts such as *Aureobasidium pullulans* (Ippolito *et al.* 2000) and *Pichia membranefaciens* (Luo *et al.* 2012) have been reported to increase the activities of defence-related compounds peroxidase (POD), chitinase (CHI) and β-1,3-glucanase. It has been revealed that plant-derived oligogalacturonides and fungal-derived chitosan oligosaccharides can activate plant defence genes in tomato leaves (Doares *et al.* 1995), whilst the susceptibility of carrots to the pathogen *Sclerotinia sclerotiorum* was also reduced when they were treated with chitosan (Molloy *et al.* 2004). Altering plant-pathogen interactions is therefore another method by which fungal infections of harvested fruit and vegetables could be potentially reduced.

1.6: Research Objectives

Reducing postharvest losses of fruits and vegetables is a highly effective way of increasing food availability without increasing the area required for crop production (Tripathi and Dubey 2004). Over the last 60 years, this has mainly been achieved through the use of postharvest chemical fungicides, particularly in the 1960s and 1970s when commercial introduction and manufacturing of these products was at its zenith (Eckert and Ogawa 1985; Eckert and Ogawa 1988; Ragsdale and Sisler 1994; Janisiewicz and Korsten 2002). Since then, however, resistance of fungi to these treatments has meant that newer and more potent chemicals have been introduced; the consequence being that as their potency has increased so have their potential side-effects as well as the cost of producing them (Tyler 1999; Sorour and Larink 2001). Furthermore, these newer products have not succeeded in eliminating the problems of pathogen resistance and, added to this, there is now increasing scientific and public concern as to their safety to both human health and the natural environment (Spotts and Cervantes 1986; Vinas *et al.* 1991; United States National Research Council 1993; Gullino and Kuijpers 1994; Holmes and Eckert 1999). In Europe and elsewhere, the regulations on the use of these synthetic chemicals are being increasingly tightened up, and many have been either banned or have been directed to be discontinued (European Commission 2011).

The withdrawal of chemical fungicides from commercial use has meant that more money, time and effort has recently been put into finding biological methods that should prove safer for man and the environment (Wilson *et al.* 1991). However, although much research has demonstrated the potential for non-chemical postharvest control treatments, very few have been introduced commercially (Wilson and Wisniewski 1989). The market in postharvest technology dictates that methods must be extremely efficient for them to be viable (Tripathi and Dubey 2004; Droby *et al.* 2009) and, although biocontrol treatments have been shown to be this effective in the lab, it has not often been possible to replicate them in commercial trials (Wilson and Wisniewski 1989). A more consolidated approach, combining not only plant physiology and mycology, but also knowledge of production and storage systems used in the distribution chain, is therefore required in order to further the development of these products (Janisiewicz and Korsten 2002).

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Our research objective was to use a multi-disciplinary approach to discovering new techniques for the preservation of harvested fruits and vegetables. The possibility of utilising plant volatiles and polysaccharides were proposed right from the start, but firstly the development of an appropriate storage and experimental environment was required. To this aim, an understanding of the basic physiological processes involved in fresh product deterioration, particularly those related to water loss, was a prerequisite to the work. Once achieved, the aim was to test plant-derived compounds as potential anti-fungal treatments as well as investigate the possibility that they could inhibit physical deterioration of harvested produce through water loss. Although commercial trials were never going to be a component of the project, our company-partner ensured that an industry-related rationale was always present and any positive outcomes could potentially be proposed for further research and development. Included in this was the idea that any biocontrol treatments revealed could possibly be utilised by the industry as a means for reducing, if not replacing, the use of synthetic chemicals.

Chapter 2: Technique Development and Water Loss in Selected Fresh Produce

2.1: Introduction

All harvested fresh produce is still alive and carrying out the numerous essential biological processes associated with life's maintenance. The energy required to continue these processes comes from respiration. The final result of this is product deterioration, so a low respiration rate is desirable (Mitchell 1992). Different commodities have different post-harvest respiration rates, and their rates of deterioration are generally proportional to these. For example, citrus fruit have a low respiration rate of 5-10 mg CO_2 kg^{-1} hr^{-1} at 5°C (Saltveit 1991) and can be stored for a period of up to 12 weeks, whilst leaf lettuce has a high respiration rate of 20-40 mg CO_2 kg^{-1} hr^{-1} and cannot be stored for any longer than 2 weeks (Aguero *et al.* 2008). Temperature is one of the major determinants of respiration rates and each 10°C reduction will reduce produce respiratory activity by a factor of 2-4 (Robinson *et al.* 1975; Burton 1977; Li and Kader 1989; Kader 1992; Wills *et al.* 1998).

Moisture loss also contributes highly to produce deterioration, as lost water cannot be replaced after harvest. Water is lost due to a vapour pressure deficit (VPD) between the internal atmosphere (which is close to saturation) of the produce and that of the external surroundings. Water vapour moves from a high concentration to a lower one, the rate of migration being a function of the resistance of the product's 'skin' (the cuticle, epidermal cells, stomata, lenticels and trichomes) as well as the VPD (Wills and Scott 1972; Wills *et al.* 1998). For instance, produce with a thick and/or waxy cuticle will lose water much less readily than something which has a very thin cuticle (Ben-Yehoshua *et al.* 1985). Transpiration and evaporation rates are also influenced by commodity factors such as surface area-to-volume ratio. Temperature is again an important factor when it comes to water loss, as warm air can hold more water vapour than cold air, thus affecting the VPD (Mitchell 1992). Relative humidity (RH) is the amount of water vapour in the air as a percentage of the maximum amount it could hold at that particular temperature. Therefore, at a given temperature, the rate of water loss will increase with decreasing RH, whilst at a given RH, the water loss of a commodity will increase as the temperature increases (see Equations 1 and 2). Maintaining produce at low temperatures and high RH are essential in keeping respiration and water loss at a minimum and thereby extending

the post-harvest life-span (Harvey 1978; Mitchell 1992; Wills *et al.* 1998; Paull 1999; Lee and Kader 2000).

$$\Psi_w = \frac{RT}{V_w} \ln (RH)$$

Equation 1: Relationship between water potential (Ψ_w) and relative humidity (RH), where R is the gas constant, T is temperature (in degrees Kelvin) and V_w is the partial molar volume of water vapour. Taken from Nobel, 2009.

$$RH = \frac{C_{wv}}{C_{wv\ (sat.)}}$$

Equation 2: Relative humidity is the concentration of water vapour (Cwv) expressed as a fraction of the saturation water vapour concentration (Cwv(sat). With this equation you get a value for RH between 0 and 1. Taken from Taiz & Zeiger, 2010.

Many authors have studied the effects of temperature and/or RH on the weight/water loss of fruits and vegetables. The information collected was then used to advise the postharvest technology industry. For instance, Cohen and Schiffmannnadel (1978) found that lemons stored best for long periods at 14°C, although for short periods lower temperatures did not induce physiological disorders such as CI. Temperature also has an effect on the flavour of oranges, as reported by Marcilla and others (2006). Here, storage temperatures of >15°C produced fruit with lower acidity and orange-flavour than those stored <15°C (Marcilla *et al.* 2006). The quality of fresh strawberries, including their antioxidant capacity, in relation to storage temperature was investigated by Ayala-Zavala and colleagues (2004), who found that overall quality was maintained longer at 0°C than at 5 or 10°C, but that the fruit stored at lower temperatures had lower antioxidant capacity, total phenolics and anthocyanin concentrations (Ayala-Zavala *et al.* 2004). The browning seen in litchi fruit, which lowers their commercial value, was also affected by temperature and RH, and could be reduced with storage conditions of 13°C and 90% RH (Jiang and Fu 1999). More recently, the influence of temperature on postharvest decay of oranges was investigated by Rab and colleagues (2012), where they found that storage at

10°C induced lower weight loss and disease incidence in the fruit than storage at either 5 or 20°C (Rab *et al.* 2012).

Physiological blemishes of citrus fruit that reduce their economic value include conditions such as rind breakdown and peel pitting. The causes of these have been found to be down to temperature and RH conditions during storage. Agusti and colleagues (2001) found that rind breakdown in oranges is affected by sudden changes in RH, and this observation was confirmed by Lafuente and Sala (2002) and Alferez and others (2003). Differences in weight loss were not characteristic of the inducement of rind breakdown, but rather the reduction in water potential in the albedo (pith) of fruit transferred from low (45%) to high (95%) RH conditions (Agusti *et al.* 2001; Lafuente and Sala 2002; Alferez *et al.* 2003). Peel pitting in grapefruit was also attributed to changes in RH by Alferez and Burns (2004), who went on to recommend the preservation of a constant water status by maintaining reasonably high RH conditions both prior to and during the post-harvest stages. The incidence of another physiological condition seen in citrus fruit, Noxan, in which hypodermis cells in the flavedo (rind) collapse leaving dried and discoloured blemishes, was also found to be reduced by raising the RH around the fruit to 96–99% (Ben-Yehoshua *et al.* 2001). Meanwhile, Porat and colleagues (2004) reported that high RH conditions reduced the development of rind disorders in oranges, tangerines and grapefruit by >40% (Porat *et al.* 2004). Other research into the effect of RH on harvested citrus fruit includes that of McCornack (1975) and Henriod (2006), who used two very different techniques for controlling RH in the storage environment. McCornack used humidistat-controlled pneumatic water atomizing nozzles to induce high RH of 90% at two temperatures (4.5°C and 21°C) as well as ambient RH (65% or 75%, respectively). Weight loss in orange fruit under the four different conditions was compared and reported to be approximately halved in fruit held either at 4.5°C or 21°C with 90% RH rather than ambient humidity (McCornack 1975). Henriod, over thirty years later, used Moisture Control Technology (MCT) liners under commercial shipping conditions that maintained the RH of the transport environment at >98%. These conditions reduced moisture loss in the fruit by 83% (Henriod 2006).

These studies in citrus all show how important RH is in the storage environment of harvested produce, but the same has been shown in other fruit too. For example, lettuce stored under low RH conditions of 70-72% lost considerably more weight and displayed a

reduction in shelf-life of 75% over those stored under optimum RH of 95-98%, even though the temperature was maintained at the optimum 0-2°C in both cases (Aguero *et al.* 2011). The quality and antioxidant capacity of strawberries stored in 75, 85 or 95% RH at 0.5, 10 and 20°C for 4 days were studied by Shin and colleagues (2007), who found that overall fruit quality declined more rapidly at 20°C, especially at 95% RH. They concluded that whilst the best temperature for long-term storage was 0.5°C, quality could be maintained at 10°C for acceptable periods of time and may be associated with better nutritional quality. Peaches, lemons and cherries were all examined by Sharkey and Peggie (1984), and their results suggested that a very high RH environment of >95% did not benefit peaches, whilst it had a beneficial effect on lemons and cherries (Sharkey and Peggie 1984).

Much of this previous work was on a much larger scale to the studies reported here, and utilised controlled-environment storage facilities for the maintenance of given RH levels. Our investigations into weight/water loss of fresh produce stored under different temperature and RH conditions were, therefore, a series of experiments into finding the best assay for regulating and maintaining a wide variety of RH levels. The method was developed via weight-loss experiments with oranges and afterwards used to compare the differences between oranges, lettuce and strawberries. These were chosen as they were very different commodities and it was thought that comparing them would be an ideal means of testing our RH regulation method. Weight loss was used as a proxy for water loss, as the weight loss observed in harvested produce that is down to respiration is relatively low. At 20° C and 50% RH, weight change through water loss is approx. 97%, whilst as 85% RH it is around 92% (Shirazi and Cameron 1993).

2.2: Materials and methods

2.2.1 Fresh produce

Three types of fresh produce were utilized: oranges (*Citrus sinensis*), strawberries (*Fragaria* x *ananassa*) and lettuce (*Lactuca sativa*). Organic produce was used whenever possible to remove any effects that synthetic chemical pre- and postharvest treatments may have.

'Valencia' oranges were sourced (South African grown) from Tesco Stores (Bangor, U.K.) and (Spanish grown) Capespan (Maidstone, U.K.). The orange fruit were used either within 24 hours of purchase, or stored at 5°C ±2°C for a period of up to one week prior to use. British organic strawberries (*Fragaria* × *ananassa* cv. Sonata) were purchased from Waitrose Stores (Menai Bridge, U.K). Living lettuce (i.e. with the roots still attached) was obtained from Lidl Stores (Bangor, U.K). Strawberries and lettuce were both used immediately upon receipt.

2.2.2 Experimental conditions

2.2.2.1 Containers

Polypropylene (PP5) boxes (16 L) with lids (Wham Products, U.K.) were purchased online (http://www.plasticboxshop.co.uk) to provide enclosed environments for the incubation of the fruit.

All experimental assays were performed at either 22°C or 5°C (as noted) in temperature-controlled conditioning rooms.

2.2.2.2 Relative humidity

Relative humidity (RH) of the incubation environment was regulated by exposing the contents of the plastic storage boxes to the evaporation from a series of solutions (Figure 2.01) of known water potentials (Greenspan 1977; Evangelou 1998). Before this procedure was adopted, a series of technical difficulties of how to achieve this were encountered and overcome. The following sections describe these.

a. Dilute NaCl Solutions

Initially, solutions of 0, 10, 20 & 30% (w/v) sodium chloride (NaCl) were made with deionised water and poured into 500 mL beakers. These were put in each of the 16 L incubation containers. The van't Hoff equation (Jenkins 2008) was used to calculate the osmotic pressures of the solutions and the corresponding equilibrium RH value. These were also measured directly with an electronic hygrometer (Oregon Scientific, ETHG880), which also measured temperature (Table 2.01).

Treatment	Temperature	NaCl conc^{n}	RH (calculated)	RH (measured)	VPD (calculated from measured RH)
1	20°C ± 2°C	0%	100%	94%	0.1413 kPa
2	20°C ± 2°C	10%	94.4%	93%	0.1637 kPa
3	20°C ± 2°C	20%	89.2%	90%	0.2338 kPa
4	20°C ± 2°C	30%	84.2%	88%	0.2806 kPa
5	5°C ± 2°C	0%	100%	96%	0.0349 kPa
6	5°C ± 2°C	10%	94.4%	95%	0.0436 kPa
7	5°C ± 2°C	20%	89.2%	92%	0.0698 kPa
8	5°C ± 2°C	30%	84.2%	89%	0.0959 kPa

Table 2.01: Treatments and RH values obtained with dilute NaCl solutions.

This method of RH regulation was abandoned after 4 weeks of measurements of water/weight loss in the orange fruit. There were discrepancies between the calculated RH values and those measured with the electronic hygrometer (Table 2.01).

b. Saturated Salt Solutions

In order to improve the assay for regulating RH, saturated solutions of a range of salts (Table 2.02) were evaluated. These were poured into 500 mL beakers and each placed inside one the experimental boxes. Greenspan (1977) provides a list of equilibrium RH for these.

Salt	Solubility g/mL	Expected RH (literature) at 5°C	Expected RH (literature) at 20°C
Potassium sulfate	0.12	98%	98%
Potassium chloride	0.40	88%	85%
Ammonium sulfate	0.79	82%	81%
Sodium chloride	0.37	76%	75%
Magnesium chloride	0.56	34%	33%
Potassium acetate	2.81	23%	23%

Table 2.02: Saturated salt solutions – expected RH values. Solubility data are from Lide (1998).

The saturated salt solutions of potassium sulfate (K_2SO_4), potassium chloride (KCl), sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) were used at both 20°C and 5°C. The RH measurements obtained can be seen alongside those expected from the literature (Greenspan, 1977) in Table 2.03. There were discrepancies between all compared values, but the range of RH values was very much wider than those obtained with different concentrations of NaCl (Table 2.01).

Temperature	Salt	Formula	Expected RH	Measured RH	VPD (calculated from measured RH)
20°C ± 2°C	Potassium sulfate	K_2SO_4	98%	87%	0.3039 kPa
20°C ± 2°C	Potassium chloride	KCl	85%	81%	0.4442 kPa
20°C ± 2°C	Sodium chloride	NaCl	75%	79%	0.491 kPa
20°C ± 2°C	Magnesium chloride	$MgCl_2$	33%	45%	1.2859 kPa
5°C ± 2°C	Potassium sulfate	K_2SO_4	98%	89%	0.0959 kPa
5°C ± 2°C	Potassium chloride	KCl	88%	82%	0.157 kPa
5°C ± 2°C	Sodium chloride	NaCl	76%	77%	0.2006 kPa
5°C ± 2°C	Magnesium chloride	$MgCl_2$	34%	50%	0.436 kPa

Table 2.03: Saturated salt solutions in beakers – expected RH values (Greenspan, 1977) versus measured values.

In order to ensure equilibrium, ASTM International (2002) recommends a maximum of 25 cm^3 volume of humid air for every cm^2 of solution surface area, so the 16 L containers required a solution surface area of >640 cm^2. The surface area of the bases of the boxes was ~800 cm^2. Therefore, 800 mL of each solution poured directly into the boxes not only provided sufficient surface area to meet the recommendation but also gave a standard 1 cm depth. To avoid these solutions coming into direct contact with the fruit, small (24 X 15 X 6 cm) PP5 plastic baskets (Wham Products, UK) were obtained from Tesco Stores and placed upside down in the boxes to provide platforms on which the fruit could stand.

2.2.3: Air Circulation

The use of salt solutions suffered from the feature of stratification, whereby the air head space was not mixed and uniform throughout the experimental boxes, but probably comprised a steep humidity gradient. This was evident from the lack of agreement between the expected RH values (Greenspan, 1977) and those obtained by measurements with the hygrometer (Table 2.03). A method for circulating the air was obtained by the dismantling of four battery-powered toy vehicles (Inductive Truck, Shantou Gold Light Toy Factory, China) bought from a local cut-price store, and the subsequent removal of 8 X 1.5V miniature motors. One motor each was secured into the sides of the four boxes inside the 20°C storage room and connected to a 6 V power supply. 5 cm diameter propellers were made from cardboard and attached to the motor spindles, which then fanned the air inside each container. These were subsequently replaced with 3 V miniature motors and 100 mm diameter 3-blade flexible plastic propellers (Rapid Electronics, UK). These were installed into the containers for the remainder of the project and the measured RH levels when combined with the saturated salt solutions in beakers can be seen in Table 2.04.

Salt (in beakers)	Formula	Measured RH at 20°C (with fans)	VPD (calculated from measured RH)
Potassium sulfate	K_2SO_4	91%	0.2104 kPa
Potassium chloride	KCl	82%	0.4208 kPa
Sodium chloride	NaCl	78%	0.5144 kPa
Magnesium chloride	$MgCl_2$	42%	1.356 kPa

Table 2.04: Saturated salt solutions in beakers combined with the air circulation system – measured RH values.

Each of these treatments (diluted NaCl solutions, saturated salt solutions, and air circulation system) was tested on the same batch of oranges. While not strictly independent experiments, it is noted that the treatments are non-destructive. Moreover, the overall record of the behaviour of the fruit provided a clear indication, not only of the differences in water/weight loss due to changes in RH and temperature, but also the influence of the experimental system (Table 2.05).

Days	Treatment	RH Range at 20°C	RH Range at 5°C
1-28	Dilute NaCl solutions (in beakers)	88-94%	89-96%
29-45	Saturated Salt Solutions (in beakers)	45-87%	50-89%
36-45	Saturated Salt Solutions (in beakers) + Air Circulation System	42-91%	N/A

Table 2.05: Treatments used on one batch of oranges, showing the development of the techniques to regulate RH.

The final assay technique, using saturated salt solutions covering the bases of the incubation containers in conjunction with the air circulation system (Figure 2.01), was utilised with a fresh batch of fruit. A few different saturated salt solutions were used as these gave the most wide-ranging RH values of the compounds in the literature (Greenspan, 1977) that were regarded as safe. The saturated salt solutions of K_2SO_4, $(NH_4)_2SO_4$, $MgCl_2$ and $KC_2H_3O_2$ poured directly into the boxes and used in conjunction with the air circulation system, gave RH values as shown in Table 2.06. These measurements were the most in accordance with those from the literature (Table 2.02).

Salt	Measured RH at 20°C (with fans)	VPD (calculated from measured RH)
Potassium sulphate (K_2SO_4)	97%	0.0701 kPa
Ammonium sulphate $(NH_4)_2SO_4$)	82%	0.4208 kPa
Magnesium chloride ($MgCl_2$)	36%	1.4963 kPa
Potassium acetate ($KC_2H_3O_2$)	28%	1.6834 kPa

Table 2.06: Saturated salt solutions covering base of boxes – measured RH values. These four solutions were chosen for use, as they provided wide-ranging and reliable RH levels for experimentation.

2.2.4: Data Collection and Analysis

Oranges were labelled with different coloured adhesive tape (Write-On™ Label Tape) and the weight of each individual fruit was taken (Mettler Toledo, AB204). Three fruit were placed in each box – eight boxes in all. Half the boxes were incubated at 20°C ± 2°C, whilst the other half was kept at 5°C ± 2°C. Relative humidity was controlled, as noted in Table 2.05.

Each individual orange was taken out and re-weighed every 24 hrs (except on the weekends) throughout the experiment. To minimize any effects of change of temperature/humidity on the fruit whilst removed from their boxes to be weighed, only one orange from each box was taken at a time. Each individual fruit was, therefore, only out of its incubation conditions for a maximum of 8 mins. Water/weight loss in strawberries and lettuce were studied on just one occasion in this way, just to get a comparison of the effects of temperature and RH on different types of fresh produce.

Data was collated and weight loss rates were calculated with Microsoft Office Excel 2010 (Microsoft Corporation, Washington, U.S.A). One-way analyses of variance (ANOVA) were then performed both on weekly data and overall, and tests for normal distributions of residuals and homogeneity of variances conducted. *Post hoc* least significant difference (LSD, $p = 0.05$) between means for multiple comparisons was used to find any significant differences between the weight loss rates. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses.

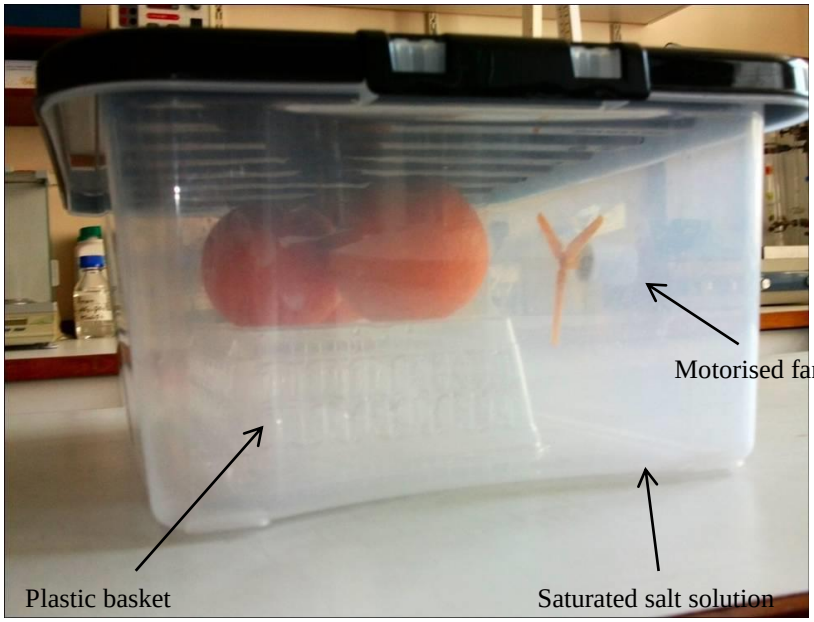

Figure 2.01: Container used to analyse water loss from fresh produce (oranges illustrated). Relative humidity (RH) was set using saturated salt solutions, motorised fans were utilised for air circulation and plastic baskets provided platform for the fruit.

2.3: Results

2.3.1: Weight Loss (RH regulated by dilute NaCl solutions) Days 1-28

All fruit showed a weight loss over the 28 days of the experiment. Table 2.07 displays the cumulative weekly mean percentage weight losses for the fruit under the eight different treatments (temperature & RH conditions) listed in Table 2.01. In the first week (days 1-7) the average weight loss of fruit maintained at 20°C was 1.6%, which decreased to 0.99% during the fourth week (days 22-28). In contrast, the average weight loss of the fruit stored at 5°C was 0.73% during the first week and had only decreased to 0.64% in week four.

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Days	**20°C 94% RH**	**20°C 93% RH**	**20°C 90% RH**	**20°C 88% RH**	**5°C 96% RH**	**5°C 95% RH**	**5°C 92% RH**	**5°C 89% RH**
1-7	1.73%[a]	1.50%[a]	1.53%[a]	1.62%[a]	0.58%[b]	0.76%[b]	0.75%[b]	0.84%[b]
8-14		2.56%[a]	2.68%[a]	2.84%[a]	1.05%[b]	1.22%[b]	1.34%[b]	1.56%[b]
15-21		3.39%[a]	3.63%[a]	3.84%[a]	1.44%[b]	1.59%[b]	1.79%[b]	2.09%[b]
22-28		4.28%[a]	4.64%[a]	4.90%[a]	1.98%[b]	2.07%[bc]	2.50%[bc]	2.92%[c]

Table 2.07: Cumulative weekly mean percentage weight loss of oranges incubated at 5 & 20°C with RH maintained by NaCl solutions (unstirred). Within each row, values followed by the same letter are not significant at p=0.05 according to LSD test.

The results of the LSD test viewed in conjunction with the cumulative mean percentage weight losses show that all the oranges stored at 5°C lost significantly ($p \leq 0.05$) less weight than all those stored at 20°C. The factor of RH had very little effect on the weight loss of the fruit maintained at the same temperatures. The only pair-wise comparison within either of the two temperature regimes that resulted in a significant difference in weight/water loss between treatments was that between 5 & 8 (5°C and RH of 96% & 89%, respectively) by the end of the 28 days. This 7% difference in RH was the greatest between any of the treatments included in the statistical analyses.

2.3.2 Weight Loss of Infected Fruit (RH regulated by dilute NaCl solutions)

Figure 2.02 shows how the three individual oranges incubated under Treatment 1 (20°C, 94% RH) lost weight during the course of the first 9 days of the experiment. Initially, the values were very similar, but after the weekend break (days 4 & 5) it can be seen that the weight/water loss of 'yellow 1' had increased compared to the others. On day 7 a visible fungal infection was observed on this individual orange in the form of white fungal hyphae, and by day 9 it had lost ~ 2X the weight/water content as the other fruit under the same temperature/RH conditions. By this time, the symptoms of fungal infection were very apparent thus making any comparisons between it and the other fruit unfeasible. It was therefore removed from the experiment, and Treatment 1 was abandoned.

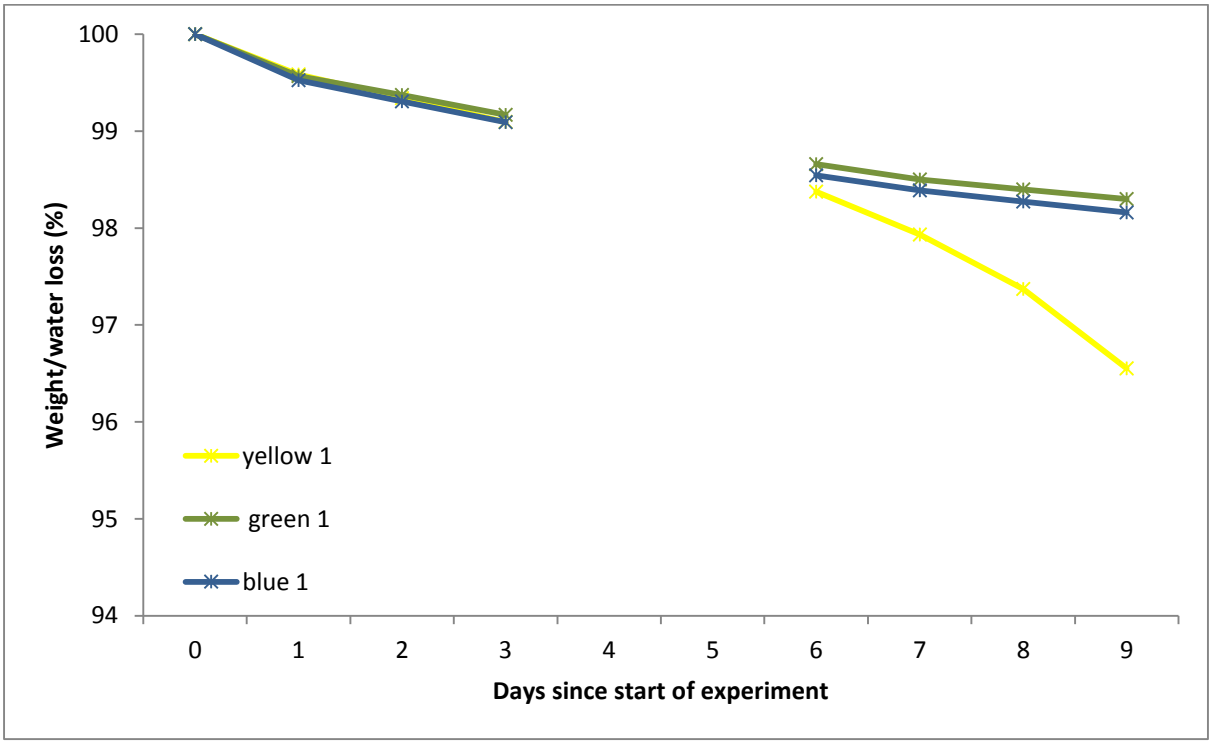

Figure 2.02: Weight loss of oranges incubated for 9 days at 20°C and 94% RH (unstirred). 'Yellow 1' data show the effect of fungal infection on one individual fruit (see text above).

2.3.3: Weight Loss (RH regulated by saturated salt solutions in beakers) Days 29-42

Weight loss continued in the fruit after the dilute NaCl solutions were exchanged for saturated salt solutions (days 29-42). Table 2.08 displays their cumulative weekly mean percentage weight losses and the results of the *post hoc* LSD multiple comparisons test. Similarly to the experiment with the NaCl solutions (Section 3.3.1), the lower temperature resulted in smaller percentage weight/water losses in the fruit which were significantly different ($p \leq 0.05$) from those values at 20°C. However, the statistics also illustrate significant differences between fruit stored under the same temperature but different RH conditions. At 20°C, Treatment 4 (45% RH) gave significantly different weight loss results than Treatments 2 & 3 (81 & 79% RH, respectively), whilst Treatments 5 & 8 (both at 5°C, but at 89 & 50% RH, respectively) were also significantly different at the end of the first week (days 29-35). This was not quite the case after the second week (days 36-42), where the P value for that pair-wise comparison was $p = 0.052$.

The air circulation system was installed in the treatments at 20°C due to our only having four motors when it was first utilised (days 36-42). Its addition resulted in higher cumulative mean percentage weekly weight losses in the fruit than those from the previous week – for example, the oranges under Treatment 4 lost 2.57% during the first week (45% RH) and a further 3.89% by the end of the second week (42% RH). It is apparent from these results that magnesium chloride was more effective in creating a very low RH environment when the air is stirred and there is little or no stratification effects in the enclosed atmospheres of the containers. It is also clear that both temperature and RH are important factors in determining weight loss in fresh produce, particularly in extremes.

Treatment / Days	2 20°C 81% RH	3 20°C 79% RH	4 20°C 45% RH	5 5°C 89% RH	6 5°C 82% RH	7 5°C 77% RH	8 5°C 50% RH
29-35	1.76%^{a}	1.58%^{a}	2.57%^{b}	0.38%^{c}	0.71%^{cd}	0.77%^{cd}	1.07%^{d}
36-42	4.68%^{a}	4.66%^{a}	6.46%^{b}	1.07%^{c}	1.67%^{c}	1.77%^{c}	2.29%^{c}

Table 2.08: Cumulative weekly mean percentage weight/water loss of oranges at 5 & 20°C with RH maintained by the saturated salt solutions. Within each row, values followed by the same letter are not significant at p=0.05 according to the LSD test.

2.3.4 Weight Loss (RH regulated by saturated salt solutions covering bases of boxes)

In a second experiment with a fresh batch of fruit (20°C, RH regulated by saturated salt solutions covering bases of boxes, plus air circulation system) similar rates of water loss were observed over the course of three days. Figure 2.03 shows how the fruit lost weight over that time course (trendlines and equations for each are also displayed) and demonstrates how it was RH dependent when extremes were compared. Potassium sulphate (97% RH) induced significantly ($p \leq 0.05$) lower mean percentage weight losses in the fruit than any other treatment. Ammonium sulphate (82% RH) also produced significantly different results. Magnesium chloride and potassium acetate (36 & 28% RH, respectively) were not significantly different to each other, but were and resulted in considerably greater water/weight loss rates when compared to either of the other two RH levels.

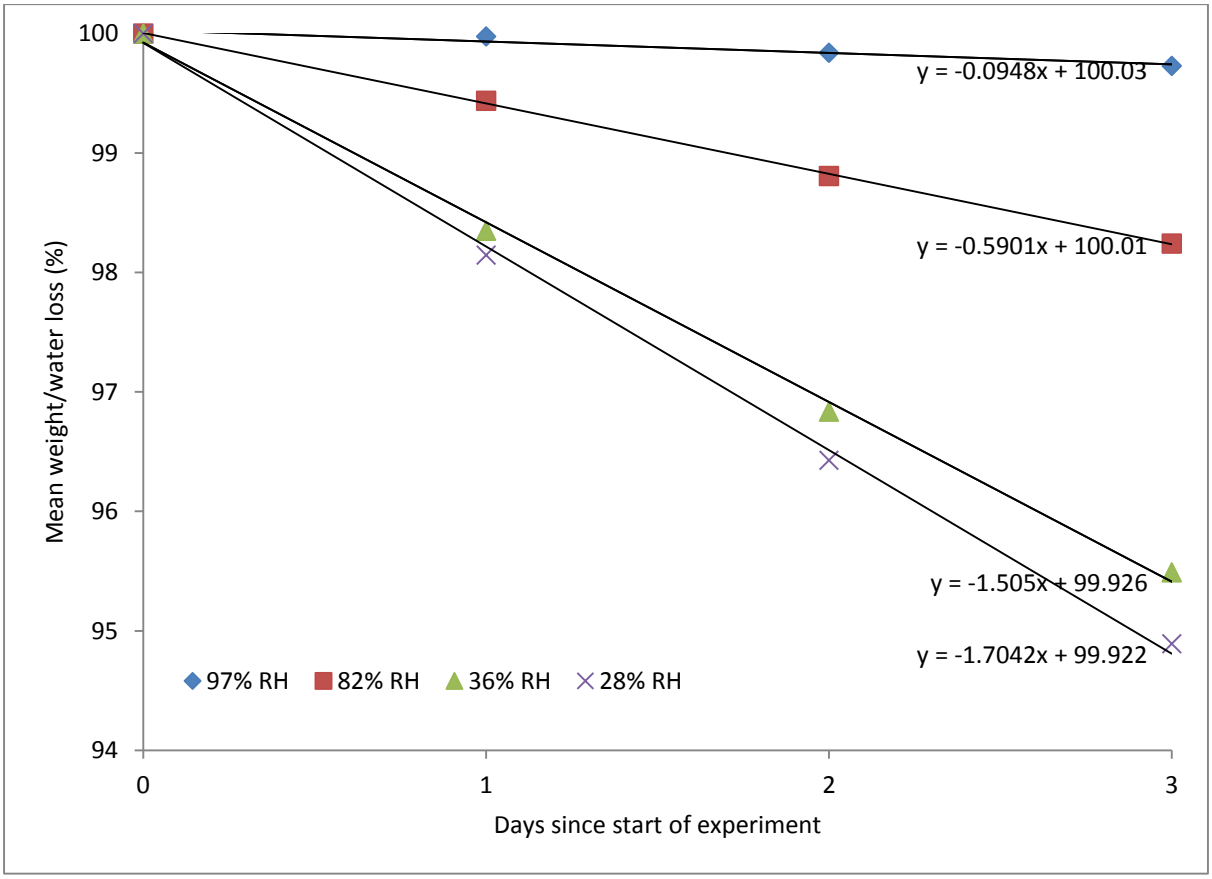

Figure 2.03: Rates of weight/water loss of oranges incubated at 20°C and various RH conditions maintained by saturated salt solutions poured directly into the experimental boxes (stirred).

This experiment demonstrated how a combination of different saturated salt solutions and a method for circulating the air in an enclosed space can be used to regulate RH in an experimental system. It also confirmed the capability of RH to influence the water loss (via loss of weight as a proxy) of fresh produce.

2.3.5 Weight Loss (RH regulated via different methods) Days 1-45

The sequential effect of using different salts to regulate RH along with the air circulation system can be demonstrated by pooling all the data obtained from the weight loss of the one batch of oranges used whilst developing the assay techniques (Figure 2.04). The weight/water loss gradients for each treatment were all relatively steep for the first few days of the experiment, but they then settled down and remained constant throughout the period in which the dilute NaCl solutions were being utilised. With the introduction of the saturated salts, the rates of weight/water loss altered somewhat. For example, Treatment 4 more than doubled its average rate of loss from 160 mg/100g/day to 370 mg/100g/day when the RH dropped from 88 to 45%. The rates then changed still further in the fruit incubated at 20°C when the fans were installed. For example, the rate for Treatment 4 became 560mg/100g/day.

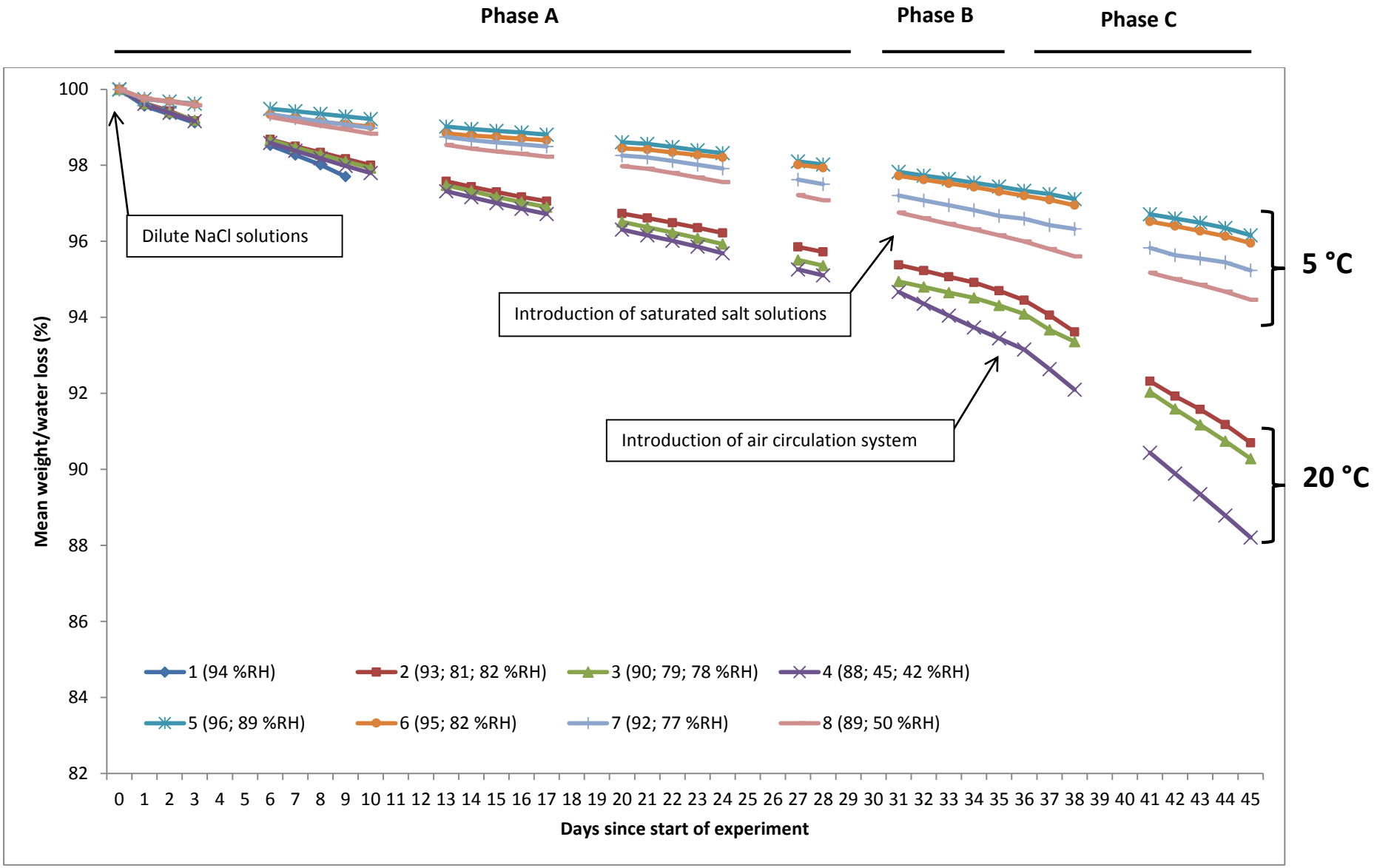

Figure 2.04: Weight/water loss of oranges over the course of 45 days, illustrating how the introduction of saturated salt solutions (for RH regulation) for all treatments (after 28 days) and the introduction of an air circulation system for treatments at 20°C (after 35 days) affected the rates of weight/water loss in the fruit.

2.3.6: Weight Loss in Strawberries and Lettuce Compared to Oranges

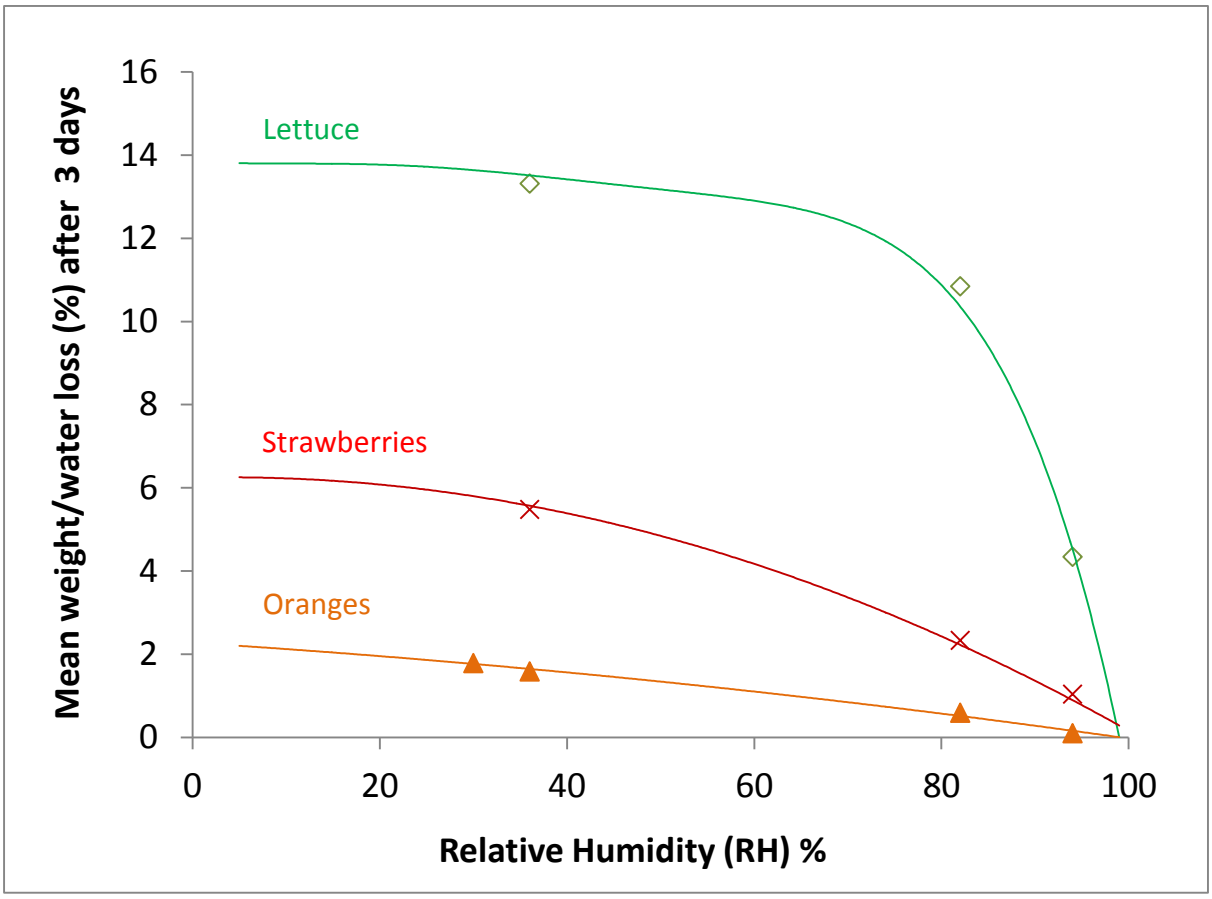

Figure 2.05: Weight/water losses of three different fresh commodities after three days of incubation at the same temperature (20°C) and various RH levels. Trendlines fitted using 2nd order polynomial regression.

Using the full experimental procedure (saturated salts covering bases of boxes, plus air circulation) oranges, strawberries and lettuce were incubated under different RH conditions (97, 82 & 36% RH, plus 28% RH for oranges only) for three days. All these showed weight loss that was dependent on RH (Figure 2.05), and the relative rates of loss varied considerable between the commodities. At 97% RH, lettuce had lost significantly more weight/water than either strawberries or oranges ($p = 0.006$ and $p = 0.029$, respectively according to the Games-Howell *post hoc* test). In contrast, oranges and strawberries were not statistically different to each other at 97% RH, but at 82% RH they were ($p = 0.005$). In summary, lettuce, with its large surface area to volume ratio, loses water much more readily than either strawberry or orange when measured at the same RH. In contrast, oranges are much more effective at retaining their water content.

2.4: Discussion

We have developed a facility that allows us to maintain harvested produce under controlled temperature and humidity conditions (Figure 2.06). A small fan was found to be essential to ensure uniform conditions. By analysing weight loss in harvested produce maintained under different environmental conditions, we have shown how water loss rates are dependent on both temperature and relative humidity (RH).

Figure 2.06: Containers set up in a (dark) controlled temperature room. Different relative humidities (RH) were maintained in each container using saturated salt solutions. Air circulation in each container was achieved by using motorised fans linked to a power supply on each set of shelves.

Temperature is probably the most important factor, as shown by the significant differences observed in the weight/water loss of oranges even when RH levels were relatively similar (Figure. 2.04, Phase A). Only when the dilute NaCl solutions were replaced with various saturated salt solutions and the fans were introduced to circulate the air in the containers, did the influence of RH have an effect on the fruit (Figure 2.04,

Phases B and C). It is, however, evident that maintaining fresh produce at low temperatures and high RH levels is the most effective way of reducing weight loss (Figure 2.04, Treatment 5), as higher temperatures and low RH increases water deficits.

As can be seen in Figure 2.05, crop species vary considerably in their water loss. This is due to evaporation rates being dependent both upon the area of exposed surface and the nature of that surface. In loose leafy vegetables like lettuce the exposed surface is large, the thin nature of the curticle makes them highly susceptible to loss and the structure of the produce is such that dried-out leaves cannot protect the others. Rates of evaporation from any given commodity are directly proportional to the VPD of its immediate surroundings, whilst the amount of evaporation which can occur is limited by the amount of water the air can hold. Therefore, a reduction in water loss can be achieved either by reducing the VPD and limiting the rate, or by reducing the volume of air in the immediate environment (e.g. with the use of packaging) and limiting the amount (Robinson *et al.* 1975). However, the "biology" of different commodities will ultimately dictate the design of any post-harvest procedures, as it is qualitatively different between produce. For example, while a treatment to reduce water loss might have the same mechanism in both orange and lettuce, it is clearly more of a practical issue for lettuce. Quantifying these practical demands will allow systematic use of biological information to optimise the requisite effects on both the biotic and abiotic stress responses of the produce.

It must not be forgotten that temperature and RH also affect the microorganisms that may be living on any particular fruit or vegetable. As a general rule, bacteria are more prevalent on vegetables and fungi are more frequent on fruit, due to their having a lower surface pH, but the growth of both are heavily influenced by environmental conditions (De Roever 1998; Teixido *et al.* 1999a; Plaza *et al.* 2004). Throughout a crop's life cycle, there may be large numbers of different microbial species associated with it, most of which will be natural epiphytes rather than pathogens (de Jager *et al.* 2001). However, once they have been harvested, the microbial population will start to change further and more opportunistic pathogenic species will invade (Lacey 1989; Korsten 2006). Growth rate is highly dependent on temperature, as is spore germination in fungi, and the optimum temperature for each will vary widely amongst different species. However, even though the growth of most fungi is completely halted at temperatures approaching 0°C

they are still alive and will continue growth and spore production immediately upon transference into more ambient conditions (Eckert and Sommer 1967). Moreover, whilst a high RH is necessary for produce susceptible to serious evaporative loss, it also favours microbial rotting, so it is essential to couple high RH with the lowest acceptable temperature. It may also be possible to store commodities more resistant to evaporation at slightly lower RH conditions in order to reduce the risks of microbial infection.

The postharvest industry often uses other methods to preserve fruit and vegetables for longer periods of time. These include the practice of waxing citrus fruit in order to reduce water loss and maintain freshness. However, research into the movement of water, ethylene and respiratory gases in harvested oranges and grapefruits has revealed that this may not be the best option (Ben-Yehoshua *et al.* 1985). This paper showed that waxing was only restrictive to ethylene, O_2, and CO_2, due to closed stomata only allowing the diffusion of these gases, whilst water moved through the cuticle and was not affected by the wax to any great degree. This sort of information is important to the industry, as reports of off-flavours and aromas have been associated with the waxing of citrus fruit. Other techniques, such as wrapping fruit individually in plastic film, could reduce transpiration whilst allowing gas exchange, and thereby reduce the risk of negative effects on the produce (Ben-Yehoshua *et al.* 1985).

In conclusion, we have developed our facility to assay different treatment methods to extend the post-harvest life-span of fresh produce. These large chambers allow the testing of a wide range of application devices (dipping, spraying, volatile, slow release etc) under different environmental conditions. We have also shown that measuring weight loss as a proxy for water loss can be a valuable way of ascertaining a commodity's freshness. The evaluation of weight loss is used by the industry and there are values of maximum permissible loss at which a commodity becomes unsaleable for different fresh produce (Robinson *et al.* 1975). Water losses not only represents direct losses of weight in commodities which are sold by weight, but also leads to wilting and produce degradation. Consequently, an actual weight loss of 5% may effectively be a loss of 100% because the produce is no longer fit for sale. It is therefore vital that fresh fruit and vegetables both retain moisture and resist pathogen infection, and the remainder of our work contained in this thesis is dedicated to that aim.

Chapter 3: The Effect of Volatile Compounds on Water Loss and *Penicillium* Infection in Citrus Fruits

3.1: Introduction

Green and blue moulds in citrus fruit are the result of infection by *Penicillium digitatum* Sacc. and *P. italicum* Wehmer, respectively (Hume 1957; Eckert 1977; Eckert and Ogawa 1985). These two citrus fruit pathogens cause economic losses in pre- and postharvest phases, the severity of which varies depending upon production area, cultivar, climatic conditions, extent of damage and postharvest handling practices. Blue mould is the major postharvest disease on cold stored citrus fruits, but green mould may cause 60–80% of decay under ambient conditions (Plaza *et al.* 2003; Plaza *et al.* 2004; Lahlali *et al.* 2006b).

These pathogens are necrotrophs that enter the fruit through rind wounds caused by insect pests during the pre-harvest stage and by handling during the postharvest and transportation stages (Kavanagh and Wood 1967; Brown 1989; Droby *et al.* 2008). Nutrients and the emission of volatile compounds contained within the plant stimulate the germination of latent spores which proceed to grow and colonise the fruit tissue (Stange *et al.* 2002; Droby *et al.* 2008). The result of infection by *P. digitatum* is that the fruit first softens and then begins to shrink before turning into a hollow mummified shell, whereas *P. italicum* reduces the fruit to a slimy mass (Spotts and Cervantes 1986; Sanderson and Spotts 1995). Both species are quickly disseminated, although the development of disease partially depends on storage conditions and the physiological status of the fruit, as well as any inherent defence mechanisms (Rodov *et al.* 1995; Vilanova *et al.* 2012). Storing the produce at low temperatures will delay fungal growth as both of these *Penicillium* species grow best at temperatures of 22-25°C and high RH of >95% (Hocking and Pitt 1979; Lacey 1989; Plaza *et al.* 2003; Plaza *et al.* 2004; Lahlali *et al.* 2006b). The recommended conditions for the transport and storage of citrus will slow pathogen development, but as soon as the fruit are removed from storage at the point of sale the fungi will resume their growth and spore production. Therefore, there is still a requirement for additional disease suppression to prevent the produce from rapidly deteriorating (Korsten 2006; Droby *et al.* 2009; Nunes 2012).

Currently, both *Penicillium* species are controlled with pre- and postharvest applications of fungicides such as sodium ortho-phenylphenate (SOPP), thiabendazole (TBZ) and imazalil (IMZ) (Kinay *et al.* 2007). These fungicides are effective in controlling pre-existing, established or new infections, but the pathogens have also been reported to develop resistance with long term doses of IMZ, TBZ, or SOPP in the citrus pack houses of California (Holmes and Eckert 1999). For example, the intense use of TBZ has compromised its efficacy due to the development of resistant pathogen populations (Baraldi *et al.* 2003). Additionally, environmental hazards, health risks and consumer concerns regarding the use of fungicides have meant that the development of alternative control measures is now sought (Eckert *et al.* 1994).

Partially due to growing environmental awareness and public concerns about food safety, the demand for organically-produced produce has increased rapidly lately (Schifferstein and Ophuis 1998; Williams and Hammitt 2001). Organic produce is perceived to be more wholesome, as the use of agricultural chemicals (including pesticides, herbicides, fungicides and fertilizers) is associated with unknown effects on health (Wilkins and Hillers 1994; Miles and Frewer 2001; Bourn and Prescott 2002; Saba and Messina 2003). Organic farming revolves around the concept of sustainability (Dahlberg 1991; Hansen 1996; Howe 1997; Rao and Rogers 2006) and involves the use of self-regulating ecological and biological processes (Francis and King 1988; Carter 1989; Macrae *et al.* 1990; Stolze and Lampkin 2009). However, all organic farmers, including those that grow citrus crops, encounter higher production costs than conventional farmers, as well as lower yields and greater losses, which mean that prices for the consumer have to be higher too (Miles and Frewer 2001; Moll and Igual 2006). Consequently, in order for the organic market to expand further there is a requirement for environmentally friendly means of aiding the production and marketing of foods so that costs and wastage are both lowered (Porat *et al.* 2000; Miles and Frewer 2001; Moll and Igual 2006).

In recent years there has been increasing interest in natural plant extracts as alternatives to synthetic chemicals to control food pathogens (Rota *et al.* 2008). Reviews have been published discussing several plant compounds with antifungal activity, including volatiles consisting of naturally occurring aldehydes, acetate esters, alcohols, and terpenes (Cowan 1999; Lanciotti *et al.* 2004; Tripathi and Dubey 2004). Of these, the aldehydes have been shown to be the most potent, being not only inhibitory but also lethal to fungal conidia

and mycelia (Archbold *et al.* 1999; Utama *et al.* 2002). For example, volatile C_5-C_9 aldehydes in mature citrus fruit inhibited *P. digitatum* (Davis and Smoot 1972); benzaldehyde protected peaches from Rhizopus rot (Wilson and Wisniewski 1989) and completely inhibited isolated cultures of *Alternaria alternata, Botrytis cinerea,* and *Colletotrichum gloeosporioides* (Vaughn *et al.* 1993), whilst acetaldehyde vapour decreased decay in raspberries and strawberries (Prasad and Stadelbacher 1974; Avissar *et al.* 1990).

Volatile compounds are also produced in plant tissue in response to mechanical or biological injury (Hatanaka 1993). These are usually six- and nine-carbon aldehydes or alcohols formed via the lipoxygenase (LOX) hydroperoxide lyase enzymatic pathway (Hildebrand 1989). This pathway is activated immediately following wounding and a number of its volatile products have been shown to be metabolized by strawberry fruit, resulting in little or no residue (Hamilton-Kemp *et al.* 1996). The natural origin of these compounds, their metabolism by fruit, and their volatile nature may enhance consumer acceptance if their efficacy against fungal pathogens was shown to be sufficient for commercial application

The aim of the work described on this chapter was to explore the use of volatile compounds as potential aids in enhancing the storage- and shelf-life of citrus fruit. Through subjecting the produce to maximum stress by inoculating them with *Penicillium* and maintaining them under ambient conditions for fungal growth, we hoped to reduce both water loss (using weight loss as a proxy) and disease incidence via exposure to natural citrus volatiles. Any compounds that had positive effects on the fruit (i.e. by reducing weight/water loss) or negative effects on the pathogens (i.e. by reducing fungal growth) could therefore be propositioned as natural postharvest protectants.

3.2: Materials and Methods

3.2.1: Fruit Material

Organic Spanish 'Valencia' oranges (*Citrus sinensis*) were obtained from Capespan (Maidstone, U.K) and from Dimensions Health Store (Bangor, U.K).

Non-organic Spanish 'Nadorcott', Peruvian 'Owari' and Uruguayan 'Nules' mandarins (*Citrus reticulata*) were purchased from Tesco Stores (Bangor, U.K).

3.2.2: Pathogens

3.2.2.1: Initial isolation and identification.

P. digitatum and *P. italicum* were first isolated from naturally infected orange fruit during initial weight-loss experiments (See Section 2.3.2). There were two distinct colonies of different fungal spores on the fruit – blue and green. These were cultured on potato dextrose agar (PDA). The media was prepared by adding PDA powder (Oxoid Microbiology Products, U.K) to deionised water (39 g/l), mixing thoroughly and then autoclaving at 121°C for 20 mins. Once sterilized, the agar was cooled to 50°C in a water bath prior to being poured into sterile 90 mm plastic Petri dishes to approximately 3 mm depth. A mycological inoculation loop was sterilised by holding it in the flame of a portable gas cartridge stove (Campingaz) until it glowed red and then allowed to cool for approximately 10 seconds. The loop was then used to scrape off a sample of spores from the outer (actively growing) edge of one of the colonies and transfer it to the centre of a PDA plate (Section 4.2.1.1). The procedure was repeated twice so that three agar plates were inoculated with spores from the same fungal colony. The loop was then sterilised again and the procedure repeated with three spore samples from the second colony. All six inoculated plates were then sealed with Parafilm and incubated at 22°C for five days in a darkened conditioning room.

To identify the fungal species, slides were made of the five-day-old cultures. Using a sterile inoculation loop, spore samples were transferred to glass microscopy slides and a drop of distilled water added before a cover slip was placed on top. The spores were then examined under an optical microscope at x 400 magnification and compared to those in an identification guide (Ramirez 1982). These observations together with information on

the major pathogens of citrus fruit (Hume 1957; Eckert 1977; Eckert and Ogawa 1985) led to the conclusion that the fungi were *Penicillium digitatum* Sacc (green spores) and *P. italicum* Wehmer (blue spores).

3.2.2.2: Maintenance of cultures

To maintain the viability of the fungal cultures, they were subcultured onto fresh media at least once a month. The method for sub-culturing was identical to that used to isolate the samples, except that spores were taken from the outer edge of a plated sample and transferred to another Petri dish containing freshly prepared PDA media (Section 3.2.2.1). Loss of viability was noted when subcultured *Penicillium* failed to grow on fresh media. Replacement samples were obtained when a delivery of organic oranges from Capespan (Kent, U.K.) arrived with a number of already-infected fruits.

3.2.2.3: Fungal suspensions

For experimental use, mixed fungal spore suspensions were required. These were prepared by pouring approximately 20 mL distilled water into a minimum-five-day-old plated culture. The contents were then swirled gently to favour detachment of conidia. A 20 mL disposable sterile luer slip syringe (Plastipak, U.S.A) was then used to transfer the conidia suspension obtained to a sterile glass vial and the procedure was repeated with the other *Penicillium* species. A further volume of distilled water was then added to the vial to give a final volume of 50 mL and the contents were mixed for approximately 10 s using a vortex mixer.

3.2.3: Chemicals

The majority of volatile compounds used throughout the research are found naturally in citrus fruit (Njoroge *et al.* 2005; Barboni *et al.* 2009; Carmen Gonzalez-Mas *et al.* 2011; Espina *et al.* 2011) and were purchased from Sigma (U.K). These were (-)-linalool, (-)-terpinen-4-ol, (-)-α-pinene, (-)-β-pinene, (+)-linalool, (+)-α-pinene, (R)-carvone, (S)-carvone, 1-octen-3-ol, citral, decanal, (E)-2-hexenal, geranyl acetate, limonene, methyl jasmonate, methyl salicylate, myrcene, neryl acetate, nonanal, octanal, valencene, β-caryophyllene and γ-terpinene (Table 3.01). All chemicals were ≥ 81% pure. The essential oils of oranges and lemons were also used – these were obtained from Paramount Citrus (California, U.S.A). Citrus volatiles were chosen after our commercial

partner alerted us to a paper indicating that these compounds might be toxic to fruit flies (Papachristos *et al.* 2009) and it was believed that they may have potential antifungal properties also.

Volatile Compound	Chemical Formula	Chemical Class	Molar Mass (g)
1-octen-3-ol	$C_8H_{16}O$	Alcohol	128.2
(-)-linalool (licareol)	$C_{10}H_{18}O$	Alcohol	154.2
(+)-linalool (coriandrol)	$C_{10}H_{18}O$	Alcohol	154.2
(-)-terpinen-4-ol	$C_{10}H_{18}O$	Alcohol	154.2
(E)-2-hexenal	$C_6H_{10}O$	Aldehyde	98.1
octanal	$C_8H_{16}O$	Aldehyde	128.2
nonanal	$C_9H_{18}O$	Aldehyde	142.2
decanal	$C_{10}H_{20}O$	Aldehyde	156.3
citral	$C_{10}H_{16}O$	Aldehyde	152.2
methyl salicylate	$C_8H_8O_3$	Ester	152.1
geranyl acetate	$C_{12}H_{20}O_2$	Ester	196.3
neryl acetate	$C_{12}H_{20}O_2$	Ester	196.3
methyl jasmonate	$C_{13}H_{20}O_3$	Ester	224.3
(R)-carvone	$C_{10}H_{14}O$	Ketone	150.2
(S)-carvone	$C_{10}H_{14}O$	Ketone	150.2
myrcene	$C_{10}H_{16}$	Terpene	136.2
γ-terpinene	$C_{10}H_{16}$	Terpene	136.2
(-)-α-pinene	$C_{10}H_{16}$	Terpene	136.2
(-)-α-pinene	$C_{10}H_{16}$	Terpene	136.2
(-)-β-pinene	$C_{10}H_{16}$	Terpene	136.2
limonene	$C_{10}H_{16}$	Terpene	136.2
valencene	$C_{15}H_{24}$	Sesquiterpene	204.4
β-caryophyllene	$C_{15}H_{24}$	Sesquiterpene	204.4

Table 3.01: List of volatile chemical compounds used throughout research. Chemical formulas, the chemical class to which each belong plus molar masses are included (information from http://www.chemspider.com). These details were used to designate each compound a colour which was then used in all infographics.

3.2.4: Incubation Conditions

See Section 2.2.3 - 22°C and 97% RH delivered by saturated salt solution of potassium sulfate (K_2SO_4).

3.2.5: Inoculation of Fruit

Fungal spore suspensions of *P. digitatum* and *P. italicum* were prepared and mixed together in a glass vial (see Section 3.2.2.3). The concentrations of spores in the suspensions were adjusted to 10^6 - 10^7 spores/mL using a hemocytometer. Each individual fruit was mounted on a small, previously weighed and labeled, 90 mm plastic Petri dish lid and weighed.

Initially, fruit punctured 8 times were infected by full immersion for 60 s in a tank containing a spore suspension. This resulted, in every case, in an uncontrollably heavy infection and it was impractical to work with such material. Henceforth, the fruit were punctured at their stem-ends with a sterile clinical lancet and a 10 µL drop of the mixed spore suspension was pipetted onto each wound site. The fruit were then placed in the boxes (5 in each).

3.2.6: Volatile Delivery Method

In initial experiments, a sheet of 7.2cm wide Lay-flat tubing (www.polybags.co.uk) was cut into 7.2 cm lengths and one of the open ends of each sealed with a heat sealer to make small packets. In a fume cupboard, 2 ml of each test solution was pipetted into one of the packets and immediately sealed with the heat sealer. Each sachet was then attached with double-sided tape to the inside lid of one of the boxes containing the inoculated orange fruit. This method was tried several times, but no results were obtained and the use of these sachets was discontinued. Hereafter, 200 µL of each volatile compound was pipetted into individual 35 mm plastic Petri dishes. Each one was then placed into one of the boxes containing the inoculated fruit.

3.2.7: Data Collection and Analysis

The inoculated fruit were placed in incubation boxes (See Section 2.2: 22°C, 97% RH) into each of which was then placed an open Petri dish containing a volatile compound.

Another container, to which inoculated fruit were added without any volatile compound, was also included in the assay as a control. After seven days the fruits were removed from the boxes and re-weighed to ascertain how much weight they had lost. The extent of fungal pathogen growth was taken by using a steel caliper (Tool Box, U.K) to measure the diameter (mm) of the area on each fruit where spores/hyphae could be visibly detected. These were recorded as lesion size (mm) and mean values calculated for each different treatment. The means obtained from the control (i.e. those without the addition of a volatile compound) were then used as the base-line value and the means from the treatments expressed as percentages of the control. The assay was performed five times with various volatile compounds, as we only had a maximum of twenty boxes set up at any one time and there were 25 volatiles in total. The total minimum number of replicate fruit for each treatment and control was fifteen.

The mean values for weight loss (g) and lesion diameter (mm) were calculated for each treatment in each experiment. The means obtained from the control treatments were then used as the reference value of zero and the means from the volatile treatments converted into percentage differences compared to the control.

Mean values and standard errors were calculated with Microsoft Excel 2010 (Microsoft Corporation, Washington, U.S.A) and then subjected to univariate analysis of variance (ANOVA) using the General Linear Model (GLM). The equality of variances were checked using the Levene's Test and, if equal, the least significant difference (LSD, $p = 0.05$) between means for multiple comparisons was calculated. If equal variances could not be assumed, then the non-parametric Games-Howell test was used for multiple comparisons between treatments. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses.

3.3: Results

3.3.1 Organic Oranges and Volatiles

Overall results varied, but the addition of a small number of the volatile compounds consistently reduced lesion size (Figure 3.01). These were neryl acetate and methyl salicylate (esters), γ-terpinene, (-)-α-pinene and limonene (terpenes) and the oils of both lemon and orange. Actual percentage decreases were variable for all, but γ-terpinene, limonene and lemon oil all produced a reduction of over 90% on one occasion. Statistically, however, no treatment was significantly different to the controls at $p = 0.05$.

The only compound to consistently reduce weight/water loss in the organic oranges was orange oil (Figure 3.02). Two volatiles, γ-terpinene and geranyl acetate, both consistently increased this variable. However, no treatment was significantly different to the controls at $p = 0.05$ according to the Games-Howell *post hoc* test.

3.3.2: Non-organic oranges and volatiles

A number of volatile compounds consistently reduced the growth of the *Penicillium* pathogens (Figure 3.03). These were nonanal (aldehyde), neryl acetate (ester), (S)-carvone (ketone), myrcene and (-)-β-pinene (terpenes), valencene and β-caryophyllene (sesquiterpenes), plus orange oil. The most effective of these was (-)-β-pinene which reduced lesion size > 40% on every occasion. However, this was not significantly different to the controls according to the Games-Howell non-parametric *post hoc* test.

There were also a number of volatile treatments which consistently reduced the weight/water loss of the citrus fruit (Figure 3.04). These were 1-octen-3-ol (alcohol), (E)-2-hexenal and octanal (aldehydes), neryl acetate (ester), myrcene (terpene), valencene (sesquiterpene), plus orange oil. The most effective of these were (E)-2-hexenal, neryl acetate and orange oil, all of which produced a mean reduction in weight/water loss of ~40%. However, none of the tested compounds were significantly different to the controls according to the Games-Howell post hoc test.

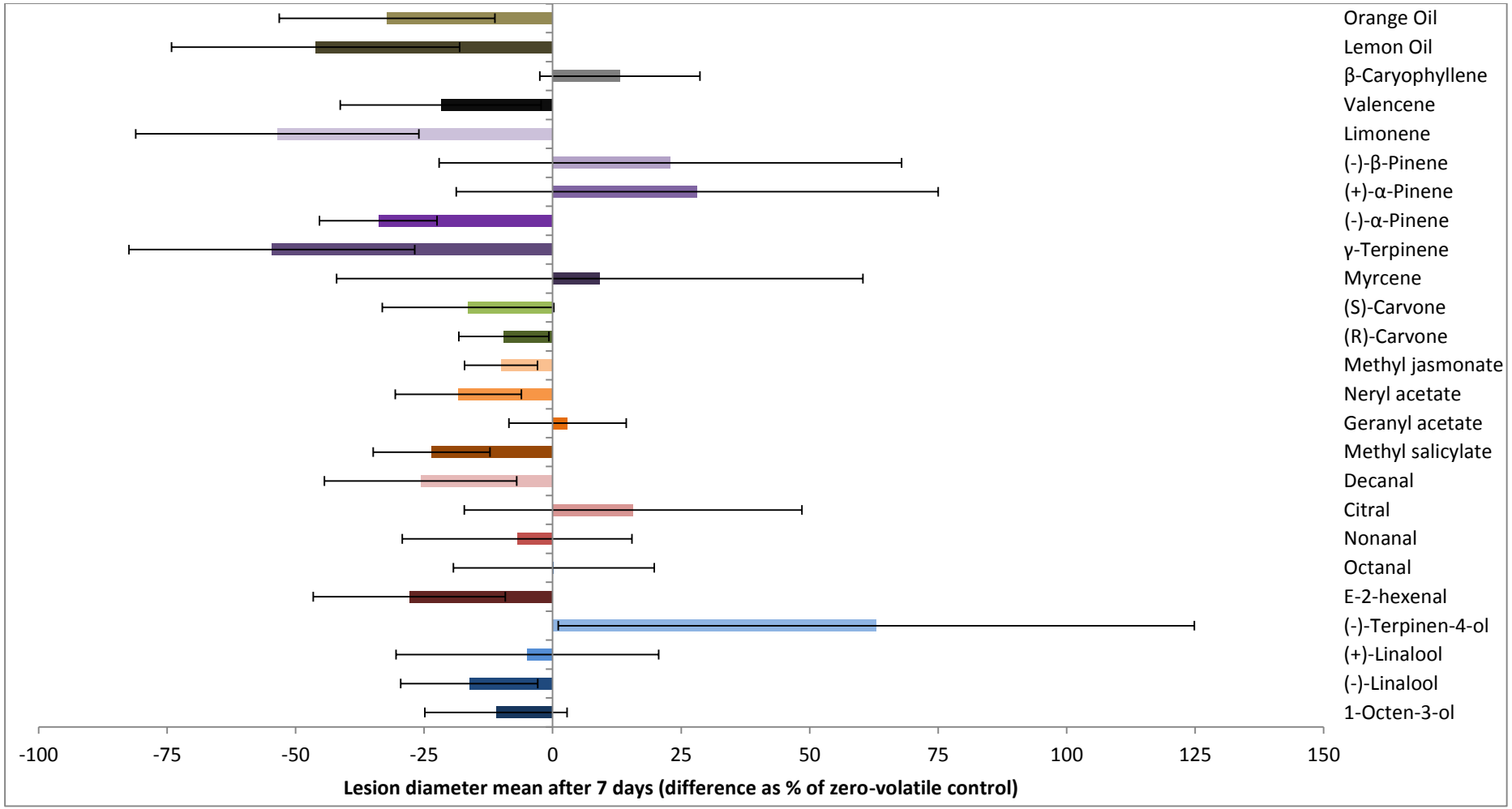

Figure 3.01. Effects of volatile compounds on the infection of *P. digitatum* and *P.italicum* on organic oranges compared to control fruit. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones; Purples = terpenes; Greyscale = sesquiterpenes. The two brown shades represent the two essential oils. Different shades of the same colour (darkest → lightest) denote individual compounds within each chemical group from lowest molar mass → highest. Error bars = ± 1 S.E.

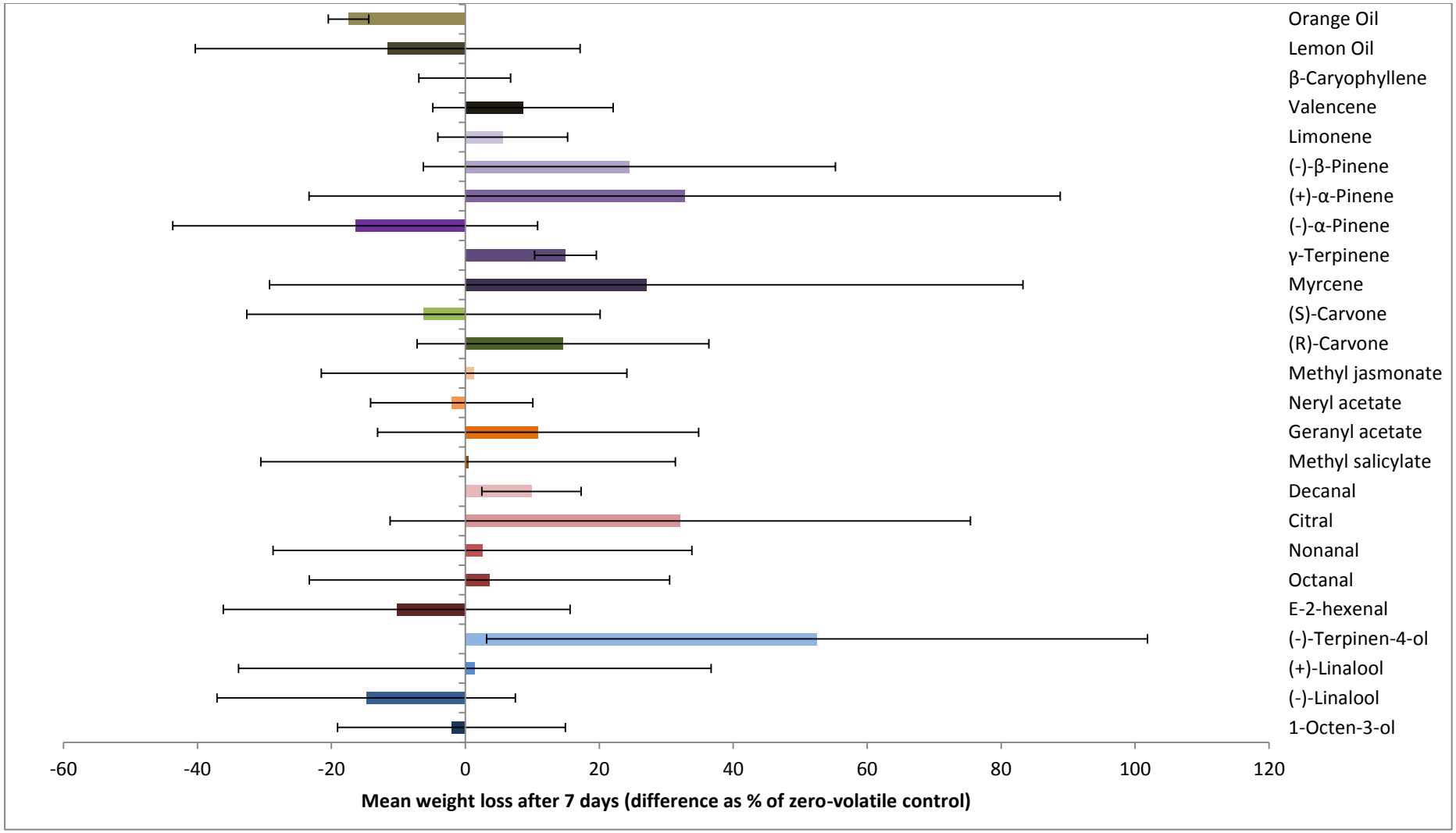

Figure 3.02. Effects of volatile compounds on the weight/water loss of organic oranges inoculated with *P. digitatum* and *P. italicum* compared to untreated fruit. Other details as in legend for Figure 3.01.

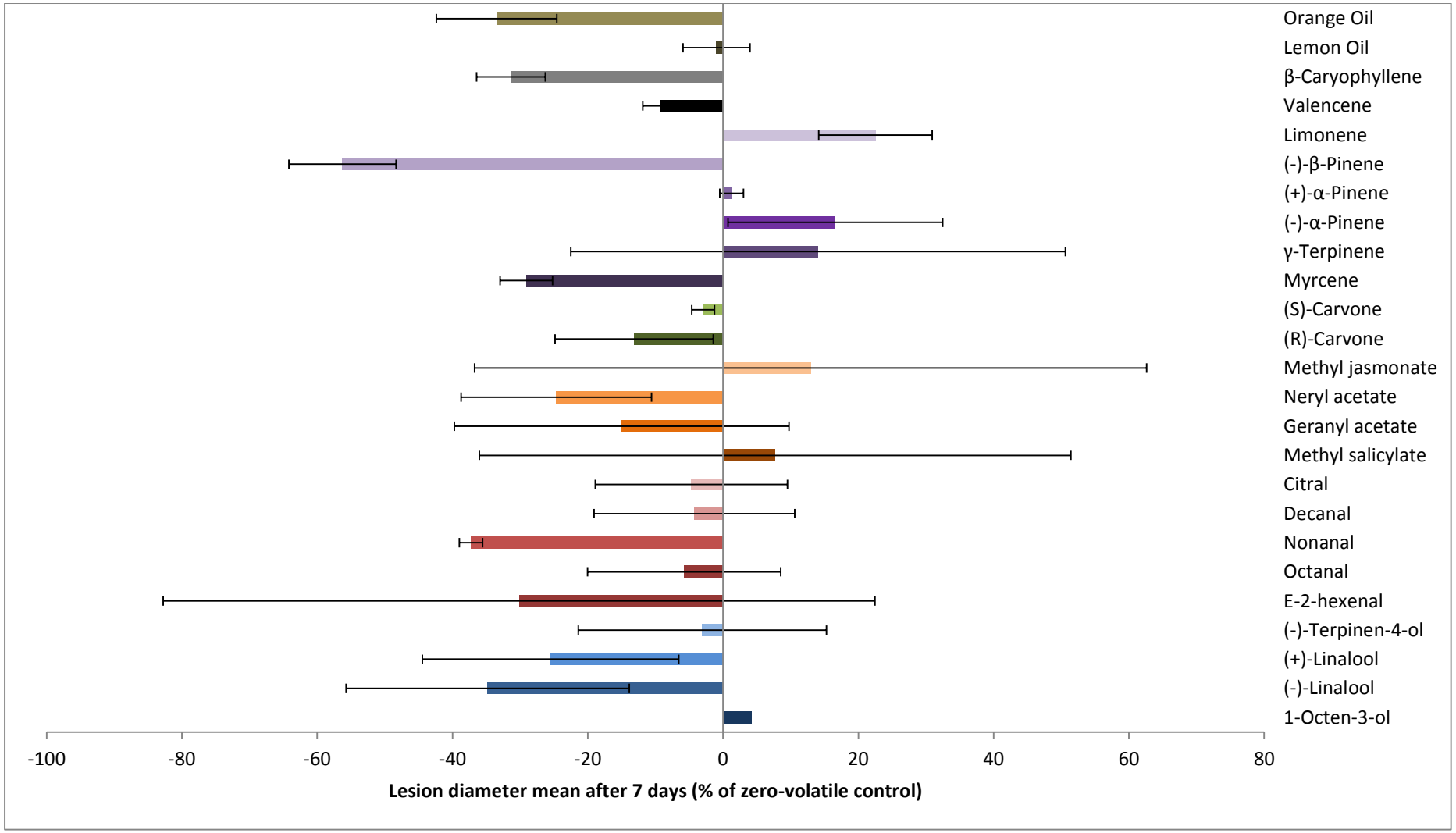

Figure 3.03: Effects of volatile compounds on the infection of *P. digitatum* and *P.italicum* on non-organic mandarin oranges compared to untreated fruit. Other details as in legend for Figure 3.01.

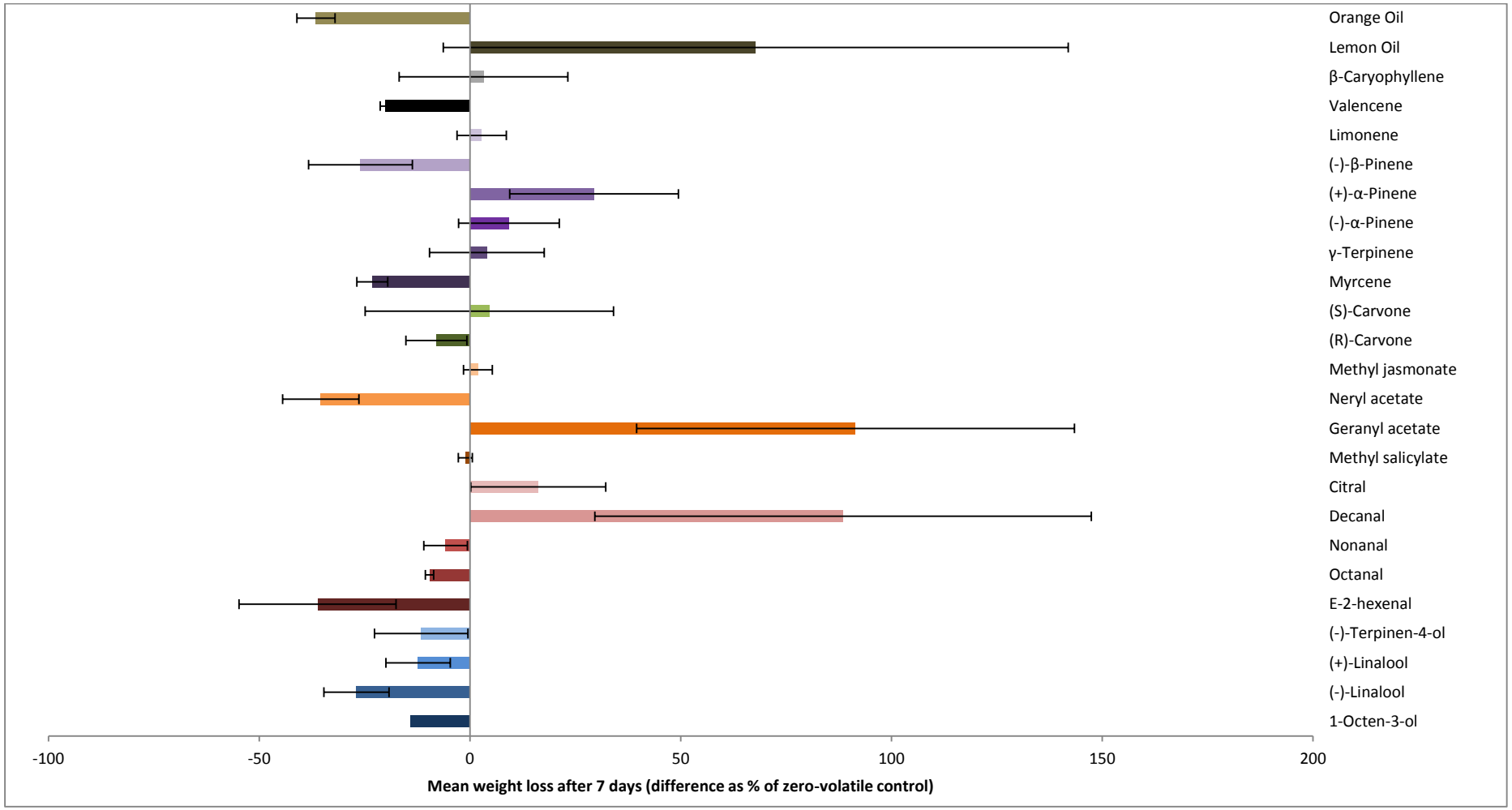

Figure 3.04: Effects of volatile compounds on the weight/water loss of non-organic mandarin oranges inoculated with *P. digitatum* and *P. italicum* compared to untreated fruit. Other details as in legend for Figure 3.01.

3.4: Discussion

The *in vivo* experiments with volatile compounds and citrus fruit produced some mixed results. There were only two volatiles that consistently reduced *Penicillium* growth in both organic and non-organic fruit – orange oil and neryl acetate (Figures 3.01 & 3.03). These two treatments also reduced weight loss in every experiment with non-organic citrus fruit (Figure 3.04), but only orange oil was completely reliable when it came to organic oranges (Figure 3.02). There were a number of other compounds that had positive effects on fruit health, but these were not the same on the two differently-produced citrus crops.

Orange oil has been shown to be effective against both gram-negative and gram-positive bacteria as well as food spoilage fungi such as *Aspergillus niger* (Prabuseenivasan *et al.* 2006; Sharma and Tripathi 2006; Sharma and Tripathi 2008; Viuda-Martos *et al.* 2008; Lin *et al.* 2010). To our knowledge it has not previously been seen to be active against *Penicillium* species of fungi, but in our studies it was highly effective against these pathogens and also reduced water/weight losses. However, its individual components (see below) were not quite so consistently successful, suggesting that it is a combination of one or more of the orange oil constituents acting in an interactive or additive manner that have the greatest effect. If this is indeed the case, it would be more beneficial in postharvest protection because pathogen resistance would not be easily established. As lemon oil was not as effective as that of orange in our investigations, this would imply that the most active components are perhaps found in greater concentrations in the latter.

The volatile constituents of citrus essential oils are a mixture of monoterpene (such as limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives, including aldehydes (such as citral), ketones, acids, alcohols (such as linalool) and esters (Flamini *et al.* 2007). Carson and Riley (1995) demonstrated that oxygenated monoterpenes had higher antimicrobial activity than did hydrocarbons, whilst Aggarwal and colleagues (2002) found that the oxygenated monoterpenes carvone and limonene oxide were active against a number of pathogenic fungi and bacteria. In our studies, it was an ester, neryl acetate, which was extremely effective at reducing infection as well as water/weight loss in oranges. The only instance in which it was not consistent was in reducing water loss in organic fruit. Neryl acetate is related to citral, which is a mixture of two terpenoids - the double bond isomers geranial and neral (Akhila 1985; Claon and Akoh 1993). Neral can

be reduced to form nerol, which can then be condensed with acetic acid to form neryl acetate (Grieco 1972; Iijima *et al.* 2006; Lozano *et al.* 2012). However, the positive results obtained with neryl acetate seem unlikely to be linked to its structural similarity with citral, as the other closely related compound in our studies, geranyl acetate, only moderately reduced *Penicillium* infection in non-organic citrus and actually induced greater weight/water loss in the fruit than the controls, as did citral itself. It is also improbable that the fact that neryl acetate is an ester is a contributing factor to its efficacy. Geranyl acetate has already been mentioned, but the other two esters, particularly methyl jasmonate, only performed marginally better than that. Methyl salicylate was consistently effective against *Penicillium* in organic oranges, but encouraged weight/water loss in the fruit in every experiment with non-organic fruit. The results with neryl acetate also contradict those reported by Rodov and colleagues (1995), where they found that young mature-green lemon fruit, with higher levels of citral, had significantly lower levels of *Penicillium* decay compared to the older yellow fruit that contained increased levels of neryl acetate (Rodov *et al.* 1995).

Looking at some of the other individual volatile constituents that we examined that were either active against the *Penicillium* pathogens or reduced water/weight loss in the fruit, there were a number that were successful in either organic or non-organic produce, but not both. For example, methyl salicylate, γ-terpinene, (-)-α-pinene, limonene and lemon oil were all consistently effective against the pathogens in organic oranges (Figure 3.01), whilst nonanal, (S)-carvone, myrcene, (-)-β-pinene, valencene and β-caryophyllene displayed 100% efficacy in non-organic fruit (Figure 3.03). These observations are both interesting and perplexing, as are the results on water/weight loss (Figure 3.04) where an almost completely different set of compounds (namely, 1-octen-3-ol, (E)-2-hexenal, octanal, myrcene and valencene) delivered both positive and reliable effects. Besides the possibility of it being due to random variability, one explanation could be the fact that the non-organic crops have been treated with both pre- and post-harvest chemical fungicides and the volatiles are interacting with these in either a positive of negative way. Besides neryl acetate and orange oil, the two sesquiterpenes, valencene and β-caryophyllene both achieved excellent results with non-organic fruit, but were less effectual on organic oranges. Neither of these compounds has been previously reported to be active against *Penicillium in vivo* to our knowledge, but as most postharvest research of this nature is

performed on produce not treated with chemical fungicides this is non-too surprising. There are very few reports in the literature concerning the antimicrobial nature of these compounds, although β-caryophyllene has been found to be active against the bacterial pathogen *Pseudomonas syringae* both *in vitro* and *in vivo* (Huang *et al.* 2012). A significant positive correlation between the antifungal effects of different citrus essential oils and the amount of sesquiterpenes each contained has also been described (Caccioni *et al.* 1998), thus supporting our findings with valencene and β-caryophyllene.

There have been a limited number of investigations (particularly *in vivo* studies) looking into the effects of volatile compounds in the vapour phase (i.e. as fumigants). One such study was conducted by Neri and colleagues (2007), who tested (E)-2-hexenal, citral and the monoterpenoid phenol carvacrol against *Monilinia laxa*, a fungal pathogen of stone fruit. Citral and carvacrol exhibited only moderate effects on the pathogen, the highest efficacy being at a concentration of 50 µl/l, whereas (E)-2-hexenal provided 46.2-80.3% inhibition at 20 µl/l in peaches, nectarines and plums and at 10 µl/l in apricots (Neri *et al.* 2007). In our work, this aldehyde displayed high efficacy *in vivo* by reducing water/weight loss in non-organic fruit (Figure 3.04), but was not so successful in reducing pathogen infection. Citral was also one of the poorest performers in our trials, with its effects on lesion size being only reasonably good on non-organic citrus, whilst it induced greater water/weight losses in both types of fruit. Perhaps the differences observed between our results and that of Neri and colleagues is due to volatile concentration discrepancies, as ours were very much lower (0.0125 µL/mL), although there could be other explanations. For example, methyl jasmonate is purported as an elicitor of plant natural defences (Sticher *et al.* 1997; Droby *et al.* 1999; Yao and Tian 2005b; Yao and Tian 2005a), but it was only moderately effective in reducing lesion size in organic oranges (Figure 3.01) and water/weight loss in non-organic fruit (Figure 3.04) in our studies. These inconsistent observations could perhaps be due to the timing of application - i.e. post infection, rather than pre-harvest/infection – as previous successful observations with this compound have been when it was utilised prior to infection with *Penicillium* pathogens (Yao and Tian 2005b; Iqbal *et al.* 2012). We also used volatile compounds with two different enantiomers, and these often showed differing effects. For example, the (-) enantiomer of α-pinene was consistently effective against *Penicillium* in the organic oranges and also moderately good at reducing water/weight loss in these produce

(Figures 3.01 and 3.02). It performed negatively, however, in non-organic fruit (Figures 3.03 and 3.04), whilst the (+) enantiomer produced poor results in both types of produce. With the different enantiomers of carvone, our work showed that the (S) enantiomer had consistent antifungal activity against the pathogens in the non-organic fruit (Figure 3.03), whereas (R)-carvone was only moderately effective. This is in contrast to the results of a series of investigations in which (R)-carvone successfully reduced the incidence of *P. digitatum* infection and reduced water/weight loss in the fruit when incorporated into citrus coatings (du Plooy *et al.* 2009). Perhaps, in this case, our conflicting results are down to the method of delivery (i.e. fumigation, rather than incorporation into a dip or coating for the fruit) or they may be due to incompatible concentrations. These factors require further investigation.

In conclusion, our results with volatile compounds and *Penicillium* infection in citrus fruit produced more questions than answers, as the results were inconsistent on the whole, and somewhat contradictory to those of previous authors. Method and timing of application, concentrations and the possibility of better results perhaps being obtained by the combination of two or more compounds have all been flagged up as potential avenues of further investigation. Neryl acetate has nevertheless been shown to have positive effects on both the reduction of fungal growth and water/weight loss in the fruit, so any subsequent work should definitely include this compound alongside those which have had positive results elsewhere – i.e. the aldehydes, ketones and methyl salicylate. Combining two or more of these in various concentrations would be a good approach to start with, as would be the incorporation of these in some sort of natural substance to act as an antifungal and freshness-maintaining coating for harvested fruit.

Chapter 4: Effect of Citrus Volatiles on the Growth of *Penicillium digitatum* and *P. italicum in vitro*

4.1: Introduction

Green and blue mould caused by *Penicillium digitatum* and *P. italicum*, respectively, are two of the most important post-harvest diseases of citrus fruit (Holmes and Eckert 1999). Chemical control of post-harvest decay in citrus began in the 1920s with alkaline solutions of sodium carbonate and sodium tetraborate (borax) (Wills *et al.* 1998). However, the environmental issues surrounding the disposal of waste water containing borate salts, which are toxic to plants, meant that borax was superseded in the 1950s by sodium *o*-phenylphenate (SOPP) (Eckert and Sommer 1967; Eckert and Ogawa 1985). This synthetic fungicide was used to control *Penicillium* species by dipping the fruit into solutions of it and, later on, it was incorporated into wax coatings (Eckert and Ogawa 1985). However, research began to indicate that it could induce urinary-bladder carcinogenesis (Hiraga and Fujii 1981; Fujii *et al.* 1987) and it was eventually designated as a potentially carcinogenic substance under Proposition 65 in 1990 (OEHHA 2010). By this time, it had already fallen into disfavour due to increasing numbers of tolerant strains of pathogens (Beraha and Garber 1966; Holmes and Eckert 1999).

The successful long-distance transport of citrus fruit was greatly helped by treatments with biphenyl (diphenyl), starting in the 1940s (Ramsey *et al.* 1944). The treatment worked via the crystalline compound being sublimated (directly transformed from the solid to the gaseous state, without becoming a liquid) in the packed container, thus fumigating the fruit during shipment (Eckert and Ogawa 1985). It was highly effective in preventing the growth of *Penicillium* species when wraps or paper sheets used to cover the fruit were impregnated with a heated solution of biphenyl in a mixture of paraffin and mineral oil (Eckert and Sommer 1967). However, strains of *Penicillium* began to develop resistance to this treatment, which has contributed to its discontinued use in many countries (Ogawa *et al.* 1975; Eckert and Ogawa 1985; Wills *et al.* 1998).

Two benzimidazoles - thiabendazole (2-(4'-thiazolyl)-benzimidazole) (TBZ) and benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate) - were introduced as fungicide treatments in the late 1960s (Eckert and Sommer 1967; Eckert and Ogawa 1985). They had systemic activity and were used in suspension and also incorporated into wax emulsions for the coating of citrus fruit. Their ease of use and long-term antifungal effects were welcomed by the industry, but strains of *Penicillium* that were resistant to these benzimidazoles soon developed and alternative treatments had to be sought (Erwin 1973; Miller and Fletcher 1974; Wild 1983; Baudoin and Eckert 1985; Vinas *et al.* 1991).

Imazalil (IMZ) came into general use in the 1970s and has been the predominantly used fungicide in citrus fruit packing houses ever since (Siegel and Ragsdale 1978; Holmes and Eckert 1999). However, its prolonged and sometimes excessive use, combined with the continuous operation of packing houses without a break to conduct proper sanitation measures, have led to growing pathogen-resistance (Bus *et al.* 1991; Eckert *et al.* 1994; Kinay *et al.* 2007). Resistant fungal isolates are rare within citrus groves, so it is still used to control infections on fruit entering packing houses, and additional measures such as heating the fungicide solutions or adding sodium bicarbonate have helped somewhat in controlling any resistant isolates inside the buildings (Smilanick *et al.* 2005; Kinay *et al.* 2007). Newer fungicides with different mode of actions have also been used in alternating strategies with IMZ, but pathogen-resistance alongside the desire by consumers to reduce our reliance on synthetic chemicals has made the search for alternative methods to control post-harvest infections a necessity (Eckert *et al.* 1994).

Flavour compounds are structurally diverse, but they generally possess similar physical properties. They are all hydrocarbons, but they may also contain oxygen, nitrogen and sulphur in different reactive groups. Compounds larger than four carbons generally have boiling points higher than that of water and they are volatile (French 1985). There have been numerous studies examining the effects of volatile compounds on the growth of fungal pathogens of fruits and vegetables *in vitro,* and consequently the antifungal activity of many such compounds is already known (Cowan 1999; Lanciotti *et al.* 2004; Tripathi and Dubey 2004).

Both C_6 and C_9 aldehydes are found in most terrestrial plants and are formed from linoleic and linolenic acids via cleavage by hydroperoxide lyase (Matsui 2006). They have been reported to have fungicidal activity against a wide range of plant pathogens, including *Alternaria alternata* and *Botrytis cinerea* (Hamilton-Kemp *et al.* 1992; Myung *et al.* 2007; Kishimoto *et al.* 2008) *Penicillium expansum* (Song *et al.* 1996; Neri *et al.* 2006a) and *Aspergillus parasiticus* (Wright *et al.* 2000). Many authors investigating volatile compounds in the vapour phase have noted that aldehydes tend to display the highest efficacy when it comes to inhibiting the growth of fungal pathogens (Gueldner *et al.* 1985; Utama *et al.* 2002). For example, (E)-2-hexenal has been known about since 1960 when it was isolated from *Gingko biloba*, a plant well known for its disease-resistance (Major *et al.* 1960). Early *in vitro* work looking at volatile compounds as potential anti-fungal treatments revealed that C5 – C8 aldehydes that are found naturally in citrus fruit inhibited the germination of *P. digitatum* spores (Davis and Smoot 1972). A terpene aldehyde, citral, has also been widely acknowledged as an antifungal compound. It has been reported to display high efficacy against *Colletotrichum musae, C. gloeosporioides* and *Fusarium subglutinans* (Garcia *et al.* 2008), *P. digitatum, P. italicum* and *Geotrichum candidum* (Wuryatmo *et al.* 2003) as well as *Rhizopus stolonifer* and *Mucor* species (Moleyar and Narasimham 1986).

Although aldehydes have been accredited as having the highest efficacy against fungal pathogens by many authors, there are others who disagree. Gueldner and colleagues (1985) found the order of activity of naturally-occurring plant volatiles to be aldehydes > ketones > alcohols, whilst Suprapta and colleagues (1997) found that alcohols were the most effective. Alcohols such as linalool have been shown to be inhibitory to various plant pathogens (Sokovic *et al.* 2009; Liu *et al.* 2012), whilst ketones such as carvone have proven to be inhibitory towards potato pathogens (Hartmans *et al.* 1995; Oosterhaven *et al.* 1995), *C. gloeosporioides* (Regnier *et al.* 2008), *B. cinerea* (Combrinck *et al.* 2011) and *P. digitatum* (du Plooy *et al.* 2009). Plant resistance elicitors like salicylic acid, methyl jasmonate and methyl salicylate have also been reported to have direct antifungal activity against *P. digitatum* and *P. italicum* (Iqbal *et al.* 2012).

This study aimed to investigate the effects of a number of natural volatile compounds of citrus fruit on *Penicillium* moulds *in vitro*. Some of these compounds have been examined by previous authors (Hamilton-Kemp *et al.* 1992; Utama *et al.* 2002; Myung *et al.* 2007; Neri *et al.* 2007; Combrinck *et al.* 2011; Iqbal *et al.* 2012; Liu *et al.* 2012), whilst others have not to our knowledge. In addition to exploring the influences on the pathogens of the volatiles alone, possible interactive or supplementary effects were also investigated by combining them with commercially available fungicides.

4.2: Materials and Methods

4.2.1: Media

4.2.1.1: Standard agar

Mycological potato dextrose agar (PDA) powder (Oxoid Microbiology Products, U.K) was added to deionised water (39 g/l), mixed thoroughly and then autoclaved at 121°C for 20 mins. Once sterilized, the agar was cooled to 50°C in a water bath prior to being poured into sterile 90 mm plastic Petri dishes to approximately a 3 mm depth.

4.2.1.2: Amended agar – ascertaining minimum inhibitory concentrations (MICs)

The additive or synergistic effects between the experimental volatile compounds and commercial fungicides were investigated. Fungicide samples were obtained from Paramount Citrus (California, U.S.A). These were PacRite® Fungaflor 75WSG and PacRite® Fungaflor 500EC (with active ingredient imazalil at 75% and 44.6% respectively) and Shield-Brite® TBZ containing 99.5% thiabendazole as the active ingredient.

To ascertain minimum inhibitory concentrations (MICs), PDA was prepared as above. After cooling to 50°C, separate volumes were mixed with different concentrations (0, 0.001, 0.01, 0.1, 1.0, 10 and 100 µg/mL) of the three fungicides, poured into Petri dishes and allowed to set prior to use.

4.2.1.3: Amended agar – ascertaining minimum concentrations required to inhibit 50% growth of the organisms (MIC50s)

Ascertaining MICs proved not to be possible, as all concentrations of the fungicides used inhibited *Penicillium* growth to some degree (Section 4.3.1; Figure 4.03). Consequently, the minimum concentrations required to inhibit 50% growth of the organisms (MIC50s) were determined instead (Section 4.3.1). These concentrations were then used to amend fresh PDA for use in combination with the volatile compounds.

4.2.2: Pathogens

4.2.2.1: Initial isolation and identification

The naturally infected orange from the initial weight-loss experiments (Section 2.3.2) had two distinct colonies of different fungal spores growing on it – blue and green. A mycological inoculation loop was sterilised by holding it in the flame of a portable gas cartridge stove (Campingaz) until it glowed red and then allowed to cool for approximately 10 seconds. The loop was then used to scrape off a sample of spores from the outer (actively growing) edge of one of the colonies and transfer it to the centre of a PDA plate (Section 4.2.1.1). The procedure was repeated twice so that three agar plates were inoculated with spores from the same fungal colony. The loop was then sterilised again and the procedure repeated with three spore samples from the second colony. All six inoculated plates were then sealed with Parafilm and incubated at 22°C for five days in a darkened conditioning room.

To identify the fungal spores, slides were made of the five-day-old cultures. Using a sterile inoculation loop, spore samples were transferred to glass microscopy slides and a drop of distilled water added before a cover slip was placed on top. The spores were then examined under an optical microscope at x 400 magnification and compared to those in an identification guide (Ramirez 1982). These observations together with information on the major pathogens of citrus fruit (Hume 1957; Eckert 1977; Eckert and Ogawa 1985) led to the conclusion that the fungi were *Penicillium digitatum* Sacc (green spores) and *P. italicum* Wehmer (blue spores).

4.2.2.2: Maintenance of cultures

To maintain the viability of the fungal cultures, they were subcultured onto fresh media at least once a month. The method for sub-culturing was identical to that used to isolate the samples, except that spores were taken from the outer edge of a plated sample and transferred to another Petri dish containing freshly prepared PDA media (Section 4.2.1.1). Loss of viability was noted when subcultured *Penicillium* failed to grow on fresh media.

Replacement samples were obtained when a delivery of organic oranges from Capespan (Kent, U.K.) arrived with a number of already-infected fruits.

4.2.2.3: Fungal suspensions

For experimental use, mixed fungal spore suspensions were required. These were prepared by pouring approximately 20 mL deionised water into a minimum-five-day-old plated culture. The contents were then swirled gently to favour detachment of conidia. A 20 mL disposable sterile luer slip syringe (Plastipak, U.S.A) was then used to transfer the conidia suspension obtained to a sterile glass vial and the procedure was repeated with the other *Penicillium* species. A further volume of distilled water was then added to the vial to give a final volume of 50 mL and the contents were mixed for approximately 10 s using a vortex mixer.

4.2.3: Chemicals

The majority of volatile compounds used throughout the research are found naturally in citrus fruit (Njoroge *et al.* 2005; Barboni *et al.* 2009; Carmen Gonzalez-Mas *et al.* 2011; Espina *et al.* 2011) and were purchased from Sigma (U.K). These were (-)-linalool, (-)-terpinen-4-ol, (-)-α-pinene, (-)-β-pinene, (+)-linalool, (+)-α-pinene, (R)-carvone, (S)-carvone, 1-octen-3-ol, citral, decanal, (E)-2-hexenal, geranyl acetate, limonene, methyl jasmonate, methyl salicylate, myrcene, neryl acetate, nonanal, octanal, valencene, β-caryophyllene and γ-terpinene All chemicals were ≥ 81% pure. The essential oils of oranges and lemons were also used – these were obtained from Paramount Citrus (California, U.S.A).

4.2.4: Inoculation of Media and Volatile Delivery Method

Mixed fungal spore suspensions were made from previously cultured samples of *P. digitatum* & *P italicum*. The concentrations of spores in the suspensions were adjusted to 10^6 - 10^7 spores/mL using a hemocytometer. PDA was prepared according to the required procedure (Section 4.2.1) and poured into a number of Petri dishes. Once set, a 10 µL drop of fungal spore suspension was placed in the centre of the agar in each dish using a

sterile Gilson pipette and the lids immediately replaced. The fungal samples were then incubated for 24 hours at 22°C in a dark conditioning room before treatment.

In a fume cupboard, 100 µl of each volatile compound was pipetted into an aluminium-backed polyethylene slow-release packets (obtained from Agrisense, U.K.; a minimum of 3 replicate units for each compound). The packets were then immediately sealed with an impulse heat sealer (PFS-400, Willis European Ltd, U.K) and each attached to the inside lid of an inoculated Petri dish with double-sided tape (Scotch^{TM}). As a control, a minimum of 3 empty but sealed slow-release sachets were also utilized in the same manner. All dishes were labelled, sealed with Parafilm and then incubated for 5 days at 22°C in a darkened conditioning room.

4.2.4: Data Collection and Analysis

The inoculated Petri dishes (with or without the volatile compounds) were incubated at 22°C for five days. After this time, the extent of fungal pathogen growth was taken by using a steel caliper (Tool Box, U.K) to measure the diameter (mm) of the area on each plate where spores/hyphae could be visibly detected (see Figure 4.01). These were recorded as colony size (mm) and mean values calculated for each different treatment. The means obtained from the control (i.e. those without the addition of fungicide and/or a volatile compound) were then used as the base-line value of zero and the means from the treatments expressed as percentage differences of the control. The assays were performed a number of times. However, the minimum number of replicates for each treatment and the control was nine.

Mean values and standard errors were calculated with Microsoft Excel 2010 (Microsoft Corporation, Washington, U.S.A) and then subjected to univariate analysis of variance (ANOVA) using the General Linear Model (GLM). Equality of variances were checked using the Levene's Test and, if equal, the least significant difference (LSD, $p = 0.05$) between means for multiple comparisons was calculated. If equal variances could not be assumed, then the non-parametric Games-Howell test was used for multiple comparisons between treatments. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses.

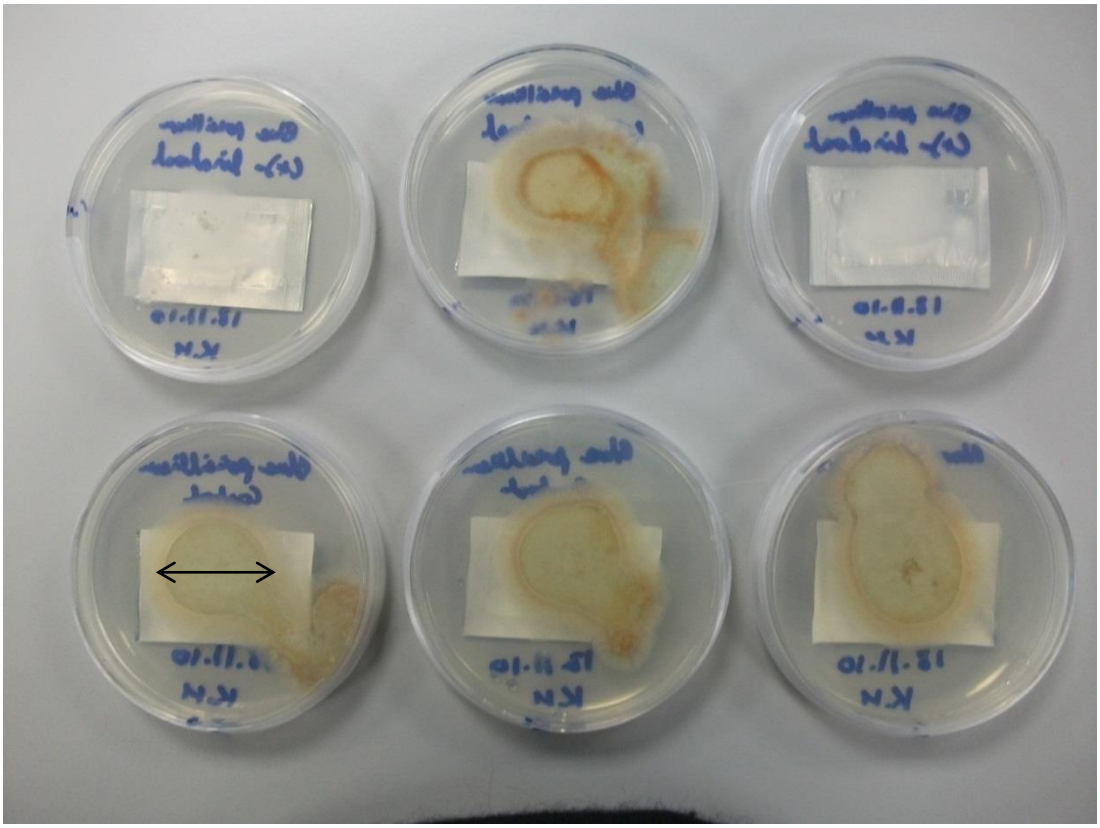

Figure 4.01: Petri dishes containing samples from *in vitro Penicillium* and volatiles assay showing inoculated PDA plates with slow-release packets. Bottom row are control plates with empty packets, whilst the top row contain (+)-linalool. Plates are upside down in order to measure the extent of the fungal growth. Arrows indicate how the fungal growth on each of these plates was measured – i.e. around the site of inoculation, not where fungal suspension has accidently spilled when plate has been moved.

4.3: Results

4.3.1: *Penicillium digitatum* and *P. italicum* and volatiles

Working alone, a number of the volatile compounds induced a significant ($p < 0.05$) reduction in the growth of the two *Penicillium* pathogens *in vitro* compared to the untreated plates (Figure 4.02). The aldehydes (E)-2-hexenal, octanal and nonanal inhibited them completely, with no *Penicillium* growth observed at all. (R)-carvone, and (+)-linalool were also very effective against the pathogens, reducing the colony sizes by ~ 80% and 60% respectively ($p = < 0.001$). The other compounds which significantly reduced pathogen growth were MeS ($p = 0.007$), (-)-terpinen-4-ol ($p = 0.002$), (S)-carvone and (-)-linalool (both $p = 0.011$).

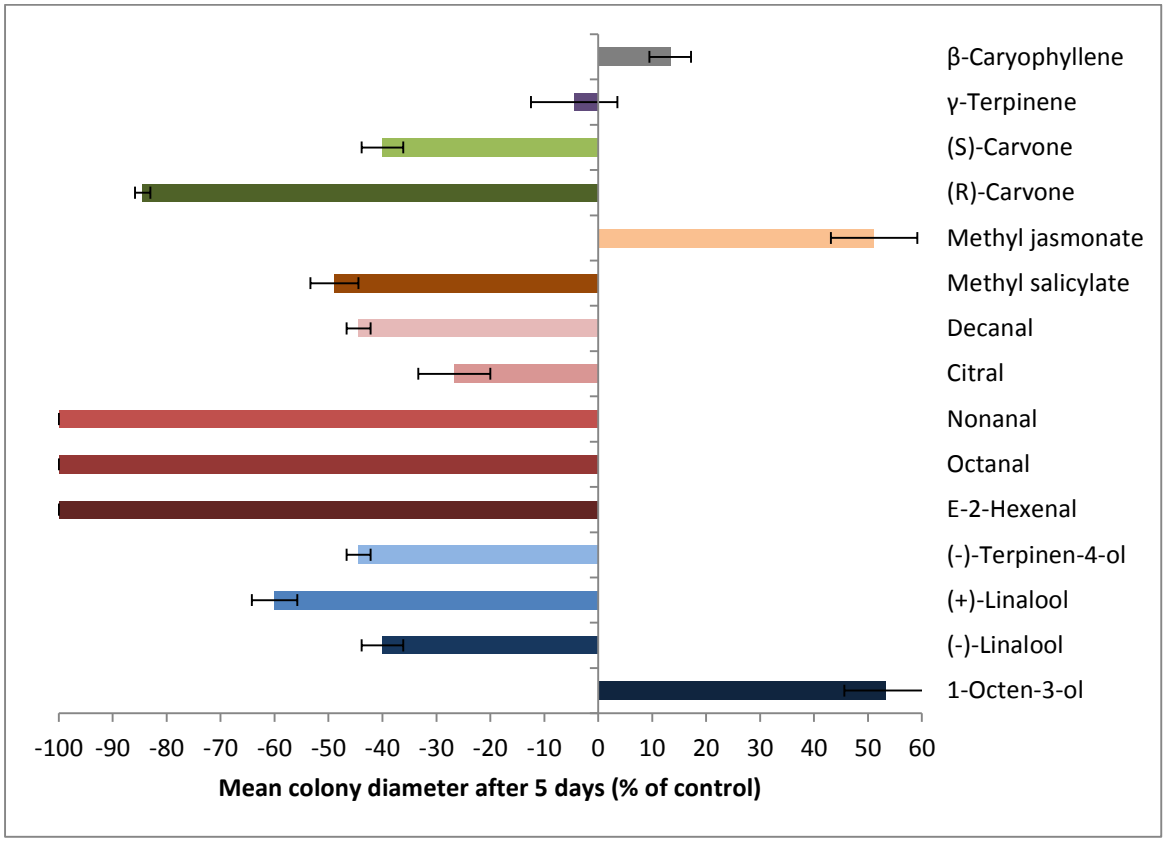

Figure 4.02: Effects of volatile compounds on the growth of *Penicillium digitatum* and *P. italicum in vitro*. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones; Purples = terpenes; Greyscale = sesquiterpenes. Different shades of the same colour (darkest → lightest) denote individual compounds within each chemical group from lowest molar mass → highest. Error bars = ± 1 S.E.

A number of volatile compounds stimulated pathogen growth. β-caryophyllene increased colony size by ~ 13%, whereas both 1-octen-3-ol and methyl jasmonate encouraged growth by > 50%. The terpene, γ-terpinene, was rather inconsistent in its effects, being slightly inhibitory in some cases and slightly stimulatory in others.

4.3.2: *Penicillium digitatum* and *P. italicum* and amended agar

Figure 4.03 displays the results of the assay to determine MICs for the three fungicides. The minimum inhibition achieved was ~19% by 0.001 µg/mL of IMZ 50, whilst the same fungicide attained 100% inhibition at the higher concentrations of 1.0, 10 and 100 µg/mL. As all of the tested fungicide concentrations inhibited the pathogen growth to some extent, MICs could not be ascertained. The alternate criterion of minimum inhibitory concentrations required to inhibit 50% of the organisms (MIC50s) was determined from Figure 4.03 instead. These were IMZ 50 = 0.07 µg/mL, IMZ 75 = 1 µg/mL and TBZ = 10 µg/mL. These concentrations were then used for the *in vitro* study combining fungicide-amended agar and volatile compounds (Section 4.3.3).

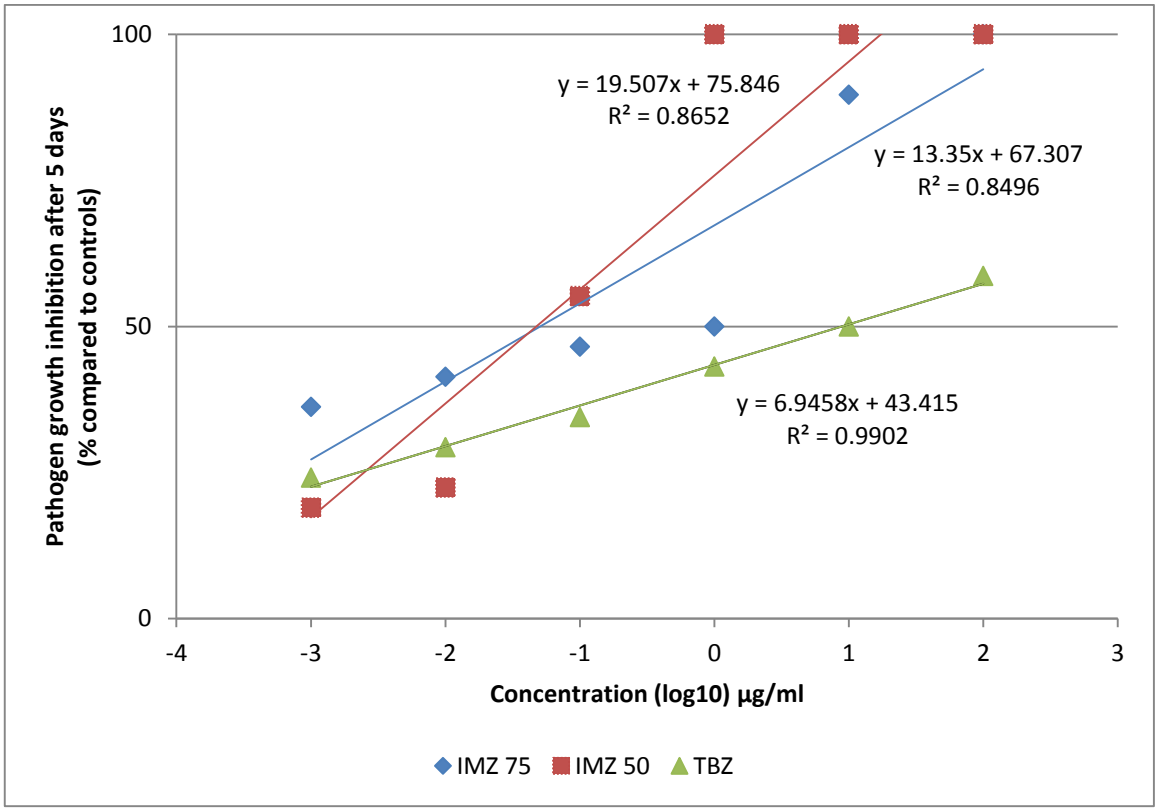

Figure 4.03: Effects of different concentrations (0.001, 0.01, 0.1, 1.0, 10 and 100 µg/mL; converted to log10 values) of PacRite® Fungaflor 500EC (IMZ 50), PacRite® Fungaflor 75WSG (IMZ 75) and Shield-Brite® TBZ on the growth of *Penicillium digitatum* and *P. italicum in vitro*. Linear trendlines, equations and R^2 values are also shown for each fungicide.

4.3.2: *Penicillium digitatum* and *P. italicum*, amended agar and volatiles

Agar amended with TBZ resulted in some reduction in fungal growth with all the volatile additions in relation to the zero-volatile control (Figure 4.04), although in one case decanal was slightly stimulatory. According to the LSD *post hoc* test, all but a few of the volatiles were significantly effective at reducing the growth of the Penicillium pathogens in vitro – those that were not were β-caryophyllene, decanal and E-2-hexenal. The highest efficacy was shown with the alcohols (-)-linalool, (+)-linalool and (-)-terpinen-4-ol, the aldehydes octanal, nonanal and citral, the ester methyl salicylate and the ketone (R)-carvone, all of which inhibited pathogen growth by > 15% on average. There were some variations in the effects however, particularly in the case of octanal where the percentage of inhibition ranged from approximately 5 – 50%. Nevertheless, it was still significantly different to the controls ($p < 0.001$).

The aldehydes and alcohols tested all increased in efficiency against the pathogens as they got larger in size (i.e. higher molar mass). In the case of aldehydes however, this observation reversed quite radically when it came to decanal.

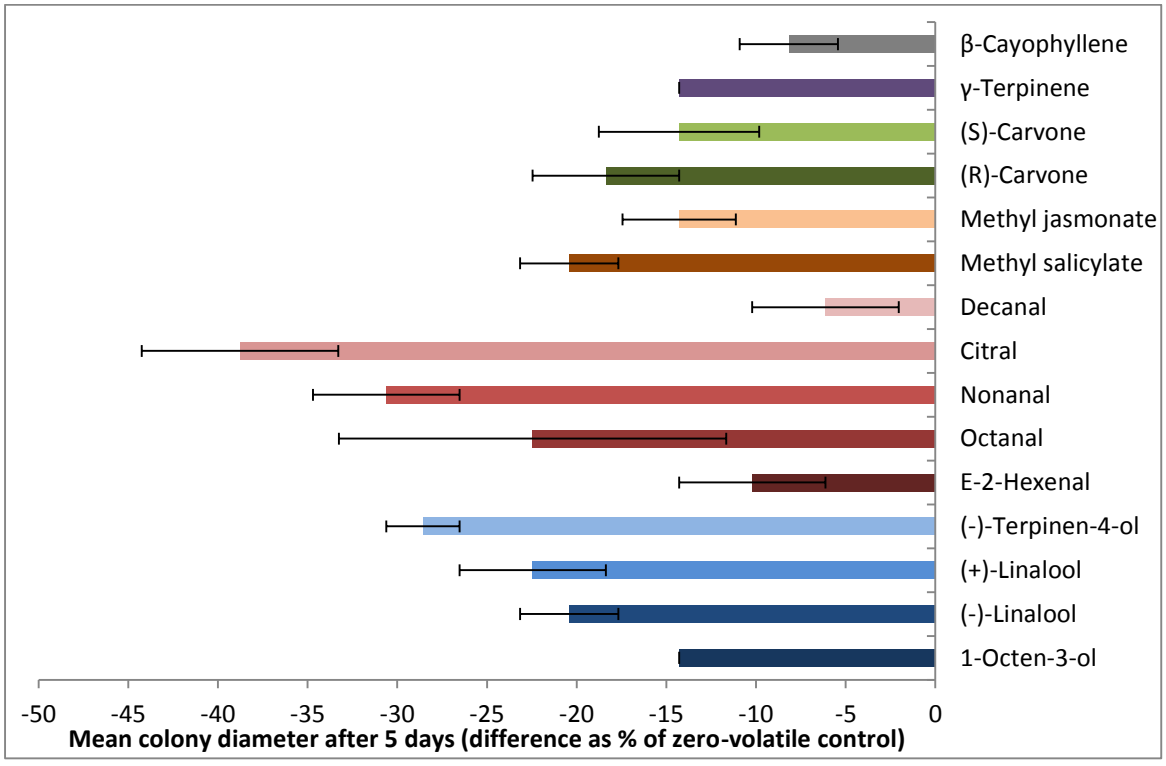

Figure 4.04: Effects of volatile compounds on the growth of *Penicillium digitatum* and *P. italicum* on PDA amended with Shield-Brite® TBZ fungicide at MIC50. Other details as in legend for Figure 4.02.

In regards to the two imazalil-amended agar studies, combining either of them with the volatile treatments significantly reduced pathogen growth in the majority of cases. This was particularly the case with IMZ50 (Figure 4.05), where only 1-octen-3-ol was not effective in decreasing the *Penicillium* growth and in fact increased it by ~ 2% compared to the fungicide alone. With IMZ 75 (Figure 4.06) there was no difference in mean colony sizes whether 1-octen-3-ol was added or not, but a number of others were not significantly different to the controls according to the Games-Howell *post hoc* test. These were β-caryophyllene, γ-terpinene, methyl jasmonate, decanal, citral, octanal, and both linalool enantiomers. The most effective with both IMZ 50 and IMZ 75 amended agar were (E)-2-hexenal, nonanal and (R)-carvone, all of which prevented the growth of the two *Penicillium* species completely (Figures 4.05 and 4.06).

Similar to the results with TBZ amended agar, octanal again produced very inconsistent effects, particularly in the case of IMZ 75 where the percentage inhibition ranged from approximately 10 – 90%.

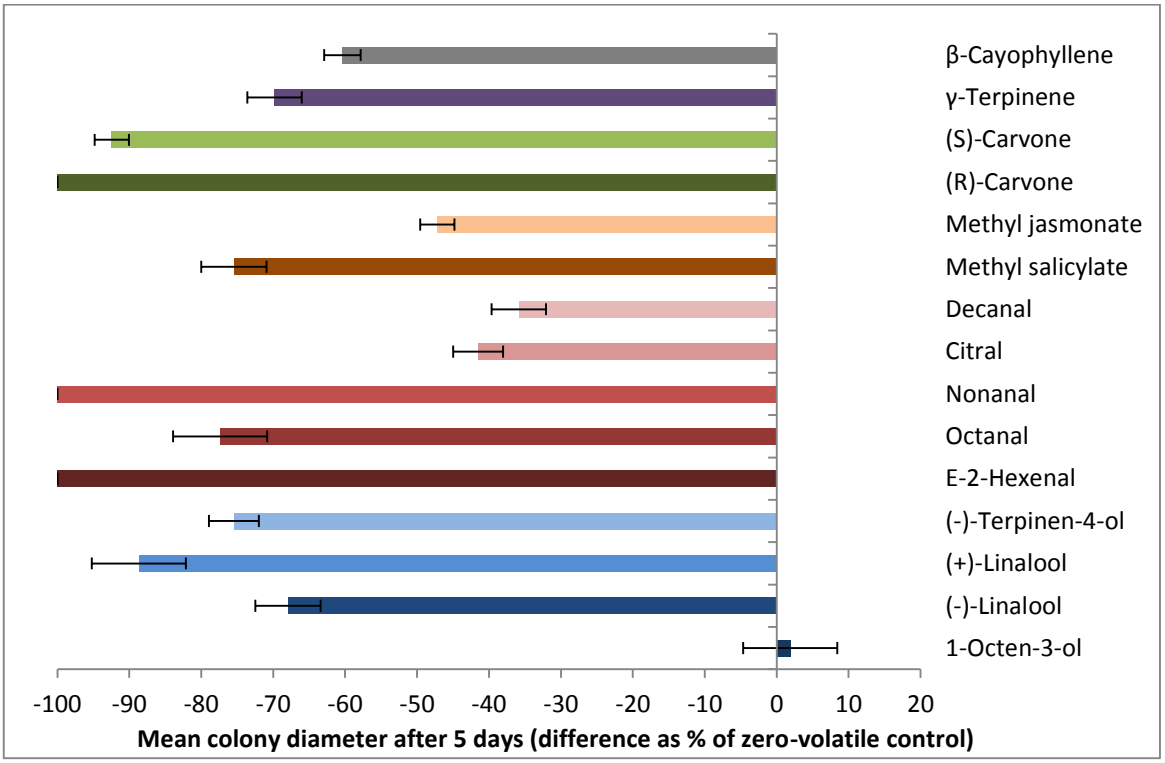

Figure 4.05: Effects of volatile compounds on the growth of *Penicillium digitatum* and *P. italicum* on PDA amended with PacRite® Fungaflor 500EC (IMZ 50) fungicide at MIC50. Other details as in legend for Figure 4.02.

No patterns were observed regarding the size of the related volatile compounds and their efficacy as with the TBZ-amended agar (Figure 4.04). However, overall the imazalil-based fungicides combined with the volatile treatments exhibited greater inhibition compared to those with the thiabendazole-based fungicide (maximum 100% in both former cases compared to a maximum of ~ 39% in the latter).

The fact that different volatiles were the most effective when combined with imazalil-based fungicides rather than thiabendazole-based is noteworthy, although aldehydes, as a group, exhibited the greatest activity in all cases.

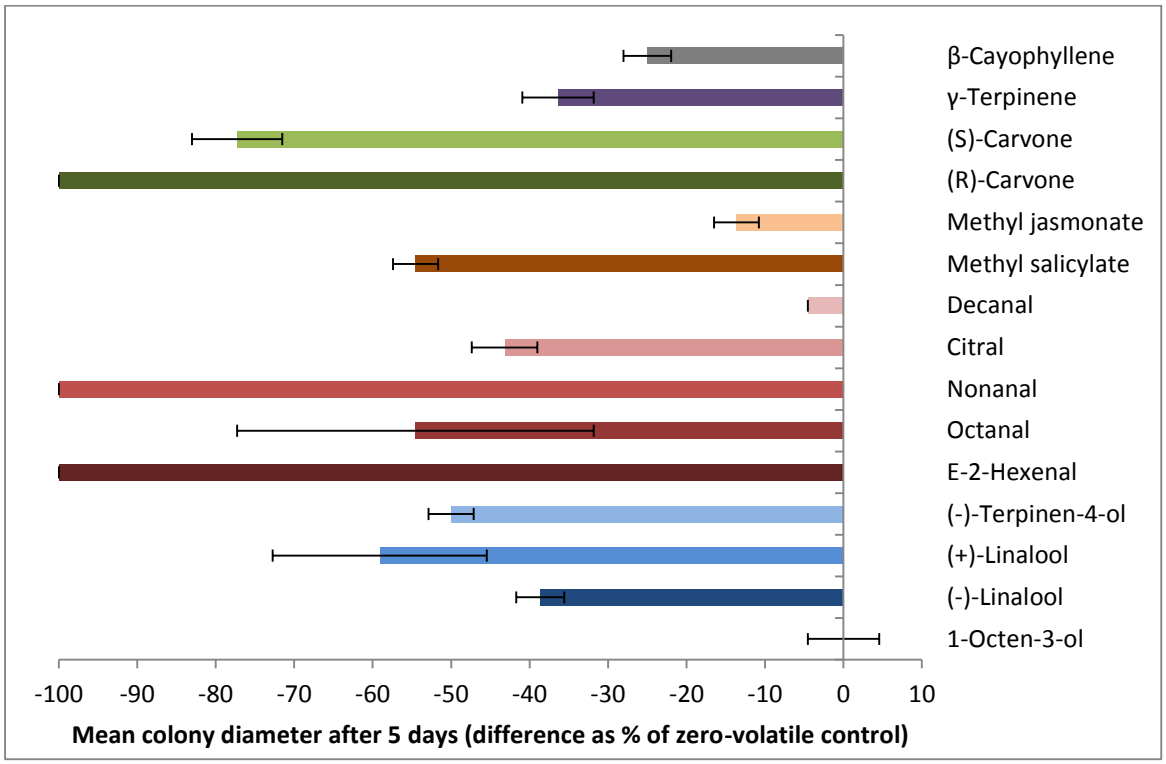

Figure 4.06: Effects of volatile compounds on the growth of *Penicillium digitatum* and *P. italicum* on PDA amended with PacRite® Fungaflor 75WSG (IMZ 75) fungicide at MIC50. Other details as in legend for Figure 4.02.

If the volatile compounds worked in parallel to the fungicides, the fact that those that proved the most effective did so whether acting alone or in amended agar is not surprising. The aldehydes, as a group, exhibited the highest overall efficacy, whilst the ketone, (R)-carvone, the alcohol (+)-linalool and methyl salicylate also demonstrated high activity against *Penicillium in vitro* in all cases. This suggests that these particular compounds have properties that affect the organisms' growth and/or development.

4.4: Discussion

The principal conclusion from the results contained in this chapter is that there were a number of compounds which appeared to inhibit or even prevent the growth of the two *Penicillium* pathogens altogether. Aldehydes, as a group, were consistently the most effective, although there were other, unrelated compounds, which exhibited high efficacy in all cases.

In their 1992 study, Hamilton-Kemp and colleagues showed that the unsaturated aldehyde, (E)-2-hexenal was more effective in reducing the growth of two other fungal pathogens, *Alternaria alternata* and *Botrytis cinerea*, than the corresponding saturated aldehyde, hexanal (Hamilton-Kemp *et al.* 1992). However, in our studies, (E)-2-hexenal was no more effective than the saturated aldehyde nonanal and in fact less so when combined with the fungicide thiabendazole (Section 4.3.2). The other unsaturated aldehyde in our studies, the monoterpene aldehyde citral, exhibited the least efficacy against the *Penicillium* pathogens when working alone and was only more effective than the saturated aldehydes tested in conjunction with thiabendazole – the opposite to (E)-2-hexenal. The only conclusion that can be drawn from this is that, in our studies, the differences in activity shown by the different aldehydes is not associated with whether they have double or single covalent bonds between the carbons and must have some other reason.

Citral, plus its isomers geranial and neral, and other related compounds were examined for their effect on *P. digitatum, P. italicum*, and *Geotrichum candidum* by Wuryatmo et al. (2003). *In vitro* investigations showed that citral and geranial were fungicidal to *P. digitatum* and *G. candidum*, while neral was only fungicidal to *G. candidum*. The citral-related compounds (citronellal, citronellol and citronellic acid) were much less effective. None of the compounds tested were very effective against *P. italicum* and, as the two *Penicillium* species were mixed in our studies, this may help explain why citral did not perform as well as the majority of our other aldehydes did. Citral was also found to be the most effective compound examined for its antifungal activity against *Colletotrichum musae*, *C. gloeosporioides* and *Fusarium subglutinans* f.sp.*ananas*, pathogens of papaya, pineapple and banana (Garcia *et al.* 2008). An earlier study that looked at the effects of

volatile compounds on five pathogenic fungi, found that citral, citronellic acid and citronellal were the most inhibitory to *Aspergillus niger*, *Fusarium oxysporum* and *P. digitatum* in a liquid medium, whilst in vapour form they were more active against *Rhizopus stolonifer* and a *Mucor* sp. (Moleyar and Narasimham 1986). Our studies only looked at the effects of the volatiles in the vapour phase, so these observations do not have any bearing on them, but it is interesting to note how the compounds behave differently when their nature is altered by incorporating them into media. Moleyar and Narasimham (1986) also came to the conclusion that the -CHO group in conjugation with a carbon to carbon double bond was responsible for the antifungal activity of citral. This is similar to that noted by Hamilton-Kemp and colleagues (1992) regarding (E)-2-hexenal, but as stated earlier the unsaturated nature of citral did not always increase its efficacy over that of the saturated aldehydes tested.

Andersen and colleagues (1994) investigated the antifungal activity of a selection of C_6 and C_9 aldehydes, ketones and alcohols on the opportunistic plant pathogen *A. alternata*. It was found that C_9 aldehydes and ketones were generally more effective in reducing spore germination and subsequent germ tube elongations than their C_6 counterparts. Unsaturated aldehydes and ketones and, to a lesser extent, alcohols were also found to have the highest efficacy against the fungal pathogen (Andersen *et al.* 1994). The most effective compound investigated was (E)-2-nonenal, which was not included in our studies. Of the two comparable compounds, (E)-2-hexenal and nonanal, the authors found that the saturated C_9 compound nonanal had a slightly higher degree of antifungal activity than the unsaturated C_6 compound (E)-2-hexenal. These findings are similar to those in our studies, seeming to indicate that the size of the compound is more important than whether it has double or single covalent bonds between the carbons.

The grouping of volatile compounds into aldehydes, ketones and alcohols (and esters) in order to test their potential antifungal properties has been used as a basis for investigations by others (Gueldner *et al.* 1985; Suprapta *et al.* 1997), but there are inconsistencies in the findings as to their order of activity. Gueldner and colleagues (1985) found it to be aldehydes > ketones > alcohols, whereby the reduction of active aldehydes and ketones to the corresponding alcohols reduced inhibitory activity. Conversely, Suprapta and colleagues (1997) found that alcohols were the most effective

against *G. candidum*, the other major citrus fruit pathogen. After looking at the effects of ten alcohols, five aldehydes and four esters on arthrospore germination and mycelial growth of *G. candidum*, they found that six of the ten alcohols were > 60% inhibitory towards the pathogen, whilst only one aldehyde (citral) was > 50% inhibitory. Besides citral, the only other corresponding compound in our studies was linalool (which enantiomer was not specified), but this had no effect on arthrospore germination or mycelial growth. However, one finding to come out of their research which is comparable to our studies is the observation that the inhibitory action of the alcohols increased with the increase of chain length from C_6 to C_9. They also note that alcohols of shorter chain length than C_6 or longer than C_9 had only small inhibitory effects, and that this is probably related to the volatility and solubility of the compounds. In the case of aldehydes in our study, this was certainly the case as decanal had the least efficacy of all those tested. We did not study any alcohols shorter than C_8, but linalool and terpinen-4-ol (both C_{10} compounds) performed better than their conclusions would suggest.

The aldehyde (E)-2-hexenal was studied quite extensively by Neri and colleagues (Neri *et al.* 2006a; Neri *et al.* 2006b; Neri *et al.* 2006c) and found to be very effective against *Penicillium expansum*, the major pathogen of pears and apples. In their initial study (Neri *et al.* 2006a) the effects of nine volatile compounds on *P. expansum* was investigated *in vitro*. Out of three corresponding compounds in our studies ((E)-2-hexenal, citral and (R)-carvone), (E)-2-hexenal was found to have the highest efficacy, followed by citral and then (R)-carvone. These results are somewhat dissimilar to ours, as (R)-carvone was more effective than citral in all but the thiabendazole-amended agar investigation. However, as the investigations conducted by Neri and colleagues were on *P. expansum* which does not affect citrus fruit, the differences observed could perhaps be expected. Focusing specifically on (E)-2-hexenal, Neri and colleagues (Neri *et al.* 2006b) found that the volatile treatment applied immediately after inoculation was not useful to control *P. expansum*, but when applied 24–72 hours after pathogen inoculation it significantly reduced infection. They suggest that this may be because germinating conidia of *P. expansum* are probably more susceptible to (E)-2-hexenal than non-germinating conidia. In our studies, the volatile treatments were applied 24 hours after inoculation by *P. digitatum* and *P. italicum* with similar significant results. This sensitivity to treatments

applied after this time period following pathogen inoculation has also been found in other studies (Mari *et al.* 2002).

Four volatile compounds (valencene, decanal, octanal and linalool) were isolated from the oil of orange fruit (*C. sinensis*) by Liu and colleagues (2012) and their antimicrobial activity tested on *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerivisiae*, *Penicillium citrinum*, and *A niger*. Their results showed that decanal, octanal and linalool (the three compounds also used in our studies) had inhibitory and bactericidal effects on four out of the five test microorganisms (*P. citrinum* was the exception) (Liu *et al.* 2012). Similarly, decanal and nonanal were two of the six compounds found to completely inhibit mycelial growth or sclerotia formation of *Sclerotinia sclerotiorum* in other *in vitro* tests (Dilantha Femando *et al.* 2005). Out of the five aldehydes we studied, decanal was probably the least effective against *P. digitatum* and *P. italicum*, and this and nonanal have actually been found to promote the development of specialized cells which aid in infection establishment in the rust fungus *Uromyces fabae* (Mendgen *et al*. 2006). In our investigations, nonanal was highly effective against the two *Penicillium* species, as was octanal, albeit somewhat inconsistently. Linalool, particularly the (+) enantiomer, had the highest efficacy of the alcohol compounds examined in our studies, except for in the case of TBZ-amended agar (Figure 4.04) where it was terpinen-4-ol.

Linalool has been shown to possess strong antifungal activity against 17 micromycetes (including *Penicillium ochrochloron* and *P. funiculosum*) (Sokovic *et al.* 2009), whilst the essential oil of *Thymus vulgaris*, the contents of which were high in p-cymene, linalool, terpinen-4-ol and thymol, were found to be highly effective against *B. cinerea* and *R stolonifer* (Reddy *et al.* 1998). Work by Ait-Ouazzou and colleagues (2011) looking at the antimicrobial activity of hydrocarbon monoterpenes and oxygenated monoterpenes (including linalool and terpinen-4-ol) against a number of bacterial organisms, also found these two compounds to be amongst the most effective. The authors noted that oxygenated monoterpenes showed higher inhibitory activity on microbial growth than did hydrocarbons (Ait-Ouazzou *et al.* 2011), an observation also recorded by Carson & Riley (1995). This was also the case in our studies, with the terpene alcohols proving to be generally more effective than γ-terpinene. Moreover, Ait-Ouazzou and colleagues stated that linalool was slightly more effective than terpinen-4-ol, which also agrees with the

results of our studies. In contrast, linalool (the (R) or (+) enantiomer) was also amongst twelve compounds evaluated against the plant pathogenic fungi *Rhizoctonia solani, F. oxysporum, P. digitatum* and *A. niger* by Marei and colleagues (2012). It was only found to be moderately inhibitory, particularly against *R. solani*, whilst the other corresponding compound to our studies, (R)-carvone, was one of these least inhibitory to all four pathogens (Marei *et al.* 2012). These findings do not equate with our results regarding these two compounds against citrus *Penicillium* pathogens as they were both amongst the most effective across all four investigations.

Both (R)- and (S)-carvone were used in a study by Combrinck and colleagues (2011), looking at their effects on five plant pathogens (*Lasiodiplodia theobromae, C. gloeosporioides, Alternaria citrii, B. cinerea* and *P. digitatum*). Pure (R)-carvone inhibited all the pathogens, excluding *A. citrii*, whilst (S)-carvone was less effective except for against *A. citrii* (Combrinck *et al.* 2011). The same authors had previously evaluated these compounds against *Botryosphaeria parva* and *C. gloeosporioides* and found them to have high fungistatic activity (Regnier *et al.* 2008), as did (R)-carvone against *P. digitatum* (du Plooy *et al.* 2009). These results are consistent with ours, in that the (R)- enantiomer of this ketone was the most inhibitory of the two. The fact that these authors also found it to have high efficacy against *P. digitatum* supports our findings further.

Natural disease resistance to plant pathogens may be induced and enhanced by physical, biological and chemical elicitors, and suppression of *P. digitatum* in grapefruit by methyl jasmonate (MeJ) has been reported to occur through the accumulation of phytoalexins (Droby *et al.* 1999). Pre-harvest treatments with salicylic acid (SA) and MeJ also reduced lesion diameters on sweet cherry fruit caused by *Monilinia fructicola*, but only SA worked *in vitro* against the pathogen (Yao and Tian 2005b). Methyl salicylate (MeS) has also been shown to exhibit antifungal activity against both *M. fructicola* and *B. cinerea in vitro* (Wilson *et al*. 1987). Cao and colleagues (2008) treated loquat fruit with MeJ 24 hours prior to inoculating it with *Colletotrichum acutatum*. They also tested the compound *in vitro* against the fungus. The volatile treatment resulted in lower disease

incidence and smaller lesion diameters than in control fruit and also inhibited spore germination, germ tube elongation and mycelial growth of *C. acutatum in vitro*. These results suggested that the control of the disease by MeJ is both directly, because of the inhibitory effect of the compound on pathogen growth, and indirectly because of the induced disease resistance triggered in the fruit (Cao *et al.* 2008). However, Quaglia and colleagues (2011) reached slightly different conclusions following their investigations with several potential elicitors, including MeJ, and their effect on *P. expansum* infection in apples. Their findings suggested that the treatments triggered resistance responses in the fruit, but that these did not limit the infection as such, whilst direct applications of the compounds did have inhibitory effects on the pathogens. They thus concluded that some natural compounds could potentially be used as alternatives to chemical fungicides, but that induction of resistance did not appear to be a viable approach (Quaglia *et al.* 2011). Our work looked at two such elicitors, MeJ and MeS, and found marked differences between the actions of the two, particularly when working alone (Figure 4.02). Here, MeJ stimulated the growth of the *Penicillium* pathogens, increasing the mean colony size by ~ 50% over the zero-volatile control mean, whereas MeS displayed ~ 50% inhibitory activity. Our results suggest that MeS, a volatile derivative of the plant hormone salicylic acid (Lee *et al.* 1995) and known to be induced by insect herbivory in many plant species (Dicke *et al.* 1990; Dicke *et al.* 1998), has direct antifungal action on *Penicillium* growth.

The other compounds utilised in our studies have not been studied to any great extent for their antifungal properties. The alcohol 1-octen-3-ol, was not effective against the *Penicillium* pathogens, except for a slightly inhibitory activity when combined with TBZ-amended agar, and has previously been reported to have antibacterial activity against *Salmonella typhimurium*, *Bacillus cereus*, *Escherichia coli* and *Vibrio parahaemolyticus* (Kim and Shin 2004). The monoterpene hydrocarbon, γ-terpinene, was tested *in vitro* against *B. cinerea*, *P. italicum*, *P. expansum*, *Phytophthora citrophthora* and *R. stolonifer*, but displayed very little antifungal activity (Camele *et al.* 2012). This is in contrast to a study by Espinosagarcia & Langenheim (1991), in which γ-terpinene was inhibitory to *B. cinerea* (amongst other redwood pathogens). As stated earlier, this monoterpene hydrocarbon was generally not as effective in reducing *Penicillium* growth as the oxygenated monoterpenes in our studies, particularly when acting alone, but in conjunction with the commercial fungicides it did display quite high efficacy. It certainly

performed better than the sesquiterpene β-caryophyllene in our investigations, which has been shown to have weak inhibitory activity *in vitro* against *P. italicum* (Li *et al.* 2010) and good inhibitory effects on *Pseudomonas syringae* (Huang *et al.* 2012).

To conclude, some natural volatile compounds appear to play a role in limiting the growth of plant pathogens *in vitro*. Certain groups of compounds, particularly aldehydes, have greater and more consistent activity so it seems there are specific structural features that enhance antifungal efficacy. In our studies, the most effective compounds with the most consistent inhibitory effects across three of the four experiments were nonanal, (E)-2-hexenal and (R)-carvone. The investigation with TBZ-amended agar did not produce such corresponding results, but the results of this study were variable in regards to all the compounds tested. This could perhaps be due to the action of the fungicide itself on the pathogens, as resistance to TBZ first occurred about 2 years after its introduction in 1968 (Holmes and Eckert 1999) and the subsequent years will have meant that this will have increased further still. Nevertheless, the efficacy of the most active compounds in our studies is highly convincing and their potential as natural biocontrol agents is worthy of further investigation.

Chapter 5: The Effect of Volatile Compounds on *Botrytis cinerea* Infection and Water Loss in Strawberry Fruits

5.1: Introduction

Although the demand for organic produce has increased rapidly over recent years, the marketing of soft fruit like strawberries (*Fragaria* × *ananassa*) via organic production methods is still very limited due to the lack of acceptable postharvest treatments and the highly-perishable nature of the crop (Nunes *et al.* 1995; Crecente-Campo *et al.* 2012). Strawberries have a high respiration rate (approximately 15 mg CO_2 kg^{-1} hr^{-1} at 0°C) and a thin epidermis with many stomata and long, pointed, thick-walled hairs (Kader 1992) and their quality declines very quickly once harvested. Weight/water losses in the fruit of > 2.5% result in softening of the flesh and shrivelling of the calyx and skin, making them overripe and less acceptable for consumers. At 20°C and 85-95% RH this occurs in 2.5-3 days (Shin *et al.* 2007). To help combat this, the fruit are usually chilled to between 1-4°C immediately after harvest, but maximum storage/transport time even at optimum temperature is still only 5 days, severely limiting their marketing lifespan (Wills *et al.* 1998).

Besides the physical deterioration of the fruit, strawberries are also highly susceptible to grey mould rot caused by the phytopathogen *Botrytis cinerea* (Eckert and Ogawa 1988; Wills *et al.* 1998; Rosslenbroich and Stuebler 2000). Pre-harvest applications of fungicides have been used to control postharvest fungal decay (Ogawa *et al.* 1975; Freeman and Pepin 1977; Hewitt 1998; Rosslenbroich and Stuebler 2000). However, strains of *B. cinerea* have developed resistance to many classes of fungicides, and chemicals with different modes of action are almost constantly being sought (Eckert and Ogawa 1988; Vali and Moorman 1992; Palou *et al.* 2007). Besides being sprayed with pre-harvest fungicides, fresh strawberries are often stored and transported in modified atmospheres containing 10% CO_2 or more as a method of reducing decay (Goulart *et al.* 1992; Palou *et al.* 2007). The fruit can tolerate high CO_2 levels without loss of quality, but the positive effects are lost as soon as they reach their marketing destination (Harvey *et al.* 1980; Elkazzaz *et al.* 1983; Nunes *et al.* 1995; Agar *et al.* 1997).

Fruit contain many volatile compounds (Pyysalo 1976; Hamilton-Kemp *et al.* 1996; Shaw *et al.* 2001; Lota *et al.* 2002; Tu *et al.* 2002; Barboni *et al.* 2009), and some have been shown to have antifungal activities *in vivo* (Pauli and Knobloch 1987; Vaughn *et al.* 1993; Dixit *et al.* 1995; Song *et al.* 1996; Suprapta *et al.* 1997; Neri *et al.* 2006a; Neri *et al.* 2007; Amiri *et al.* 2008). Although many volatiles have demonstrated efficacy against fungal pathogens in harvested fruit, none are used commercially to prevent or delay decay. This could be down to the failure to maintain sufficient concentrations in the gas headspace surrounding the fruit, or the realisation that the compounds have detrimental effects on fruit quality. For example, fumigation with 1% acetaldehyde vapour for 30-60 minutes was shown to inhibit *B. cinerea* infection in strawberries (Prasad and Stadelbacher 1974), but a later study (Pesis and Avissar 1990) showed that it caused changes in the appearance and quality of the fruit. However, other studies with different compounds have not reported such negative side-effects, such as that of hexanal on *B. cinerea* and *Penicillium expansum* development on inoculated apple slices (Song *et al.* 1996) and (E)-2-hexenal on fungus-inoculated peach, nectarine, and plum fruit (Neri *et al.* 2006a).

The work described here was conducted to test a selection of naturally-occurring volatile compounds in *in vivo* experiments on both organic and non-organic strawberries. The reason for using the two differently-produced fruit was so we could test if the compounds were sufficiently protective on their own, or whether they could just enhance the efficacy of commercially-applied fungicides. The fruit were inoculated with *B. cinerea* and subjected to volatile treatments whilst being stored under conditions optimum for fungal growth (Hunter *et al.* 1972; Dennis and Cohen 1976; Kerssies *et al.* 1995; Lahlali *et al.* 2006a). They were then evaluated for extent of pathogen infection and loss of moisture to ascertain which, if any, compounds could potentially be used to extend the shelf-life of these popular fruit.

5.2: Materials and Methods

5.2.1: Fruit Material

Organic British strawberries (*Fragaria* x *ananassa*; variety, Elsanta) were purchased from Waitrose Stores (Menai Bridge, U.K.).

Non-organic British strawberries (Elsanta) were purchased from Tesco Stores (Bangor, U.K.).

5.2.2: Pathogens

B. cinerea was first isolated from naturally infected strawberry fruit during initial weight-loss experiments (See Section 2.3.6). These were cultured on potato dextrose agar (PDA) and sub-cultured at least once a month to check viability. Loss of viability was noted on a couple of occasions, so spores from other naturally infected organic strawberries were isolated and cultured in the same way (See Section 4.2.2).

5.2.3: Chemicals

The majority of volatile compounds used throughout the research are found naturally in citrus fruit (Njoroge *et al.* 2005; Barboni *et al.* 2009; Carmen Gonzalez-Mas *et al.* 2011; Espina *et al.* 2011) and were purchased from Sigma (U.K). These were (-)-linalool, (-)-terpinen-4-ol, (-)-α-pinene, (-)-β-pinene, (+)-linalool, (+)-α-pinene, (R)-carvone, (S)-carvone, 1-octen-3-ol, citral, decanal, (E)-2-hexenal, geranyl acetate, limonene, methyl jasmonate, methyl salicylate, myrcene, neryl acetate, nonanal, octanal, valencene, β-caryophyllene and γ-terpinene All chemicals were ≥ 81% pure. The essential oils of oranges and lemons were also used – these were obtained from Paramount Citrus (California, U.S.A).

5.2.4: Incubation Conditions

See Section 2.2.3 - 22°C and 97% RH delivered by saturated salt solution of potassium sulfate (K_2SO_4).

5.2.5: Inoculation of Fruit

A fungal spore suspension of *B. cinerea* was prepared by pouring approximately 20 mL distilled water into a minimum-five-day-old plated culture. The contents were then swirled gently to favour detachment of conidia. A 20 mL disposable sterile luer slip syringe (Plastipak, U.S.A) was then used to transfer the conidia suspension obtained to a sterile glass vial. A further volume of distilled water was then added to the vial to give a final volume of 50 mL and the contents were mixed for approximately 10 seconds using a vortex mixer. The concentrations of spores in the suspensions were adjusted to $10^6 – 10^7$ spores/mL using a hemocytometer. Individual strawberry fruits were sorted into groups of equal numbers and approximately the same weight – the numbers of groups dependent on how many treatments were being implemented and how many strawberries there were. There were, however, a minimum number of fifteen individual fruit in each. Each group of strawberries were then placed in small (24 X 15 X 6 cm) PP5 plastic baskets (Wham Products, UK) and then weighed. A 10 µL drop of the spore suspension was then pipetted onto the calyx (stem) end of each individual fruit. The baskets of strawberries were then placed in the incubation boxes.

5.2.6: Volatile Delivery Method

To dispense the volatile compounds to the inoculated fruit, 200 µL of each was pipetted into individual 35 mm Petri dishes. Each one was then placed into one of the boxes containing the strawberries for experimentation 24 hrs after inoculation.

5.2.7: Data Collection and Analysis

The inoculated fruit were placed in incubation boxes (See Section 2.2.3: 22 °C, 97% RH) into each of which was then placed an open Petri dish containing a volatile compound.

Another container, to which inoculated fruit were added without any volatile compound, was also included in the assay as a control. After three days the baskets of fruit were removed from the boxes and re-weighed to ascertain how much weight/water they had lost. The size of each lesion induced by the fungal pathogen on each individual fruit was also measured and recorded. The assay was performed eight times with various volatile compounds – three times with organic strawberries and five times with non-organic. The total minimum number of replicate fruit for each treatment across all experiments was fifteen.

The mean values for weight loss (g) and lesion diameter (mm) were calculated for each treatment in each experiment. The means obtained from the control treatment were then used as the base-line value and the means from the volatile treatments converted into percentage differences compared to the control.

Mean values and standard errors were calculated with Microsoft Excel 2010 (Microsoft Corporation, Washington, U.S.A). The data for non-organic strawberries was then subjected to univariate analysis of variance (ANOVA) using the General Linear Model (GLM). Equality of variances were checked using the Levene's Test and, if equal, the least significant difference (LSD, $p = 0.05$) between means for multiple comparisons was calculated. If equal variances could not be assumed, then the non-parametric Games-Howell test was used for multiple comparisons between treatments. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses. The data for organic strawberries was not subjected to statistical analysis as there was not enough information to do so with any credibility.

5.3: Results

5.3.1: Organic Strawberries and Volatiles

The ketone, (R)-carvone was the only volatile compound that was consistently effective at reducing the growth of *B. cinerea* in inoculated organic strawberries (Figure 5.01). It was one of a few compounds (the others being (+)-linalool and (-)-α-pinene) that were used in three separate experiments. The remaining results for the effect of the volatiles on inoculated organic strawberries were mixed, as the majority of the compounds were only used twice due to the difficulties of obtaining organic strawberries from local suppliers. Many of the compounds (β-caryophyllene, valencene, limonene, (+)-α-pinene, γ-terpinene, myrcene, geranyl acetate, nonanal, octanal, (-)-terpinene-4-ol) induced mean lesion sizes compared to the control that were higher on one occasion and lower on the other. The others (neryl acetate, (-)-β-pinene, citral, (E)-2-hexenal and (-)-linalool) may have displayed consistent efficacy against the pathogen, but these were only utilized twice so the data obtained was not conclusive.

Most of the volatile compounds promoted water/weight loss in the fruit compared to the controls (Figure 5.02). In particular, the ester neryl acetate had exceptionally negative effects on this parameter, with a 270% increase in water/weight loss in the strawberries subjected to this treatment. The volatile that had the greatest positive effects was the monoterpene (-)-β-pinene, with a reduction of > 60%. The analysis of water/weight loss in inoculated organic strawberries treated with volatiles was only done in a single experiment with the majority of compounds. Of the four compounds that were utilized twice, two of them decreased water/weight loss in the fruit on both occasions (citral and (+)-linalool), whilst (-)-α-pinene had the opposite effect.

The shortage of data with organic strawberries was mainly due to the difficulties of obtaining enough organic fruit for experimentation. Regarding measuring weight/water loss, this was exacerbated by initial complications in devising a method for weighing ‘mouldy’ fruit.

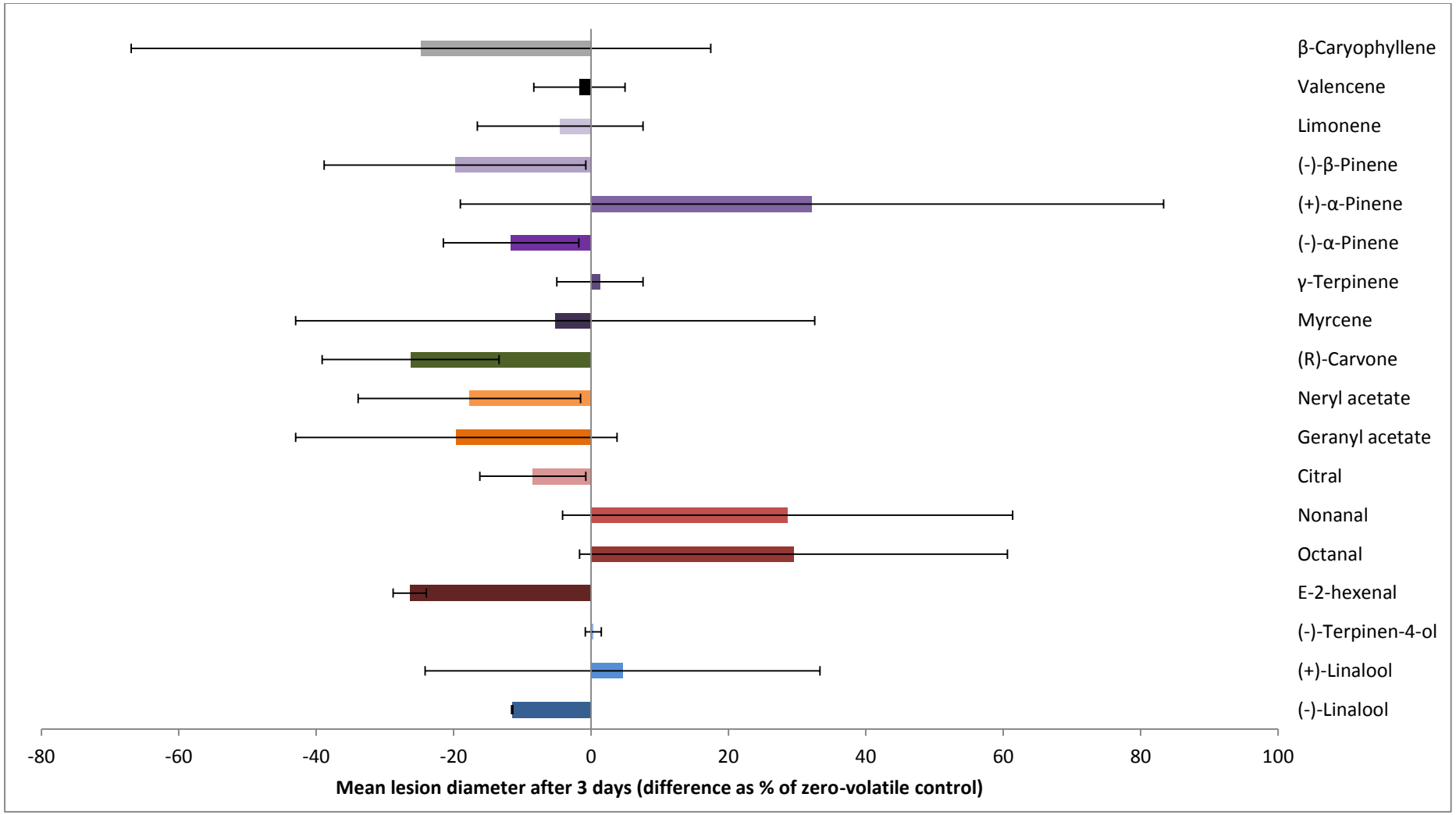

Figure 5.01: Effects of different volatile compounds on the infection of *B. cinerea* on organic strawberries compared to untreated fruit. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones; Purples = terpenes; Greyscale = sesquiterpenes. Different shades of the same colour (darkest → lightest) denote individual compounds within each chemical group from lowest molar mass → highest. Error bars = ± 1 S.E.

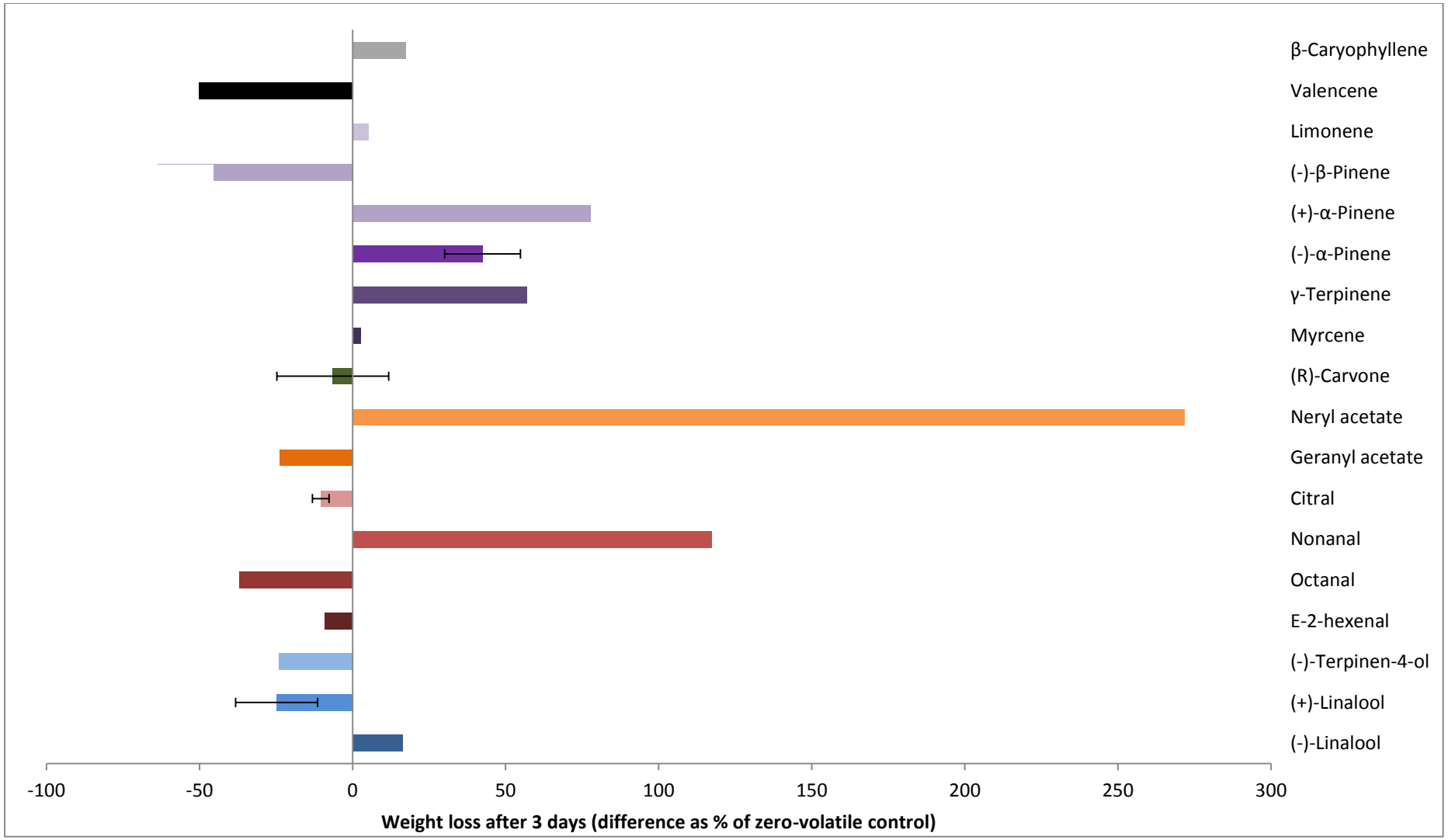

Figure 5.02: Effects of volatile compounds on the weight/water loss of organic strawberries inoculated with *B. cinerea* compared to untreated fruit. All values except those for (-)-α-pinene, (R)-carvone, citral and (+)-linalool are absolute and not means. Other details as in legend for Figure 5.01.

5.3.2: Non-organic strawberries and volatiles

Regarding mean lesion sizes of *B. cinerea* in inoculated non-organic strawberries (Figure 5.03), there were a number of volatile compounds which consistently reduced its growth compared to the controls. These were the alcohol (+)-linalool, the aldehyde citral, the ketone (R)-carvone, the monoterpenes (-)-α-pinene and (-)-β-pinene, plus the ester methyl salicylate. The most effective overall was (+)-linalool, which inhibited fungal growth by an average of > 50% and was significantly different than the controls (LSD, $p = 0.021$). Conversely, a couple of compounds induced infection in the fruit in a consistent manner. These were γ-terpinene and 1-octen-3-ol. The rest of the compounds did not have reliable effects in either direction.

As for weight/water loss of the volatile-treated fruit compared to the controls (Figure 5.04), there were also a number of compounds that consistently reduced this parameter, though these were not the same as those that reduced lesion size. They were the alcohols 1-octen-3-ol and (-)-terpinen-4-ol, the aldehydes (E)-2-hexenal, octanal and decanal, the ester neryl acetate, the monoterpene myrcene, the sesquiterpene valencene and both lemon and orange oil. However, none of these were significantly different to the controls according to the Games-Howell *post hoc* test. Neryl acetate had the highest efficacy, with an average reduction in weight/water loss compared to the controls of ~46%, which was in complete contrast to the results on this parameter in organic strawberries. The remaining volatiles were not consistent in their effects – sometimes promoting water/weight loss in the fruit, whilst on other occasions repressing it in comparison to the controls.

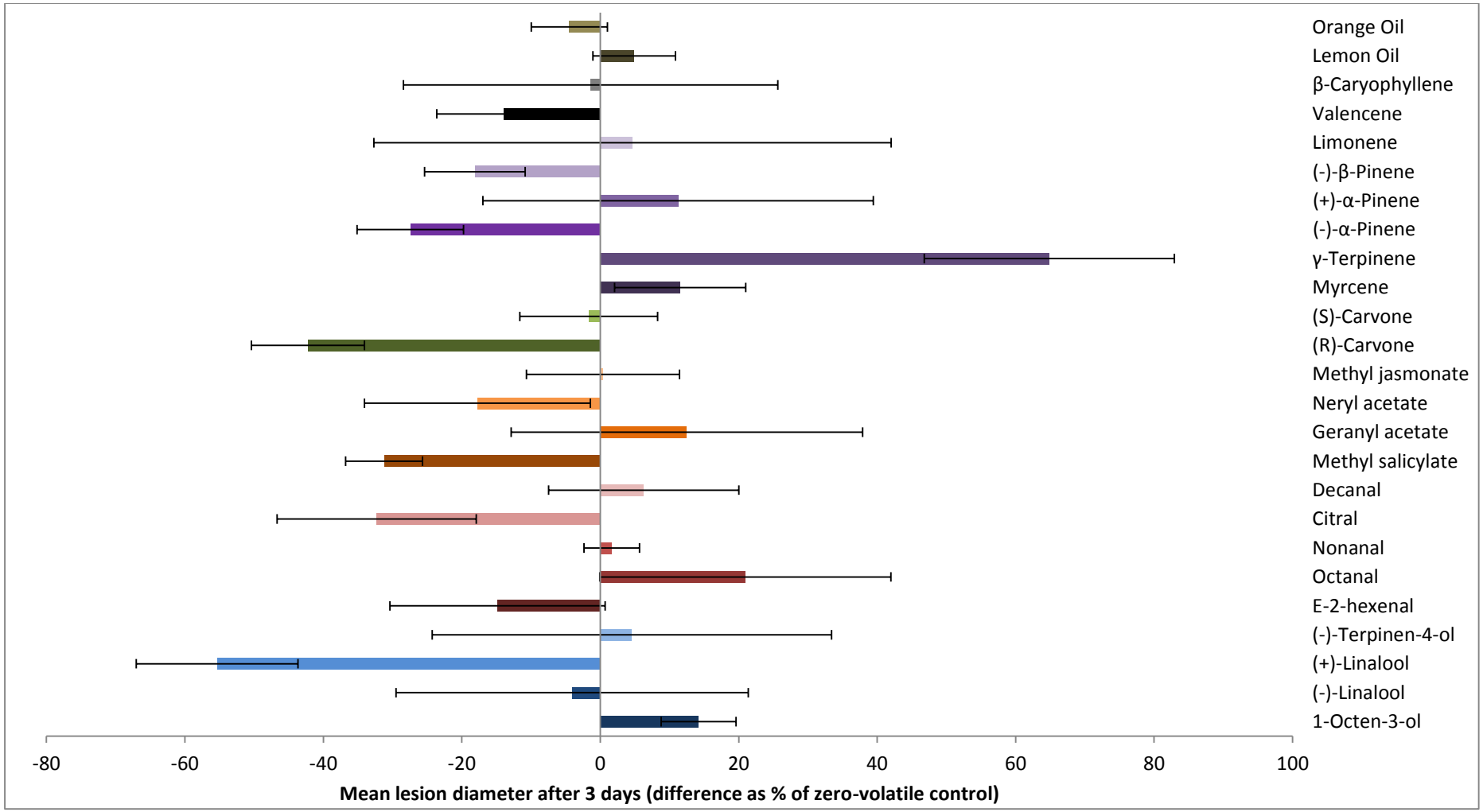

Figure 5.03: Effects of volatile compounds on the infection of *B. cinerea* on non-organic strawberries compared to untreated fruit. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones; Purples = terpenes; Greyscale = sesquiterpenes. The two brown shades represent the two essential oils. Different shades of the same colour (darkest → lightest) denote individual compounds within each chemical group from lowest molar mass → highest. Error bars = ± 1 S.E.

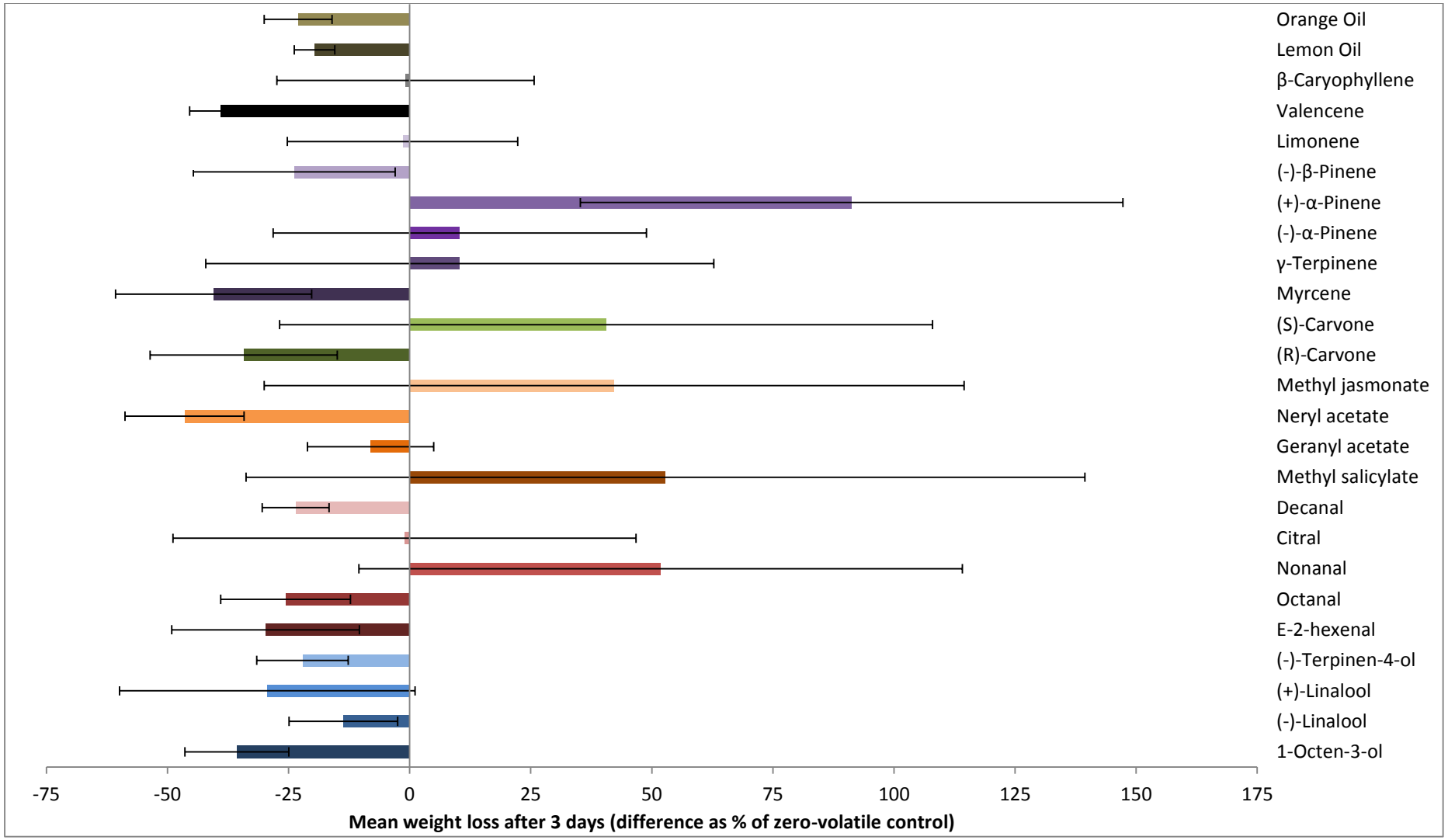

Figure 5.04: Effects of volatile compounds on the weight/water loss of non-organic strawberries inoculated with *B. cinerea* compared to untreated fruit. Other details as in legend for Figure 5.03.

5.4: Discussion

The *in vivo* experiments with volatile compounds and strawberries produced some mixed results. Only (R)-carvone consistently reduced *B. cinerea* growth in both organic and non-organic fruit (Figures 5.01 & 5.03). It was also effective in reducing water/weight loss in the non-organic fruit (Figure 5.04). If we disregard the lack of data for organic strawberries (particularly in reference to water/weight loss), there were a number of compounds that had positive effects. These were (-)-linalool, (+)-linalool, (E)-2-hexenal, neryl acetate and (-)-β-pinene. Focusing just on non-organic fruit, a couple of the other compounds displayed very good efficacy in either one or the other aspects of fruit health, and had good (but not consistent) effects in the other. These were valencene and orange oil.

Vaughn and colleagues (1993) evaluated the effects of volatile compounds on strawberries and raspberries inoculated with *A. alternata*, *B. cinerea*, and *C. gloeosporioides*, and found that (E)-2-hexenal exhibited good antifungal activity, but also induced phytotoxic effects on the fruit (Vaughn *et al.* 1993). Similar consequences were also observed by Archbold and others (1997), who looked at a number of different volatiles and their *in vivo* effects on *B. cinerea*-inoculated strawberries, blackberries and grapes. This particular aldehyde inhibited fungal growth in all three types of fruit, and had deleterious effects on strawberries but not blackberries or grapes (Archbold *et al.* 1997). The other comparable compound, MeS, was effective against *B. cinerea* on strawberry and blackberry and did not negatively affect the fruit, but failed to inhibit the fungus in grapes. Our studies delivered almost the opposite results, with (E)-2-hexenal reliably reducing water/weight loss in the fruit and lacking some consistency in regards to minimising fungal growth, whilst MeS prevented the growth of *B. cinerea* and yet promoted negative effects on the strawberries. Archbold and colleagues (1997) also selected (E)-2-hexenal as a model test volatile to examine what interactions it was having in the three types of fruit. The fruit metabolised the compound, the major reactions being the reduction of the aldehyde to an alcohol and saturation of the carbon-carbon double bond adjacent to the carbonyl. However, strawberry metabolised more of the compound and yielded more esters (such as hexyl acetate and (E)-2-hexenyl acetate) than either blackberry or grape, which may have been a contributing factor to the phytotoxic effects

observed. The volatile concentrations used in our experiments (0.0125µL/mL) were a little different to those in both of these previous studies. Archbold and colleagues (1997) used (E)-2-hexenal at 2 µL in 250 mL (0.008 µL/mL) and methyl salicylate at 12 µL in 250 mL (0.048 µL/mL), whilst Vaughn and others (1993) used (E)-2-hexenal at 0.4 µL/mL. Perhaps this made some contribution to the different effects we observed, but as our value was somewhere in the middle of the two this seems unlikely. The fungal suspension concentrations were also alike, the only difference in this respect being their application. We inoculated each individual fruit with a 10 µL drop, whereas both previous investigations sprayed the suspensions onto the fruit. Neither of these inconsistencies in method adequately explains our differing results, but our combined findings certainly present evidence that both these compounds show potential as post-harvest treatments for strawberries and perhaps other fruits too.

A more recent study also examined the effect of (E)-2-hexenal on *B. cinerea* in order to elucidate its mode of action (Myung *et al.* 2007). The aldehyde was radiolabeled and exposed to fungal cultures, after which both protein and lipid fractions of the fungus were examined. Radiolabel was recovered in protein-enriched but not lipid-enriched fractions and was incorporated into conidia at higher levels than mycelia. Changes in the profile of > 33% of all proteins were observed. In our work, the unsaturated aldehydes, (E)-2-hexenal and citral, were the most effective in reducing the growth of the fungal pathogens, corresponding to a report by Hamilton-Kemp et al. (1992). In this study, (E)-2-hexenal (a C_6 unsaturated aldehyde) was shown to inhibit hyphal growth of *B. cinerea* more effectively than C_9 aldehydes, which the authors put down to the compound's size and its carbon-carbon double bond. Treatment with C_6 aldehydes has also been reported to increase resistance of Arabidopsis against *B. cinerea*, causing lignification of plant tissues and an accumulation of antifungal compounds (Kishimoto *et al.* 2005), whilst another study showed that unsaturated aldehydes can react with thiol groups in proteins or form charge transfer complexes with electron donors (Kurita *et al.* 1979). Citral, the other unsaturated aldehyde in our studies, also showed better antifungal activity than the saturated aldehydes, and was only unsatisfactory overall in regards to water/weight loss in the non-organic strawberries. In an investigation by Camele and others (2012), citral was also found to be active against *B. cinerea,* whilst the other comparable compounds in our study, β-pinene and γ-terpinene, had no effect (Camele *et al.* 2012). Citral has also been

reported to be inhibitory to *A. flavus* (Luo *et al*. 2002). All these observations indicate that unsaturated aldehydes, such as (E)-2-hexenal and citral, are very promising potential antifungal treatments for safeguarding fresh produce after harvest. If the risks of phytotoxicity (whether it results in increased water/weight loss or other deleterious effects) can be addressed by changing the concentrations and/or application method of the volatiles to the fruit, there may be the possibility that one or a combination of these compounds could be put into commercial use in the future.

As mentioned earlier, β-pinene and γ-terpinene were also examined for their antifungal activity against *B. cinerea* by Camele and colleagues (2012), but their results were somewhat different from ours. They reported no differences with these compounds compared to the controls (Camele *et al.* 2012), whereas we had predominantly negative outcomes with γ-terpinene and positive effects with (-)-β-pinene. Their work was *in vitro*, as was that by Espinosa-Garcia and Langenheim (1991), although here they found γ-terpinene was inhibitory to *B. cinerea* (amongst other redwood pathogens) - yet another contradictory account (Espinosa-Garcia and Langenheim 1991). Our own work seems to indicate that (-)-β-pinene is perhaps having an effect on the fruit itself rather than the pathogen, and the same could be said about a number of the other volatiles, particularly neryl acetate, valencene and orange oil. The essential oil of orange contains a mixture of many different volatile compounds (predominantly limonene), so it was probably a combination of one or more of these that provided the positive effects observed (Flamini *et al.* 2007). The essential oil of *Thymus vulgaris* was also found to be highly effective against *B. cinerea* and *R. stolonijer* (Reddy *et al.* 1998), which the authors put down to the high concentrations of thymol, carvacrol and linalool. Terpinen-4-ol was also a major constituent of the oil, but this was not considered to be an antifungal compound, although the results of our studies are not consistent with that. Terpinen-4-ol had similar efficacy to that of the (-) enantiomer of linalool, so one can only presume that the linalool Reddy and colleagues refer to is the (+) enantiomer, which was the most effective out of all the tested alcohols on strawberries.

There was no pathogen growth when MeS was introduced to the atmosphere of inoculated fruit in the *in vivo* studies with non-organic strawberries. However, it promoted water/weight loss in the fruit by > 200% compared to the controls in one

experiment (Figure 5.04), so it would seem its activity may be the opposite to that of (-)-β-pinene, affecting the pathogen rather than the fruit. The investigation by Archbold and colleagues (1997) is in contrast to ours, in that MeS showed the most promise as a post-harvest treatment as it displayed high antifungal activity whilst causing no phytotoxic effects on the fruit. Concentrations of the volatile in our experimental boxes were approximately a quarter of those in their assay (0.0125 µL/mL versus 0.048 µL/mL), but one would think that it would be higher concentrations that would perhaps have deleterious effects on biological systems rather than lower. Methyl salicylate is associated with plant defence against insect herbivores (Hardie *et al.* 1994; Pare and Tumlinson 1996), whilst MeJ is a plant signalling molecule that plays a key role in plant growth and development with responses to biotic and abiotic stresses (Droby *et al.* 1999; Yao and Tian 2005b). Jin and colleagues (2009) treated peaches with MeJ and then inoculated them with *P. expansum, B. cinerea* or *R. stolonifer* spore suspension after 12 hours. Their results showed that MeJ could reduce disease incidence for all three pathogens in inoculated peaches (Jin *et al.* 2009), in contrast to ours where MeJ stimulated both pathogen growth and water/weight loss in strawberries.

The ketone, (R)-carvone was probably the most effective volatile compound in our *in vivo* work with strawberries and *B. cinerea*. (R)- and (S)-carvone, were used in an *in vitro* study by Combrinck and colleagues (2011), where it was reported that the (R)-enantiomer was the better pathogen inhibitor of the two (Combrinck *et al.* 2011). Of all the compounds tested in our work with *B. cinerea*, the (R)-enantiomer of carvone plus (E)-2-hexenal proved to be the most successful, with no negative results in any of the completed tests. Some of the other volatiles showed promise too, but the phytotoxic effects (i.e. increased weight/water loss) sometimes encountered would need to be considered in any future work. It may mean that long-term treatments could not be considered, or it may be that concentrations need to be reduced and perhaps combinations of two or more compounds utilised at the same time. Nonetheless, if appropriate application strategies can be developed, some of these volatile compounds could well be developed as antifungal treatments of the future.

Chapter 6: Effect of Citrus Volatiles on the Growth of *Botrytis cinerea in vitro*

6.1: Introduction

The post-harvest decay of fruit such as strawberries is predominantly caused by the fungal pathogen *B. cinerea* (Ceponis *et al.* 1987), and the use of fungicides is key to reducing loss of yield pre-harvest and loss of quality post-harvest (Eckert and Ogawa 1988; Carvalho 2006). However, postharvest pathogens are exhibiting growing resistance to synthetic fungicides (Spotts and Cervantes 1986; El-Ghaouth 1997), and in recent years there has been considerable pressure by consumers to reduce or eliminate the use of synthetic chemicals associated with food production (Ragsdale and Sisler 1994; Lanciotti *et al.* 2004).

Sulphur dioxide (SO_2) as a fumigant for the control of *B. cinerea* on grapes came into standard use in 1928 (Eckert and Sommer 1967) and it is still being used as such, in addition to a preservative in wine making and for dried fruit and in fruit juices (Ogawa *et al.* 1975; Eckert 1977; Wills *et al.* 1998). However, studies have shown that some people are hypersensitive to it (Gunnison and Jacobsen 1987) and since the mid-1980s its use has been eliminated or reduced (Smilanick *et al.* 1990). The fungicide dichloran (2,6-dichloro-4-nitoaniline) was introduced as a control against infection by *B. cinerea* and *Rhizopus* species in the early 1960s and was used successfully as both a spray (on strawberries) and a fruit-brushing application, particularly on peaches (Eckert and Sommer 1967; Eckert 1977). It is still registered for use against *R. stolonifer* on peaches (Adaskaveg 2002), but it has not been used on strawberries for many years. This is due to increasing fungal tolerance to these treatments, as well as unease about its toxic effects on fish when it gets into water courses (Webster and Ogawa 1970; Readman *et al.* 1997). Two benzimidazoles - thiabendazole (2-(4'-thiazolyl)-benzimidazole) (TBZ) and benomyl (methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate) - were introduced as fungicide treatments in the late 1960s (Eckert and Sommer 1967; Eckert 1977). They had systemic activity and their ease of use and long-term antifungal effects were welcomed by the industry. However, the development of strains of *B. cinerea* that were resistant to

these benzimidazoles (Erwin 1973; Miller and Fletcher 1974) and the toxicity of benomyl to invertebrates, particularly earthworms (Sorour and Larink 2001), meant that alternative treatments had to be sought. The most widely-used of these is the phenylpyrrole fungicide, fludioxonil (FLU). It is a synthetic analogue of pyrrolnitrin, a secondary metabolite produced by the bacterium Pseudomonas that has antifungal and antibiotic properties (Rosslenbroich and Stuebler 2000). It has been used for both pre- and post-harvest control of *B. cinerea* for around twenty years and works by inhibiting spore germination, germ tube elongation and mycelium growth (Leroux 1996; Rosslenbroich and Stuebler 2000). However, resistance to this fungicide has been increasing (Ziogas and Kalamarakis 2001) and alternatives are being sought, amongst them the possible use of natural volatile compounds.

Reports on the antifungal effects of some of these compounds on *B. cinerea* are numerous (Wilson *et al.* 1987; Andersen *et al.* 1994; Fallik *et al.* 1998; Archbold *et al.* 1999; Wolken *et al.* 2002; Hamilton-Kemp *et al.* 2003; Chitarra *et al.* 2004; Mercier and Smilanick 2005). Archbold and colleagues (1999) reported that (E)-2-hexenal was effective against *B. cinerea* on seedless table grapes, whilst Fallik and others (1998) found that whilst concentrations below 0.5 mol/L stimulated the pathogen *in vitro*, concentrations above it were inhibitory. Other volatile compounds, such as acetaldehyde (Prasad and Stadelbacher 1974; Avissar *et al.* 1990; Avissar and Pesis 1991) and benzaldehyde (Wilson *et al.* 1987) have also been shown to inhibit *B. cinerea* both *in vitro* and *in vivo*.

This present study aimed to investigate the *in vitro* effects of a number of volatile compounds naturally found in citrus fruit on *B. cinerea*. Some of these compounds have been examined before (Hamilton-Kemp *et al.* 1992; Vaughn *et al.* 1993; Archbold *et al.* 1997; Myung *et al.* 2007; Zhao *et al.* 2011; Camele *et al.* 2012), whilst others have not to our knowledge. In addition to exploring the influences on the pathogen of the volatiles alone, possible interactive or supplementary effects were also investigated by combining them with a commercially available fludioxonil fungicide formulation.

6.2: Materials and Methods

6.2.1: Media

6.2.1.1: Standard agar

Mycological potato dextrose agar (PDA) powder (Oxoid Microbiology Products, U.K) was added to distilled water (39 g/L), mixed thoroughly and then autoclaved at 121°C for 20 mins. Once sterilized, the agar was cooled to 50°C in a water bath prior to being poured into sterile 90 mm plastic Petri dishes to approximately 3 mm depth.

6.2.1.2: Amended agar – ascertaining MIC

The additive or synergistic effects between the experimental volatile compounds and commercial fungicides were investigated. Obtaining postharvest *B. cinerea* fungicides in the U.K. proved difficult, but eventually a fludioxonil-based fungicide (Geox 50 WG) containing 500 g/kg active ingredient was obtained from Syngenta (Basel, Switzerland).

To ascertain minimum inhibitory concentration (MIC), PDA was prepared according to Section 4.2.1.1 and mixed with different concentrations (0, 0.01, 0.1, 1.0, 10 and 100 µg/mL) of the fungicide once it had cooled. The amended agar was then poured into Petri dishes to a 3 mm depth and allowed to set.

6.2.1.3: Amended agar - MIC50

Minimum inhibitory concentration (MIC) was difficult to establish as all concentrations used inhibited *B. cinerea* to some degree (see Section 6.3.2, Figure 6.02). The minimum inhibitory concentration required to inhibit the growth of 50% of the organisms (MIC50) was therefore calculated and used to amend fresh PDA (Section 6.3.2).

6.2.2: Pathogens

6.2.2.1: Initial isolation and identification

Strawberry fruit from the weight-loss experiments (Section 2.3.6) were retained in a lidded container at 22°C for a further 48 hrs after measurements had been taken. After this time period, the majority of fruit had developed a grey, downy mould on their surface. The fungus was thereafter isolated following the procedure in Section 4.2.2.1.

To identify the fungal species, slides were made of the five-day-old cultures. Using a sterile inoculation loop, spore samples were transferred to glass microscopy slides and a drop of deionised water added before a cover slip was placed on top. The spores were then examined under an optical microscope at x 400 magnification and compared to those in an identification guide (Kendrick 2000). These observations together with information on the major pathogens of strawberry fruit (Dennis and Mountford 1975; Eckert 1977; Eckert and Ogawa 1988) led to the conclusion that the fungus was *B. cinerea*.

6.2.2.2: Maintenance of cultures

To maintain the viability of the fungal cultures, they were subcultured onto fresh media at least once a month (Section 4.2.2.2). Loss of viability was noted when subcultured *B. cinerea* failed to grow on fresh media. Replacement samples were obtained by purchasing a 250 g punnet of organic British strawberries (Waitrose, Menai Bridge, U.K) and storing them in a conditioning room at 22°C for five days until they had developed *B. cinerea* infection. Spores were thereby isolated and cultured in the same way as Section 4.2.2.1.

6.2.2.3: Fungal suspensions

For experimental use, fungal spore suspensions were required. These were prepared by pouring approximately 20 mL distilled water into a minimum-five-day-old plated culture. The contents were then swirled gently to favour detachment of conidia. A 20 mL disposable sterile luer slip syringe (Plastipak, U.S.A) was then used to transfer the conidia suspension obtained to a sterile glass vial. A further volume of distilled water was then

added to the vial to give a final volume of 50 mL and the contents were mixed for approximately 10 s using a vortex mixer.

6.2.3: Chemicals

The majority of volatile compounds used throughout the research are found naturally in citrus fruit (Njoroge *et al.* 2005; Barboni *et al.* 2009; Carmen Gonzalez-Mas *et al.* 2011; Espina *et al.* 2011) and were purchased from Sigma (U.K). These were (-)-linalool, (-)-terpinen-4-ol, (-)-α-pinene, (-)-β-pinene, (+)-linalool, (+)-α-pinene, (R)-carvone, (S)-carvone, 1-octen-3-ol, citral, decanal, (E)-2-hexenal, geranyl acetate, limonene, methyl jasmonate, methyl salicylate, myrcene, neryl acetate, nonanal, octanal, valencene, β-caryophyllene and γ-terpinene All chemicals were ≥ 81% pure. The essential oils of oranges and lemons were also used – these were obtained from Paramount Citrus (California, U.S.A).

6.2.4: Inoculation of Media and Volatile Delivery Method

Fungal spore suspensions were made from previously cultured samples of *B. cinerea*. The concentrations of spores in the suspensions were adjusted to 10^6 - 10^7 spores/mL using a hemocytometer. PDA was prepared according to the required procedure (Section 6.2.1) and poured into a number of Petri dishes. Once set, a 10 µL drop of fungal spore suspension was placed in the centre of the agar in each dish using a sterile Gilson pipette and the lids immediately replaced. The fungal samples were then incubated for 24 hours at 22°C in a darkened conditioning room before treatment.

In a fume cupboard, 100 µL of each volatile compound was pipetted into an aluminium-backed polyethylene slow-release packets (obtained from Agrisense, U.K.; a minimum of 3 replicates for each compound). The packets were then immediately sealed with an impulse heat sealer (PFS-400, Willis European Ltd, U.K) and attached to the inside lid of each inoculated Petri dish with double-sided tape (Scotch™). As a control, a minimum of 3 empty but sealed slow-release sachets were also utilized in the same manner. All dishes were labelled, sealed with Parafilm and then incubated for 3 days at 22°C in a darkened conditioning room.

6.2.5: Data Collection and Analysis

The inoculated Petri dishes (with or without the volatile compounds) were incubated at 22°C for three days. After this time, the extent of fungal pathogen growth was taken by using a steel caliper (Tool Box, U.K) to measure the diameter (mm) of the area on each plate where spores/hyphae could be visibly detected (see Section 4.2.5, Figure 4.01). These were recorded as colony size (mm) and mean values calculated for each different treatment. The means obtained from the control (i.e. those without the addition of fungicide and/or a volatile compound) were then used as the base-line value of zero and the means from the treatments converted to percentage differences compared with the control.

Mean values and standard errors were calculated with Microsoft Excel 2010 (Microsoft Corporation, Washington, U.S.A) and then subjected to univariate analysis of variance (ANOVA) using the General Linear Model (GLM). The equality of variances were checked using the Levene's Test and, if equal, the least significant difference (LSD, p = 0.05) between means for multiple comparisons was calculated. If equal variances could not be assumed, then the non-parametric Games-Howell test was used for multiple comparisons between treatments. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses.

The assays were performed a number of times. However, the minimum number of replicates for each treatment and controls was nine.

6.3: Results

6.3.1: *Botrytis cinerea* and volatiles

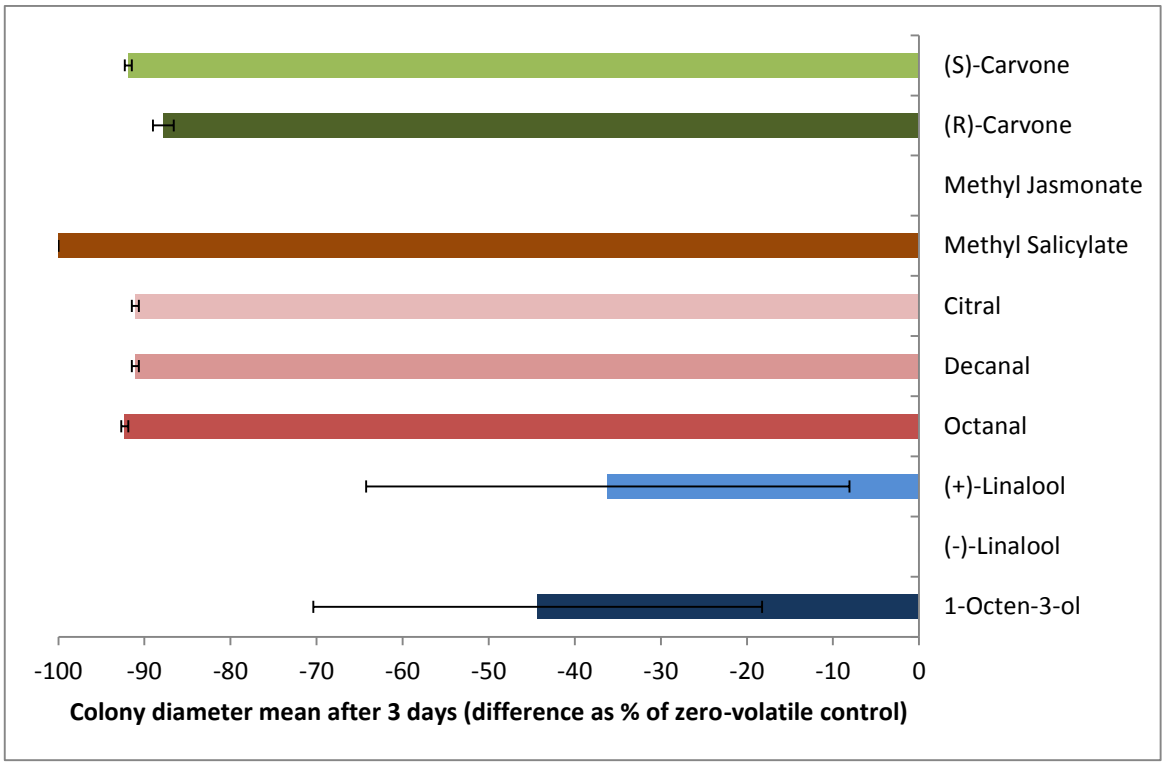

Figure 6.01: Effects of volatile compounds on the growth of *Botrytis cinerea in vitro*. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones. Different shades of the same colour (darkest → lightest) denote individual compounds from that with the lowest molar mass within its chemical group to the highest. Error bars = ±1 S.E.

A number of volatile compounds induced a significant ($p \leq 0.05$) reduction in the growth of *B. cinerea* compared to the zero-volatile control. These were the aldehydes octanal, decanal and citral, the ketones (R)-carvone and (S)-carvone, plus the ester methyl salicylate (Figure 6.01). Methyl salicylate (MeS) resulted in 100% inhibition of the pathogen, whilst the aldehydes and ketones all consistently provided ~ 90%. The alcohols tested proved to either have no effect on fungal growth at all, as in the case of (-)-linalool, or be very inconsistent in their influence. Both (+)-linalool and 1-octen-3-ol had stimulatory and inhibitory effects, as can be seen by the sizeable standard errors (Figure 6.01).

6.3.2: *Botrytis cinerea* and amended agar – MIC establishment

Figure 6.02 displays the results of the assay to determine MICs for the fludioxonil fungicide. The minimum inhibition achieved was ~46% by a concentration of 0.01 µg/mL, whilst 100% inhibition was attained at 100 µg/mL. As all the tested fungicide concentrations inhibited the pathogen growth to some extent, MICs could not be ascertained. The alternate criterion of minimum inhibitory concentrations required to inhibit 50% of the organisms (MIC50s) was determined from Figure 6.02 instead. This was calculated to be 0.02 µg/mL, and this concentration was then used for the *in vitro* study combining fungicide-amended agar and volatile compounds (Section 6.3.3).

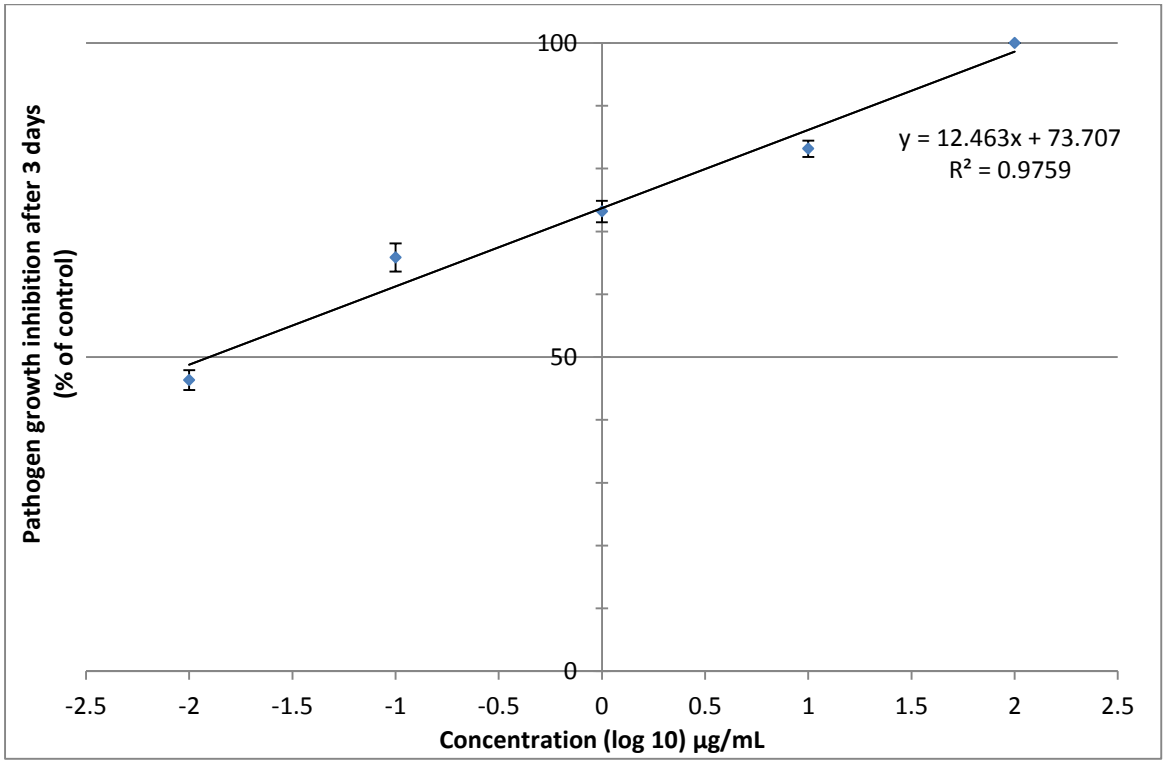

Figure 6.02: Effects of different concentrations (0.01, 0.1, 1.0, 10 and 100 µg/mL; converted to log10 values) of Geox 50 WG fungicide (FLU) on the growth of *Botrytis cinerea in vitro*. Linear trendline, equation and R^2 value shown. Error bars = ± 1. S.E.

6.3.3: *Botrytis cinerea*, amended agar and volatiles

All the volatile treatments on the fludioxonil-amended agar resulted in reduced colony sizes compared to the zero-volatile control plates (Figure 6.03), and the majority of them were significantly different ($p = 0.05$) according to the Games-Howell *post hoc* test. Those that were not were the alcohols (-)-linalool and (-)-terpinen-4-ol, the esters geranyl and neryl acetate, the terpenes myrcene, limonene and both (+)- and (-)-α-pinene, the sesquiterpene valencene and orange oil. The volatiles that had the greatest inhibitory effect on *B. cinerea* were MeS and the aldehydes, although citral, the only terpene aldehyde, did not produce 100% inhibition like the other compounds. As with the investigation into the effects of the volatiles alone (Section 6.3.1), the two ketones, (R)- and (S)-carvone were also very effective, giving ~ 80% reduced growth. Most of the other compounds, although inhibitory, were rather less so and with less consistency.

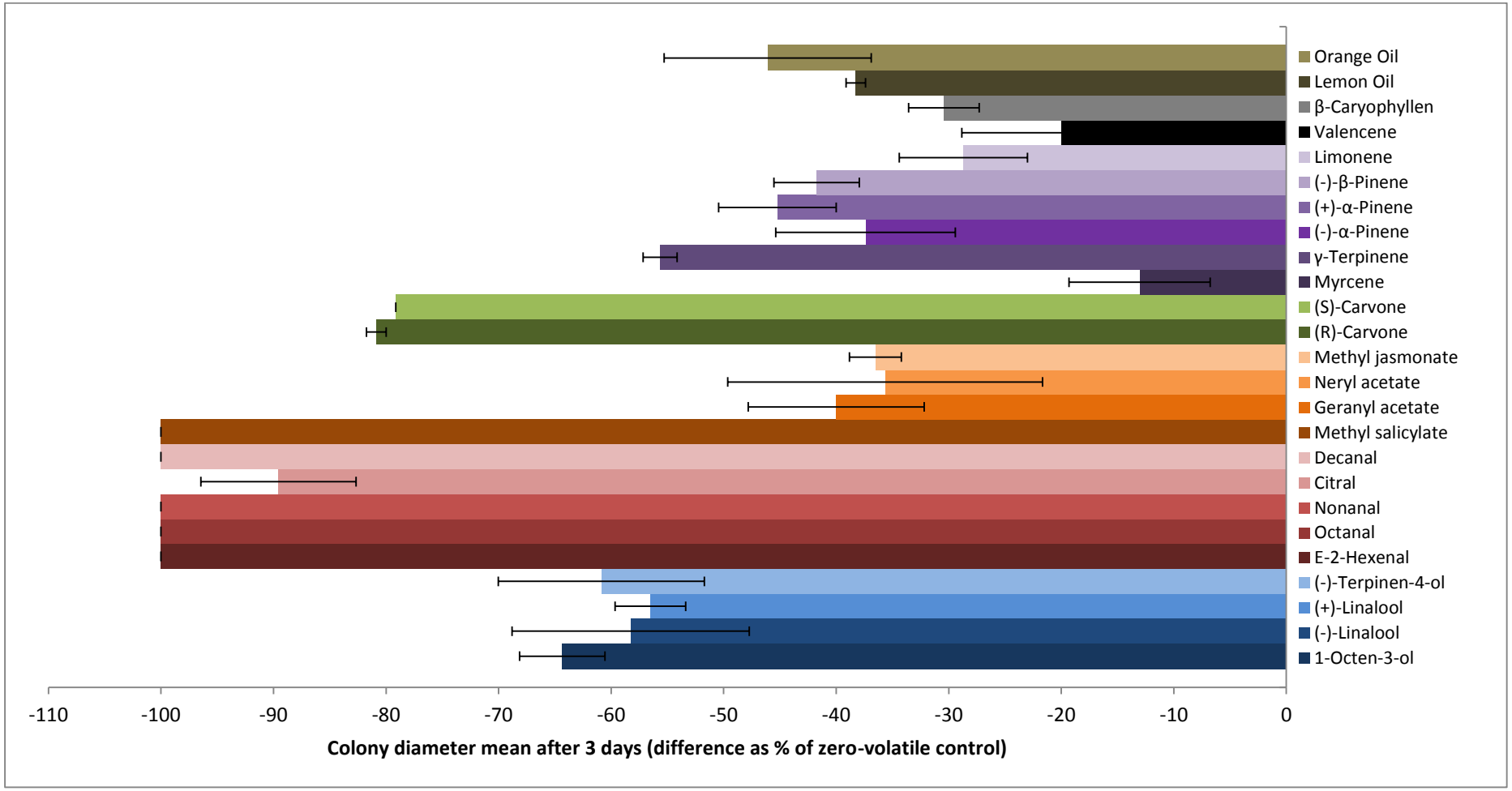

Figure 6.03: The effects of volatile compounds on the growth of *Botrytis cinerea* on PDA amended with Geox 50 WG fungicide (FLU) at MIC50. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greens = ketones; Purples = terpenes; Greyscale = sesquiterpenes; Browns = pure fruit oil extracts. Different shades of the same colour (darkest → lightest) denote individual compounds within each chemical group from lowest molar mass → highest. Error bars = ±1 SE.

6.4: Discussion

Across the results of all *in vitro* studies conducted regarding the effects of citrus volatiles on the pathogen *B. cinerea*, the principal conclusion is that there are a number of compounds which appear to inhibit or even prevent its growth altogether. As a group, the aldehydes exhibited the highest overall efficacy, whilst methyl salicylate (MeS) stood out as the most effective ester. Ketones, despite there only being two compounds in the studies, also exhibited high activity. This suggests that these particular compounds have properties that affect growth and/or development. If the volatile compounds worked in parallel with the fungicides, the fact that those that proved the most effective did so whether acting alone or in amended agar is not surprising.

Methyl salicylate was the most effective volatile compound in our studies with *B. cinerea* (Figures 6.01 and 6.03). To our knowledge, very few authors have previously examined this compound in relation to this particular pathogen, possibly due to it being mainly associated with plant defence against insect herbivores (Hardie *et al.* 1994; Pare and Tumlinson 1996). However, Archbold and colleagues (1997) inoculated a number of different fruit types with *B. cinerea* and treated them with various naturally occurring volatile compounds, including (E)-2-hexenal, nonanal, limonene and methyl salicylate. Of these, MeS showed the most promise as a post-harvest treatment as it displayed high antifungal activity whilst causing no phytotoxic effects on the fruit (Archbold *et al.* 1997). Our *in vitro* studies also demonstrated its high activity against *B. cinerea*, with 100% inhibition both when acting alone and when in combination with the fludioxonil-based fungicide.

As a group, the aldehydes were also very effective in reducing the growth of the pathogen (Figures 6.01 and 6.03), with only citral not fitting quite the same activity pattern as the others. The C_6 unsaturated aldehyde, (E)-2-hexenal has been shown to inhibit hyphal growth of *B. cinerea* by previous authors (Hamilton-Kemp *et al.* 1992; Vaughn *et al.* 1993), and in *in vitro* assays it was found to be more effective than C_9 aldehydes (Hamilton-Kemp *et al.* 1992). This was not the case in our investigations, as (E)-2-hexenal, octanal, nonanal and decanal all proved to have almost identical efficacy. Exogenous treatment with C_6 aldehydes have also been shown to increase resistance of *Arabidopsis thaliana* to *B. cinerea*, causing lignification of plant tissues and an accumulation of antifungal compounds (Kishimoto *et al.* 2005). Later, Kishimoto and

colleagues (2008) examined whether the increased production of C_6 compounds in inoculated *A. thaliana* plants led to decreased susceptibility to the pathogen due to direct fungicidal activity of the compounds or to their signalling properties. They concluded that direct fungicidal activity was predominantly responsible (Kishimoto *et al.* 2008), which correlates with the findings of our *in vitro* studies.

To characterise the interactions of C_6 aldehydes, including (E)-2-hexenal, with *B. cinerea*, Myung and colleagues (2007) exposed the pathogen to radiolabeled compounds. The results showed that the aldehydes caused changes in the protein profile of the fungus, even at sub-lethal levels (Myung *et al.* 2007). Earlier work by Kurita and others (1979) demonstrated that aldehydes have the ability to form charge transfer complexes with electron donors as well being reactive with SH groups, and this is what gives them their antifungal properties (Kurita *et al.* 1979). The mode of action of aldehydes against bacteria was investigated by Trombetta and colleagues (2002), who showed that they caused changes in the permeability of bacterial cell membranes, whilst the theory put forward by Kubo and others (2003) stated that they disrupt hydrogen bonds thus affecting the lipid bilayer of cell membranes by this action (Trombetta *et al.* 2002; Kubo *et al.* 2003). Whether their mode of action is one, or a combination, of these mechanisms, they are effective against both the generalist plant pathogen *B. cinerea* and *Penicillium* pathogens of citrus fruit (see Chapter 4), as our studies have also established.

The other aldehyde in our study, the terpene citral, was not quite as effective against *B. cinerea* as the others in its group when combined with fludioxonil-amended agar (Figure 6.03). Alone, however, it displayed very similar efficacy to that of octanal and decanal (Figure 6.01). In an investigation by Camele and others (2012), it was found that citral was active against *Phytophthora citrophthora* (a phytopathogenic oomycete) as well as *B. cinerea*, whereas the other comparable compounds in our study, β-pinene and γ-terpinene, had no effect on any of the tested pathogens (Camele *et al.* 2012). Citral has also been reported to be inhibitory to *A. flavus*, where its possible mode of action against the pathogen was to have detrimental effects to the plasma membrane of the fungus (Luo *et al.* 2002). This report on citral and those on aldehydes as a group seem to indicate that the antimicrobial activity of aldehydes is primarily focused on the disruption of cell membrane functions, which leads to the inhibition of spore germination and mycelium growth and perhaps ultimately the death of the organism.

The other compounds in our investigation that exhibited high efficacy against *B. cinerea* were the two ketones, (R)- and (S)-carvone. To our knowledge, these have not been examined for their potential effects on this pathogen, although they have been found to be effective against various other pathogenic bacteria and fungi (Oosterhaven *et al.* 1995; Aggarwal *et al.* 2002). These ketones both provided ~ 90% colony size reduction compared to the non-volatile control when working alone, and ~ 80% reduction when combined with fludioxonil-amended agar. This is comparable to the results obtained when we looked at the effects of these compounds on the two *Penicillium* pathogens of citrus fruit (see Chapter 4), so it seems clear that this is an observation worthy of further investigation.

The remaining chemical groups we examined did not perform as well in our studies. Alcohols, terpenes, sesquiterpenes and esters (excepting MeS), although effective compared to the controls on the whole, were not as consistent in their influence on *B. cinerea* and the standard errors of the means were generally very large. This was particularly the case regarding the compounds working alone (Figure 6.01), although only ten out of the whole project's twenty-five volatile selection were utilised. In regards to the assay looking at possibly additive or interactive effects with fungicide-amended agar, the best of the rest were the alcohols 1-octen-3-ol and (+)-linalool, plus γ-terpinene, all of which consistently resulted in > 50% additional inhibition of the pathogen. The fact that the two alcohols did not reliably reduce *B. cinerea* growth without acting in conjunction with fludioxonil is puzzling and suggests that these compounds are perhaps working synergistically with the fungicide rather than having supplementary effects. To our knowledge, 1-octen-3-ol is the only alcohol in our studies to have been shown to be effective against *B. cinerea* by previous authors (Zhao *et al.* 2011), where it inhibited spore germination and caused mycelia to swell and contort. This investigation concerned the volatile independently, as did that by Sikkema and colleagues (1994) where they examined the interactions of hydrocarbons with biological membranes. Four comparable terpenes were utilised in their work (α-pinene, β-pinene, γ-terpinene and limonene), in which they discovered that these compounds could permeate both artificial and bacterial membranes and make them swell. In bacterial membranes, this inhibited respiratory enzymes and led to the dissipation of both the pH gradient and the electrical potential of the cells (Sikkema *et al.* 1994). It is possible that these effects occurred in our

investigations too, however it appears that here these volatiles required the addition of the fungicide to provide efficacy greater than the sum of their individual effects.

The volatile compounds not mentioned so far were generally inhibitory, just rather less so and with less consistency (Figures 6.01 and 6.03). There have been no other investigations with these compounds and *B. cinerea* to our knowledge, but a few authors have had positive results on other microorganisms. For example, Uribe and colleagues (1985) found that both α- and β-pinene inhibited mitochondrial respiratory activity of the yeast *Saccharomyces cerevisiae*, whilst β-pinene also affected K^+ and H^+ leakage in the cell membrane (Uribe *et al.* 1985). Later studies (Uribe and Pena 1990; Uribe *et al.* 1990) confirmed this activity and also reported similar effects with limonene. The essential oils of lemon and orange have also been stated to exhibit antibacterial and antifungal properties (Caccioni *et al.* 1998; Sharma and Tripathi 2006; Sharma and Tripathi 2008; Singh *et al.* 2010; Espina *et al.* 2011), whilst treatment with MeJ has been reported to reduce decay and increase the activities of plant defence compounds in peach (Yao and Tian 2005a; Jin *et al.* 2009), sweet cherry (Yao and Tian 2005b), loquat fruit (Cao *et al.* 2008) and raspberries (Chanjirakul *et al.* 2006).

In conclusion, the present work demonstrated that several volatile compounds have the potential as postharvest treatments to control *B. cinerea*. Other mould-causing fungi, such as *Penicillium* spp, may also be controlled by these compounds (Section 4.4). Aldehydes had the highest efficacy *in vitro*, followed quite closely by ketones, so perhaps their activity has something to do with the carbonyl group they possess. However, the fact that MeS, a completely unrelated compound, also had exceptional inhibitory qualities suggests that this may not be the case, unless MeS has an entirely different mode of action on the pathogen.

It is also clear from our studies that the integration of volatile treatments with commercial products like fludioxonil could provide a means for reducing our reliance on synthetic fungicides. The establishment of alternative decay control programs would help minimize the risks of resistance development, residues on food and environmental pollution. Many studies besides ours have reported the fungicidal activity of volatiles, but further research needs to be addressed to reveal their mode of action, cost-efficacy, and possible phytotoxicity against the fruit/vegetable products they are aimed to protect.

Chapter 7: The Effects of Chitosan and/or Pectin Coatings on Orange fruit, Plus Chitosan Hydrolysis and Analysis

7.1: Introduction

Although ripening in fruits is the start of senescence, they still have innate defence responses against microbial attack that remain primed and capable of activation, at least for a time after harvest (Wilson and Wisniewski 1989). Resistance responses include compartmentalisation of the pathogen within the host tissue at the site of infection, where lignin-like substances are deposited around the wound, thus isolating the pathogen and preventing it from spreading (Brown and Barmore 1983; Baudoin and Eckert 1985). Pectinase and proteinase inhibitors produced by the plant as a defence response also play a role, as they inhibit polygalacturonases secreted by the pathogen (Albersheim and Anderson 1971).

A number of treatments have been shown to elicit defence responses in harvested produce (Wilson *et al.* 1991; Terry and Joyce 2004). These include solutions of chitosan, a polysaccharide produced commercially by partial deacetylation of chitin, the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.) and the cell walls of fungi. The degree of deacetylation in commercial chitosans is in the range 60-100%. Commercial chitosan is therefore a linear polymer of randomly distributed β-(1-4)-linked D-glucosamine (GlcN, deacetylated unit) and N-acetyl-D-glucosamine (GlcNAc, acetylated unit).

Chitosan is a biopolymer that has been reported to elicit natural innate defence responses within plants against a range of pests and pathogens (Allan and Hadwiger 1979; Choi *et al.* 2002; Bautista-Banos *et al.* 2006). For instance, chitosan has been shown to control infection by *B. cinerea* and *P. expansum* in tomatoes by eliciting their biochemical defence responses (Liu *et al.* 2007). The susceptibility of carrots to the pathogen *Sclerotinia sclerotiorum* was also reduced when they were treated with chitosan (Molloy *et al.* 2004), as was that of oranges deliberately inoculated by *P. italicum* and *P. digitatum* (Fajardo *et al.* 1998; Chien *et al.* 2007) and *Guignardia citricarpa* (Rappussi *et al.* 2009). Navel oranges treated with 2% chitosan solutions and stored at 20 °C and 85-95% RH

had a 90.2% reduction in the size of the lesions caused by *P. italicum* and *P. digitatum* than those in control fruit (Deng *et al.* 2008; Zeng *et al.* 2010). In Tankan citrus fruits, the percentage of decay caused by *P. digitatum, P. italicum* or *B. cinerea* was only 10, 18.8 and 37.5% respectively in fruits treated with 0.2% low molecular weight (LMW) chitosan, compared with 100% in control fruit (Chien and Chou 2006). The chitosan-treated fruits also exhibited reduced weight loss compared to untreated samples, as did 2% chitosan treated logan, litchi and 'Murcott' tangor fruit (Zhang and Quantick 1997; Jiang and Li 2001; Chien *et al.* 2007). Experiments conducted on wheat indicated that chitosan with a degree of acetylation (DA) of 65% had good efficacy against *Bipolaris sorokiniana* (Burkhanova *et al.* 2007), whilst chitosan formulations were also effective at controlling *Botrytis cinerea* and *Rhizopus* rot of strawberries (Romanazzi *et al.* 2013). When applied either pre- or post-harvest, chitosan was shown to be very effective in reducing storage decay of sweet cherry induced by *Monilinia laxa*, *B. cinerea* and *R. stolonifer*, whilst *in vitro* and field trials also showed its activity to be comparable to that of fenhexamid fungicides (Feliziani *et al.* 2013).

Chitosan coatings modified with essential oils have also been tested on harvested produce. On strawberries, chitosan modified with limonene was shown to reduce decay caused by *B. cinerea* whilst not causing any phytotoxic effects (Vu *et al.* 2011). Coatings containing tea tree oil gave a reduction of *P. italicum* growth of 50% on Navel oranges compared to uncoated samples (Chafer *et al.* 2012). Cinnamon oil incorporated into chitosan films exhibited good inhibitory effects against *Staphylococcus aureus*, *Aspergillus oryzae*, and *P. digitatum*, although the film alone was not very effective (Wang *et al.* 2011). The oil did display inhibitory activity by itself, but this was increased when incorporated into the film, so the authors suggested that there was an additive or interactive effect occurring that might be related to the constant release of the oil.

Pectin is another polysaccharide, and is commercially derived from the cell wall of higher plants. It has been used in food for many years as a gelling agent in jams and jellies, as well as a stabilizer in fruit juices and milk drinks. Pectin is a complex structure, consisting of three polysaccharides – homogalacturonan (HG, α-(1-4)-D-galacturonic acid units having various degree of methyl-esterification), rhamnogalacturonan I (RGI, (1-2)-α-L-rhamnosyl-(1-4)-α-D-galacturonic acid disaccharide units), and rhamnogalacturonan II (RGII, a homogalacturonan backbone with complex side chains) (Ridley *et al.* 2001).

Since the biological activities of polysaccharides are related to their structural characteristics, chemical modification has often been applied to them in order to increase their functionality. Sulfation, in which hydroxyl groups are replaced with sulfate groups, has been shown to effect physiological functions of polysaccharides such as anticoagulant, anti-tumour and anti-infection activities (Wang *et al.* 2004; Martinichen-Herrero *et al.* 2005), and when applied to pectin was shown to increase its antimicrobial effects against the bacteria *Bacillus cereus* and *Vibrio fischeri* (Bae *et al.* 2009). Pectin of apple origin was also found to be inhibitory to various food-borne pathogenic bacteria, although not quite as effective as another dietary polysaccharide, carrageenan (Yamashita *et al.* 2001).

Chitosan and pectin have also been utilised together. For example, fresh-cut papaya fruit were coated in an edible coating made of chitosan and pectin, which also incorporated β-cyclodextrin and E-cinnamaldehyde as antimicrobial compounds (Brasil *et al.* 2012). This complex multi-layered coating extended the shelf-life and increased the quality of the fruit compared to uncoated controls. Previously, polypropylene (PP) film covered by 12 alternating chitosan/pectin layers was used as packaging for tomatoes by Elsabee and colleagues (2008). It was found to be highly antifungal and maintained the freshness and quality of the produce much better than PP films alone (Elsabee *et al.* 2008).

Some authors have developed techniques for hydrolysing chitosan to produce chitooligosaccharides (COS), which are smaller and more soluble than chitosan itself. Studies on the functional properties of COS have revealed that they are largely dependent on their degree of polymerization (DP) and molecular weight as well as degree of acetylation (DA) (Kendra and Hadwiger 1984; Yamada *et al.* 1993; Kulikov *et al.* 2006). Kendra and Hadwiger (1984) showed that oligomers of high DP (5-7) inhibited fungal growth of *Fusarium solani* on pea plants better than those of lower DP; Kittur and others showed that COS with a DP of 2-6, particularly the hexamer, were more effective against *E. coli* and *B. cereus* than native chitosan; and Jeon and Kim (2000) reported that a 0.5% concentration of chitooligosaccharides with a DP of 3-6 completely inhibited the growth of *E. coli*. Regarding DA, Yamada and colleagues (1993) found that whilst $(GlcNAc)_6$ induced phytoalexin formation in cultured rice cells, GlcNAc oligomers smaller than trimers and deacetylated oligomers had very little activity.

Matrix-assisted laser desorption/ionization (MALDI) is an ionization technique used in mass spectrometry. It allows the analysis of large molecules, such as proteins, peptides and sugars as well as other organic polymers. It uses a photon-absorbing matrix which is mixed with the analyte on an electrically conducting target (usually a stainless steel plate). This is then placed in a vacuum where a UV laser beam is applied and desorption is triggered. The matrix absorbs the photon energy, leading to rapid heating and the creation of a hot plume, thus bringing the analyte into the gas phase where ionization occurs (Karas *et al.* 1987; Zenobi and Knochenmuss 1998). Time-of-flight mass spectrometry (TOF-MS) is an analytical technique whereby charged molecules can be separated and their mass-to-charge ratios determined via a time measurement. When ions are accelerated in an electric field of known strength, all ions with the same charge will have the same kinetic energy. As the speed in which they then travel will be determined by their mass only, the time that it subsequently takes for the particles to reach a detector at a known distance will separate them by mass (heavier particles move slower). Results are displayed as spectra of the relative quantities of ions as a function of their mass-to-charge ratios, which can then be used to determine the elemental composition and chemical structure of a compound (Brown and Lennon 1995; Mirsaleh-Kohan *et al.* 2008).

The aim of this study was to test the effectiveness of chitosan as a protective coating for harvested fruit, in this case oranges (*C. sinensis*). Commercial low molecular weight (LMW) chitosan was used as a convenient application in a number of experiments, after which pectin was also added as another coating treatment to test alongside volatile compounds. This was so we could investigate possible interactions between the coatings and the volatiles, or additive effects. The preparation of COS was also attempted via both acid and enzymatic hydrolysis, after which the samples were analysed via MALDI-TOF-MS to evaluate their DP and DA.

7.2: Materials and Methods

7.2.1: Chemicals

Low molecular weight (75-85% deacetylated) chitosan, pectin (from citrus fruit), inulin, HCl (37%), NaOH (37%), methanol (70%), acetone, and sodium acetate buffer solution were obtained from Sigma (U.K). Enzymes, (Pepsin from porcine gastric mucosa; Amano Lipase A from *Aspergillus niger* and Laminarinase from *Trichoderma* sp.) were also purchased from Sigma (U.K). Elga UHQ water was used throughout.

The majority of volatile compounds used throughout the research are found naturally in citrus fruit (Njoroge *et al.* 2005; Barboni *et al.* 2009; Carmen Gonzalez-Mas *et al.* 2011; Espina *et al.* 2011) and were purchased from Sigma (U.K). These were (-)-linalool, (-)-terpinen-4-ol, (-)-α-pinene, (-)-β-pinene, (+)-linalool, (+)-α-pinene, (R)-carvone, (S)-carvone, 1-octen-3-ol, citral, decanal, (E)-2-hexenal, geranyl acetate, limonene, methyl jasmonate, methyl salicylate, myrcene, neryl acetate, nonanal, octanal, valencene, β-caryophyllene and γ-terpinene All chemicals were ≥ 81% pure. The essential oils of oranges and lemons were also used – these were obtained from Paramount Citrus (California, U.S.A).

7.2.2: Fruit material

Organic Spanish 'Valencia' oranges (*Citrus sinensis*) were obtained from Capespan (Maidstone, U.K) and from Dimensions Health Store (Bangor, U.K).

7.2.3: Pathogens

P. digitatum and *P. italicum* were first isolated from naturally infected orange fruit during initial weight-loss experiments (See Section 2.3.2). These were cultured on potato dextrose agar (PDA) and sub-cultured at least once a month to check viability. Loss of viability was noted when subcultured *Penicillium* failed to grow on fresh media on one occasion, so spores from another naturally infected organic orange fruit were isolated and cultured in the same way (See Section 4.2.2).

7.2.4: Organic oranges with chitosan and/or pectin coatings

7.2.4.1: Preparation of coatings

Method adapted from Chien *at al.* (2007). Solutions of different concentrations of chitosan only (0.2% and 2%, plus 1% on one occasion) or chitosan and pectin (0.1% and 1%) were made with 5% acetic acid (to dissolve the compounds), sodium hydroxide (2 M NaOH, to adjust the pH to 5) and distilled water, along with a control solution that contained just 5% acetic acid, NaOH and distilled water. Oranges were submerged in the solutions for 90 s before being left to dry. Some fruit (1/6th of the total) were not coated in any of the solutions and were used as a second control in the experiment with both chitosan and pectin. Once all fruit were dry (after approximately two hrs) each was mounted on a small, previously weighed and labelled, plastic Petri dish and weighed.

7.2.4.2: Incubation Conditions

See Section 2.2.3 - 22°C and 97% RH delivered by saturated salt solution of potassium sulfate (K_2SO_4).

7.2.4.3: Inoculation of Fruit

Fungal spore suspensions of *P. digitatum* and *P. italicum* were prepared and mixed together in a glass vial (see Section 5.2.2.3). The concentrations of spores in the suspensions were adjusted to 10^6 - 10^7 spores/mL using a hemocytometer. Fruit to be inoculated were punctured at their calyx-ends with a sterile clinical lancet and a 10 µL drop of the mixed spore suspension was pipetted onto each wound site. The fruit were then placed in the boxes (6 in each).

7.2.4.4: Volatile Delivery Method

In a fume cupboard, 200 µL of each volatile compound was pipetted into individual 35 mm plastic Petri dishes. Each one was then placed into one of the boxes containing the inoculated fruit.

7.2.4.5: Date collection and analysis

The oranges were placed in the incubation boxes (22 °C, 97% RH). In experiments involving chitosan coatings effects only, the fruit were stored for a period of up to 14

days and re-weighed every day (except on weekends) to ascertain the extent of water/weight loss. Half of the fruit in the second of these experiments (7.3.2) were inoculated with mixed *Penicillium* spores after 48 hrs and immediately replaced in the incubation boxes. There were 3-6 replicate fruit for each treatment/control. In the experiment involving chitosan and pectin coatings plus the addition of volatile compounds, inoculated fruit were placed in the incubation boxes into each of which was then placed an open petri dish containing one of the volatiles. There were also control boxes, where fruit subjected to the six different coating treatments ((0.1% & 1% chitosan, 0.1% and 1% pectin, control (with no coating) and control+ (coated with just acetic acid, NaCl & distilled water)) were added without any volatile compound. After seven days the oranges were removed from the boxes and re-weighed to ascertain how much weight/water they had lost. The diameter (mm) of each lesion induced by the fungal pathogen on each individual fruit was also measured and recorded. The assay was performed just once with a small number of volatiles, and there were six replicate fruit for each (combined) treatment.

Mean values and standard errors were calculated with Microsoft Excel 2010 (Microsoft Corporation, Washington, U.S.A) and then subjected to either univariate analysis of variance (ANOVA) when only weight loss was being tested, or multivariate analysis of variance (MANOVA) when both weight loss and lesion diameters were being investigated. The equality of variances were checked using the Levene's Test and, if equal, the least significant difference (LSD, $p = 0.05$) between means for multiple comparisons was calculated. If equal variances could not be assumed, then the non-parametric Games-Howell test was used for multiple comparisons between treatments. The statistical software package IBM SPSS Statistics Version 19.0 (IBM Corporation, New York, U.S.A) was used for all statistical analyses.

7.2.5: Chitosan Hydrolysis and Analysis

7.2.5.1 Mass Spectrometry

A Maldi sample target plate was seeded with 2,5-dihydroxybenzoic acid (DHB) in ethanol (20 mg/mL). Chitosan and chitosan hydrolysate samples were mixed (20 mg/mL)

with DHB in UHQ H_2O (10 mg/mL). An aliquot (0.7 µl) of each sample mix was then spotted onto the prepared plate and allowed to air-dry at room temperature. All mass spectra were recorded on a Bruker Reflex IV (Bruker Daltonik, Bremen, Germany) in the positive ion mode. Spectra were the sum of 200 shots. Inulin in H_2O (5 mg/mL) was used for both mass calibration and comparison with chitosan hydrolysates.

Inulin is a linear polysaccharide mainly composed of β-(2→1) fructosyl-fructose linkages (Roberfroid 2005). It is synthesized from sucrose by repeated fructosyl transfer from a fructosyl donor, and usually has a terminal glucose unit (Ernst *et al.* 1998; Roberfroid and Delzenne 1998). Generally, plant inulin contains between 20 and several thousand fructose units (Fuchs 1991). It can be degraded enzymatically or chemically to a mixture of fructooligosaccharides with the general structure Glu-$(Fru)_n$ (GF_n, with G as glucose, F as fructose, and $_n$ indicating DP). The simplest oligomer of inulin is kestose, which has 2 fructose units and 1 glucose unit, a DP of 3 (Vijn and Smeekens 1999).

7.2.5.2: Acid Hydrolysis of Chitosan with Hydrogen Chloride

a). Method adapted from Cabrera & Van Cutsem (2005). Chitosan (2 g) was added to 50 mL distilled water in a 250 mL round-bottom flask and heated to 50°C with stirring. Hydrochloric acid (12 M, 1 mL) was then added to dissolve the chitosan, followed by a further 4 mL HCl (12 M) for hydrolysis. Solution was boiled at 100°C under reflux for 3 hrs. After the allotted time period, the solution was cooled in a water bath. Enough NaOH (19 M) was then added to adjust the pH to 7 and to form a precipitate. A Buchner flask and funnel, containing a disc of filter paper (Whatman grade 540), was then attached to a vacuum pump and the solution was filtered in increments whilst being washed with distilled water. The solution was allowed to dry on the filter paper and then transferred to a petri dish and further dried under vacuum. Sample (OT52) was finally crushed to a coarse powder with a pestle & mortar prior to analysis with MALDI-TOF-MS.

b). Method adapted from Lee *at al*. (1999). Chitosan (4 g) was added to 100 mL distilled water in a 250 mL round-bottom flask and heated to 100°C with stirring. HCl (12 M, 2.4 mL) was then added to dissolve the chitosan, followed by a further 16 mL HCl (12 M) for hydrolysis. Solution was boiled (100°C) under reflux for 3.5 hrs. After the allotted time period, the solution was cooled in a water bath, during which a small amount of precipitate was formed. Enough NaOH (19 M) was then added to adjust the pH to 7 and

to induce further precipitation. A Buchner flask and funnel, containing a disc of filter paper (Whatman grade 540), was then attached to a vacuum pump and the solution was filtered in increments whilst being washed with distilled water. Solution was allowed to dry on the filter paper and then transferred to a petri dish and further dried under vacuum (Precipitate I). Methanol (70%, 315 mL) was added to the supernatant and left overnight in the fume cupboard prior to being rotary evaporated under reduced pressure at 60°C. Acetone was then added at a ratio of 9:1 acetone/solution to induce precipitation of chitosan remaining, which was then filtered and dried as previous to obtain Precipitate II. Both samples (OT53, 1st & 2nd fractions) were finally crushed to a coarse powder with a pestle and mortar prior to analysis with MALDI-TOF-MS.

7.2.5.3: Enzyme Hydrolysis of Chitosan with Pepsin

Method adapted from Roncal *et al.* (2007). Sodium acetate buffer solution (100 mM, pH 4.5, 200 mL) was heated in a 250 mL round-bottom flask to 40°C. Chitosan (2 g) was then added with stirring and left for 1 hr to dissolve. Enzyme (Pepsin from porcine gastric mucosa) was then added to the solution in an enzyme/substrate ratio of 1:100 (w/w) and incubated under reflux for 1.5 hrs. Solution was then boiled for 30 mins to deactivate the enzyme. After cooling, a Buchner flask and funnel, containing a disc of filter paper (Whatman grade 540), was attached to a vacuum pump and the solution was filtered to remove as much enzyme as possible, after which enough NaOH (12 mM) was added to adjust the pH to 7. Filtrate was then concentrated to ~1/10 of its initial weight by rotary evaporation under reduced pressure at 60°C. A precipitate was obtained by adding ethanol at an alcohol/solution ratio of 9:1, which was removed and transferred to a petri dish. Sample (OT54) was then dried under vacuum and crushed with a pestle and mortar prior to analysis with MALDI-TOF-MS.

7.2.5.4: Enzyme Hydrolysis of Chitosan with Amano Lipase A

Method adapted from Roncal *et al.* (2007). Sodium acetate buffer solution (100 mM, pH 4.5, 200 mL) was heated in a 250 mL round-bottom flask to 40°C. Chitosan (2 g) was then added with stirring and left for 1 hr to dissolve. Enzyme (Amano Lipase A from *Aspergillus niger*) was then added to the solution in an enzyme/substrate ratio of 1:100 (w/w) and incubated under reflux for 3.5 hrs. Solution was then boiled for 30 mins to deactivate the enzyme. After cooling, a Buchner flask and funnel, containing a disc of

filter paper (Whatman grade 540), was attached to a vacuum pump and the solution was filtered to remove as much enzyme as possible. Filtrate was very dark brown so powdered activated carbon (5 g) was added with stirring and left to decolourise the solution. Excess carbon was then removed via filtration through a layer of diatomaceous earth (Celite). Solution was then concentrated to ~1/10 of its initial weight by rotary evaporation under reduced pressure at 60°C. A precipitate was obtained by adding acetone at a ratio to the solution of 9:1, which was removed and transferred to a petri dish. Sample (OT55) was then dried under vacuum and crushed with a pestle and mortar prior to analysis with MALDI-TOF-MS.

7.2.5.5: Enzyme Hydrolysis of Chitosan with Laminarinase

Method adapted from Xie *et al.* (2009). Sodium acetate buffer solution (100 mM, pH 4.5, 100 mL) was heated in a 250 mL round-bottom flask to 45°C. Chitosan (1 g) was then added with stirring and left for 1 hr to dissolve. Enzyme (Laminarinase from *Trichoderma* sp.) was added to the solution in an enzyme/substrate ratio of 1:100 (w/w) and incubated under reflux for 21 hrs. Solution was then boiled for 30 mins to deactivate the enzyme. After cooling, solution was transferred to an Erlenmeyer flask via filtering through a funnel containing a cotton wool ball to remove as much enzyme as possible. Filtrate was then concentrated to ~1/10 of its initial weight by evaporation under reduced pressure at 60°C. A precipitate was obtained by adding ethanol at an alcohol/solution ratio of 9:1, which was removed and transferred to a petri dish and further dried under vacuum (Precipitate I). Remaining solution was then concentrated again to ~1/10 of its initial weight by rotary evaporation under reduced pressure at 40°C. Acetone was then added at a ratio of 9:1 acetone/solution to induce precipitation of chitosan remaining, which was then filtered and dried as previous to obtain Precipitate II. Both samples (OT56, 1st & 2nd fractions) were finally crushed to a coarse powder with a pestle and mortar prior to analysis with MALDI-TOF-MS.

7.3: Results

7.3.1 The effects of chitosan coatings on weight/water loss in orange fruit

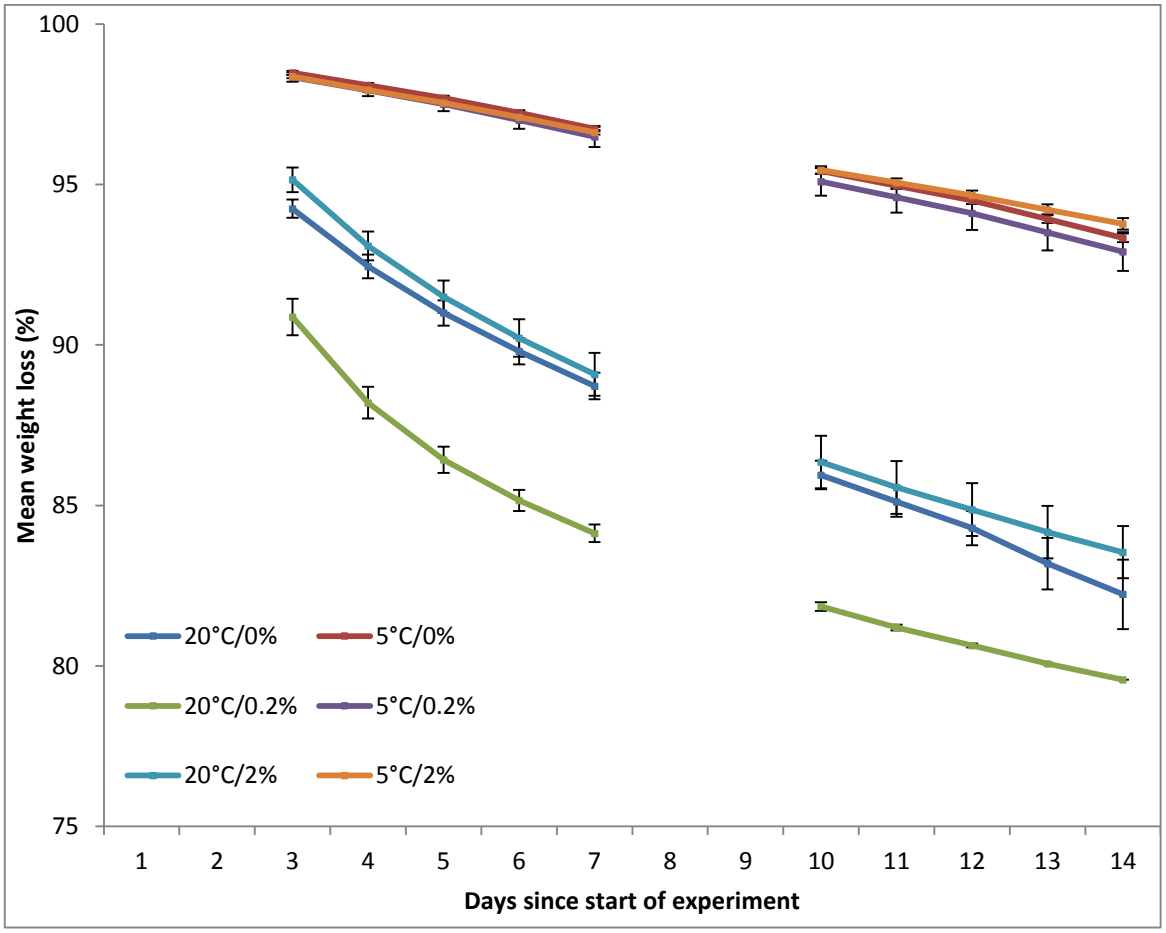

Figure 7.01: Weight loss of oranges over the course of 14 days when coated in different concentrations of chitosan and stored at 5 and 20°C. Legend key indicates temperature at which fruit were incubated and concentration of chitosan coating. Error bars = ± 1 S.E.

The effects of coating fresh organic oranges with different concentrations of chitosan can be seen in Figure 7.01. Besides the obvious effect of the two different temperatures, with the fruit at 5°C losing weight/water at a much lower rate than those at 20°C, the chitosan coatings also induced different loss rates, particularly at 20°C. Oranges coated with 0.2% chitosan lost > 9% of their original weight in the first three days (approx. 3% per day) and by the end of the two weeks had lost > 20%, compared to those coated with 2% chitosan, which lost < 5% in the first three days (approx. 1.6% per day) and < 17% by day fourteen. The fruit coated with 0.2% chitosan lost significantly less weight than either those with

2% chitosan or no coating at all, according to the LSD *post hoc* test (p = 0.005 and p = 0.024, respectively). Weight loss differences at 5°C were much harder to detect, but similar effects with the 0.2 and 2% concentrations of chitosan could be seen by the end of the two weeks, with 0.2% chitosan inducing > 7% weight/water loss and 2% chitosan giving > 6%. However, none of the comparisons at 5°C gave significant values according to the LSD *post hoc* test. The control fruit (i.e. those coated with just acetic acid and H_2O solutions) had weight losses somewhere between the two chitosan concentrations at both temperatures, but was only statistically lower than coated fruit at the 0.2% concentration at 20°C.

7.3.2: The effects of chitosan coatings on weight/water loss and Penicillium infection in orange fruit

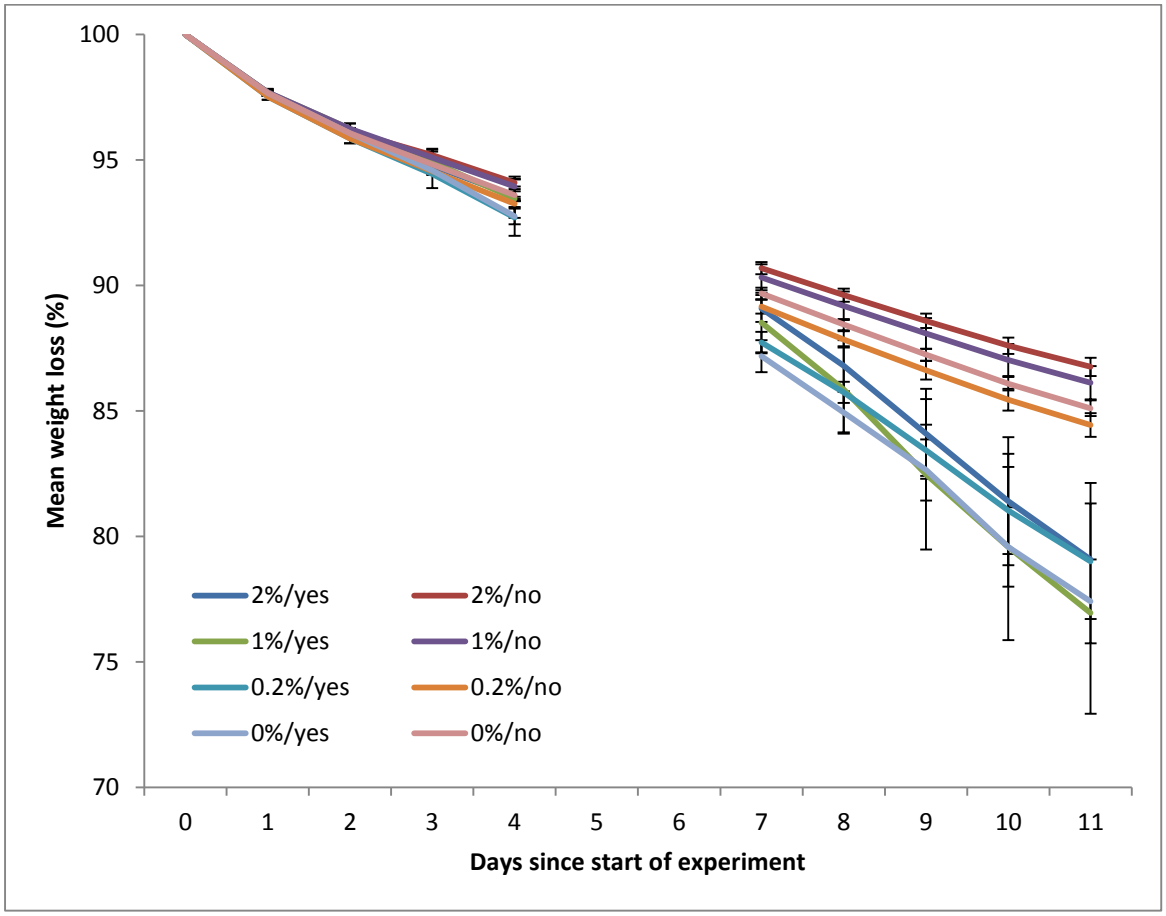

Figure 7.02: Weight loss in oranges treated with different concentrations of chitosan and subsequently infected (or not) with *Penicillium digitatum* and *P. italicum*. Legend key indicates concentration of chitosan coating on the fruit and whether they were inoculated with *Penicillium* spores after 48 hours. Error bars = ± 1 S.E.

Oranges were coated with different concentrations of chitosan and incubated for 11 days at 20°C. They were weighed every day to ascertain weight/water loss rates, and after 48 hrs half of them were inoculated with a mixed spore solution of *P. digitatum* and *P. italicum.* Figure 7.02 displays the effects that different chitosan coatings had on the weight/water loss of both inoculated and non-inoculated oranges.

Firstly, it can be seen that during the first 48 hrs there was very little difference between any of the differently treated fruit, although the 1% coated fruit were losing weight at a slightly lower rate (approx. 1.88% per day) than the control (0%) fruit (1.97% per day) whilst the 0.2% coated fruit were exhibiting a slightly higher rate of loss (2.07% per day). However, once half of the fruit had been inoculated, not only did the infected oranges begin to lose weight at a much higher rate, but also the differences between the treatments became more apparent. By the end of the eleven days, the infected fruit had all lost > 20% of their initial weight, whilst the non-infected oranges had lost < 15% on average. The different chitosan coatings also had effects on the losses in both the infected and non-infected fruit, with the 2% chitosan coatings inducing the lowest weight/water loss in both, whilst 0.2% chitosan gave the highest rate in the non-infected fruit and 1% (plus the control) gave the highest in the infected produce. Although its effect on infection wasn't measured directly, it can be seen that chitosan did not alter the differences in weight loss in the infected compared to the non-infected fruit. At 2% concentration, weight loss in non-infected oranges was 13.25% after 11 days compared to 14.9% in untreated fruit – a difference of 1.65% - whilst 2% chitosan-treated and infected oranges lost 21% of their original weight compared to 22.6% in the non-treated fruit – a difference of 1.6%. This would suggest that the chitosan treatments had no effect on infection caused by *Penicillium* pathogens, which is supported by the fact that the statistical analyses revealed no significant differences compared to the controls at either temperature.

7.3.3: The effects of chitosan or pectin coatings plus volatile compounds on weight/water loss and *Penicillium* infection in oranges fruit

Coating organic oranges with different concentrations of chitosan or pectin had different effects on the infection caused by *P. digitatum* and *P. italicum* (Figure 7.03). The addition of different volatile compounds sometimes amplified these effects and other times altered

them completely. For example, the highest reduction in pathogenicity (lesion size) was exhibited by 1% pectin in combination with (+)-linalool (-49.8%), but 1% pectin alone actually increased lesion size compared to the control fruit by 8.4%. Another instance concerns 0.1% chitosan, which alone brought about a 47.8% reduction in the *Penicillium*-induced lesions, whilst in combination with methyl salicylate it encouraged pathogenicity by 15.3% compared to the control. Not one coating type resulted in 100% positive results, whether acting alone or in combination with one of the volatile compounds, however neryl acetate reduced pathogen infection whether in addition to a coating or not. Unlike the investigation with chitosan coatings alone (Figure 7.02), these results indicate that chitosan does have an effect on infection in orange fruit caused by *Penicillium* pathogens, as both the 0.1 and 1% coatings decreased mean lesion sizes by > 30% as stand-alone treatments. However, none of the statistical analyses revealed any significant differences between any of the combined volatile/coating treatments and the controls, where the fruit were left uncoated and without the addition of a volatile compound.

Figure 7.04 shows how the different coatings, both alone and in combination with volatile compounds, affected the weight/water loss in the inoculated oranges. As with the results on pathogenicity, the effects are complicated with no one coating providing 100% positive outcomes. The best result was seen with 0.1% chitosan in combination with orange oil, which reduced weight/water loss by 60.8% compared to the control. It also performed well with the other volatiles (e.g. -45.3% with (E)-2-hexenal), but alone it increased weight loss by 30.6%. The only volatile to produce 100% positive results and reduce weight/water loss on all occasions was MeS, however none of the treatments were significantly different to the controls according to the Games-Howell *post hoc* test.

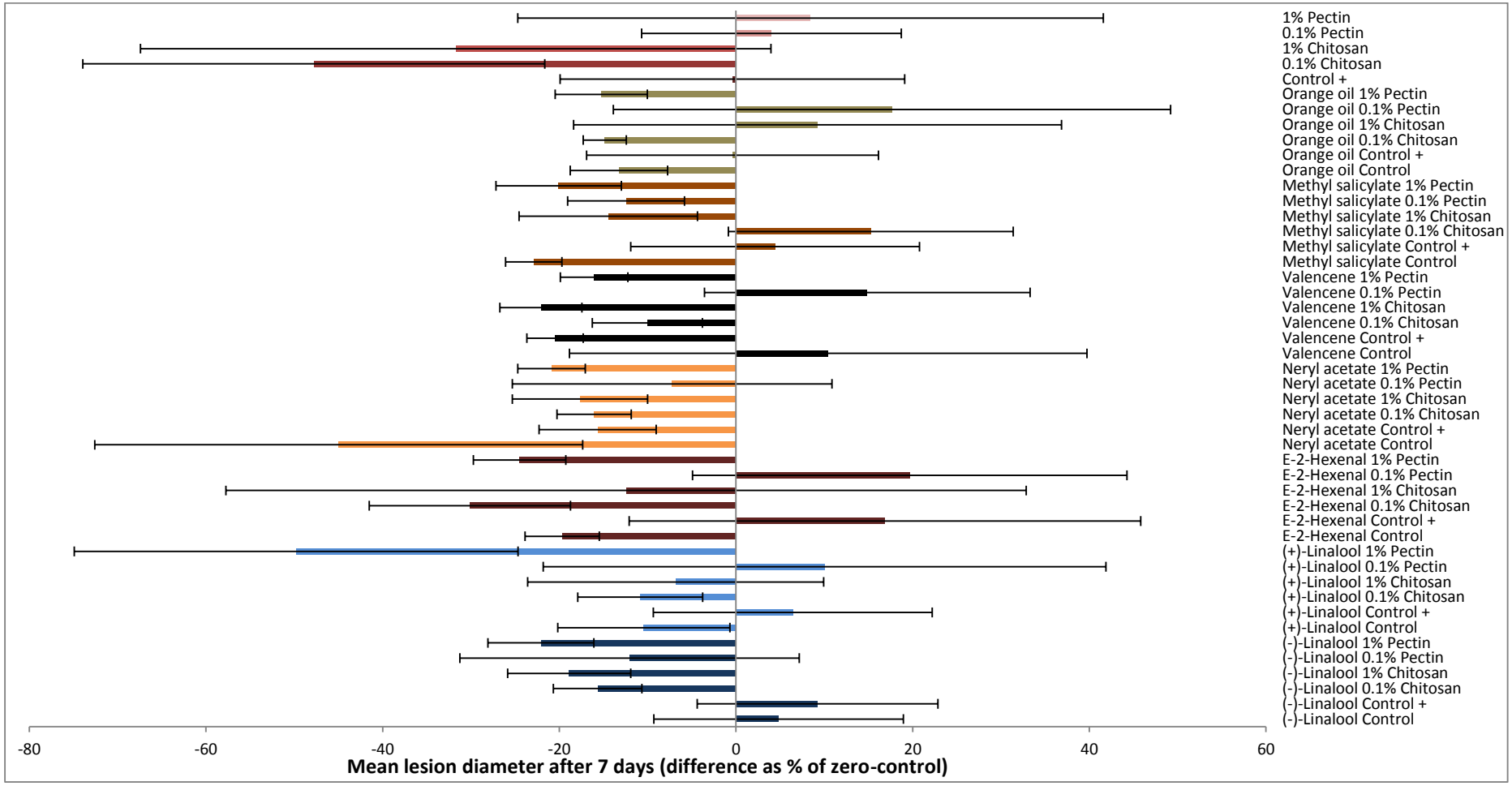

Figure 7.03: Effects of chitosan and pectin coatings of different concentrations plus volatile compounds on *Penicillium* infection in organic oranges compared to untreated fruit. Each bar represents the result from one treatment with six replicate fruit in each. Different chemical groups are represented by different colours (Blues = alcohols; Reds = aldehydes; Oranges = esters; Greyscale = sesquiterpenes. Brown represents the essential oils of orange, whilst the purple shades denote treatments not incorporating volatile compound additions. Error bars = ± 1. S.E.

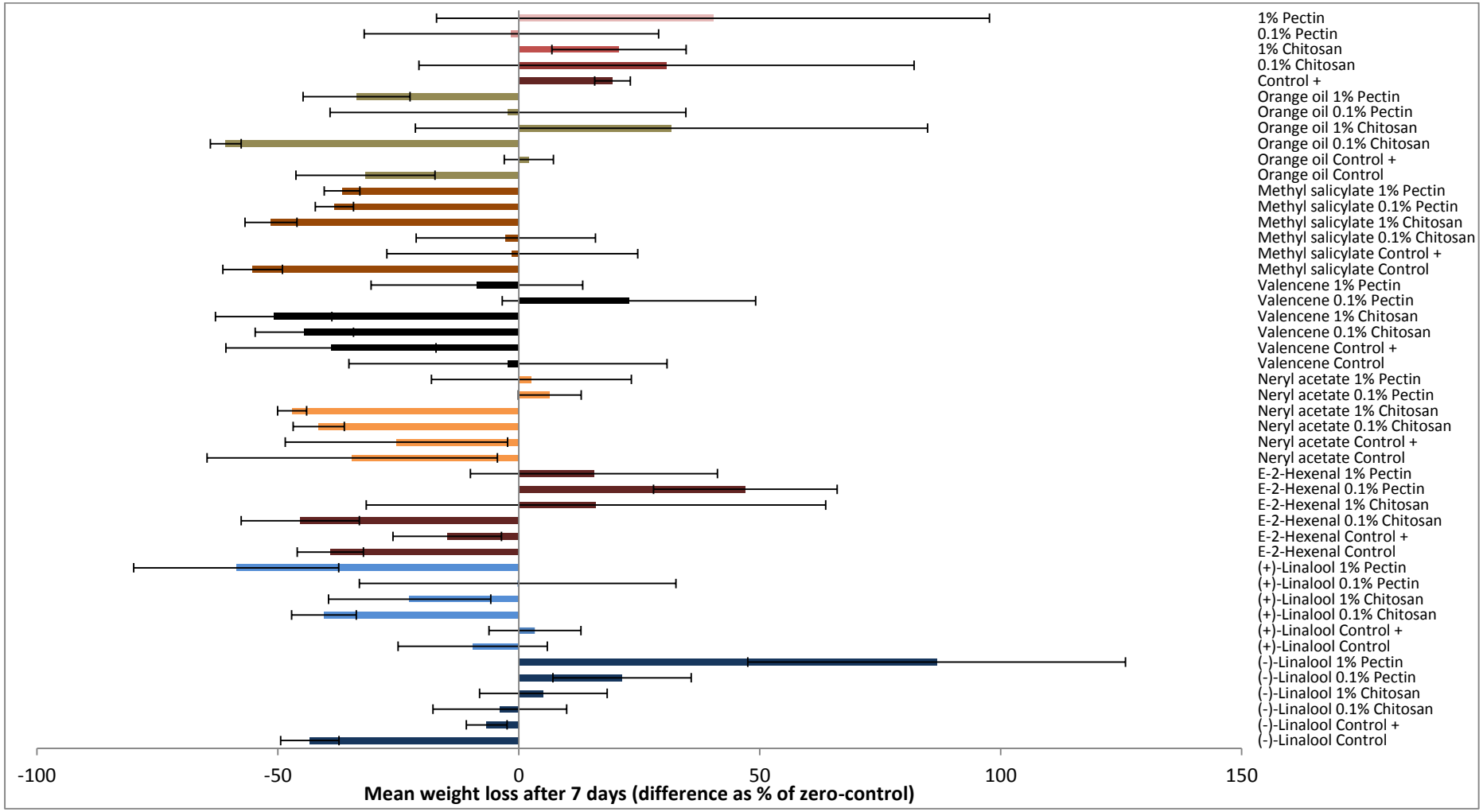

Figure 7.04: Effects of chitosan and pectin coatings of different concentrations plus volatile compounds on the weight/water loss of organic oranges inoculated with *Penicillium digitatum* and *P. italicum* compared to untreated fruit. Other details as in legend for Figure 7.03.

7.3.4: MALDI-TOF-MS Analyses

7.3.4.1: Inulin and Commercial Chitosan

As with all instrumentation, it is important for the MALDI-TOF-MS to be calibrated. Accuracy in measurements is essential, and the use of internal or external 'standards' (i.e. compounds with known molecule or molecule fragment masses) aids in the conversion of a recorded time-of-flight spectrum to a mass spectrum. As a simple polysaccharide, inulin and its known mass values can be used to establish a relationship between the measurement technique used by the instrument and the unknown values of the tested compound, in this case chitosan.

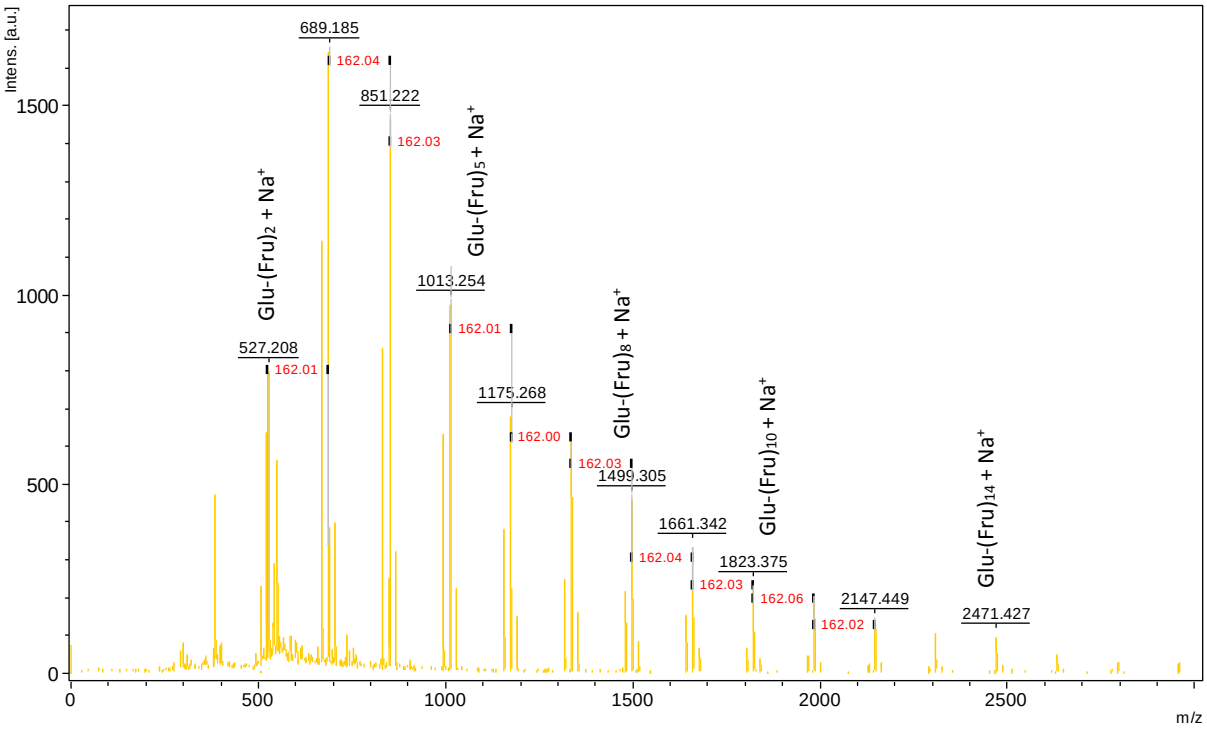

Figure 7.05: Mass spectrum of inulin produced via MALDI-TOF-MS.

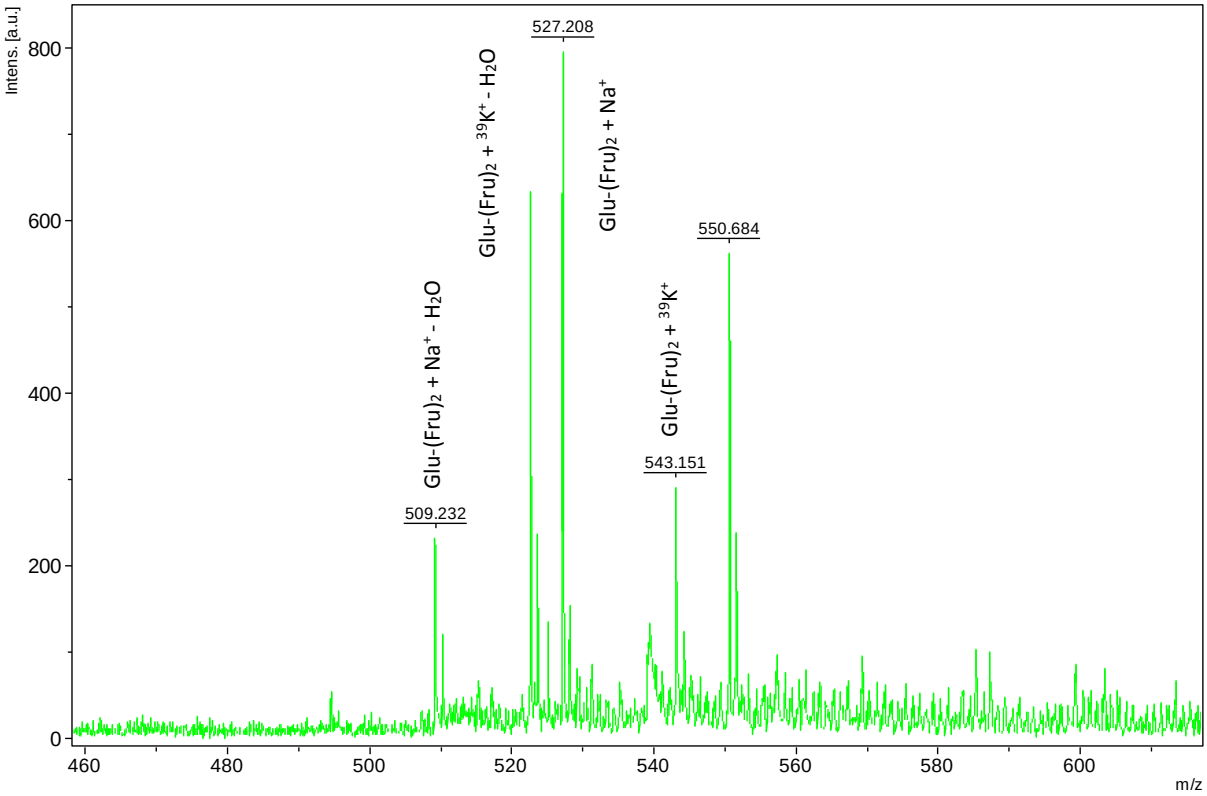

Figure 7.06: Mass spectrum of inulin trimer produced by MALDI-TOF-MS.

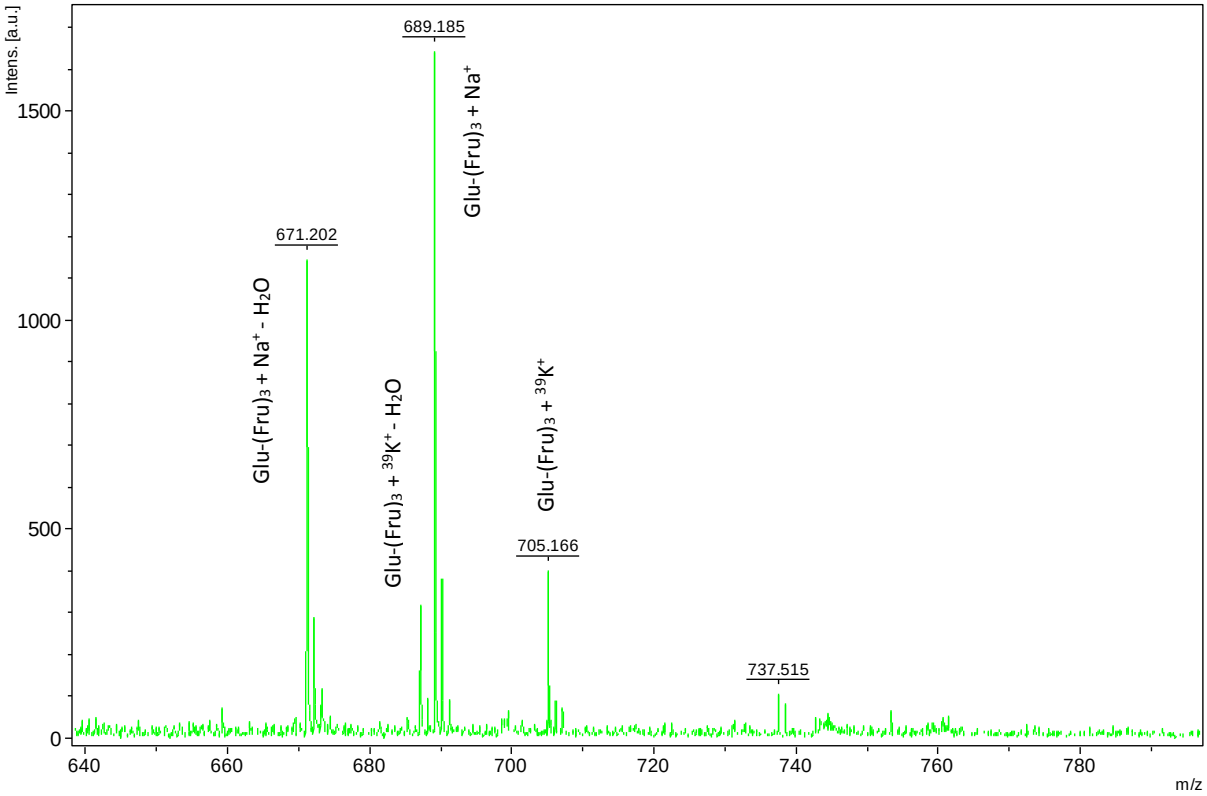

Figure 7.07: Mass spectrum of inulin tetramer produced by MALDI-TOF-MS.

Figure 7.05 shows the mass spectrum of inulin. Each assigned peak is M + Na^+, with M being the structure $(C_{12}H_{22}O_{11}).(C_6H_{10}O_5)_n$, and each successive peak representing a glucofructan with a fructose more than that of the previous peak (i.e. 162 mass units).

Figures 7.06 and 7.07, respectively, show the inulin trimer and tetramer in greater detail. In both of these, the M + Na^+ - H_2O peaks are also assigned, as are the M + $^{39}K^+$ and M + $^{39}K^+$ - H_2O peaks. The loss of a H2O molecule is a common feature in MALDI-TOF-MS and occurs as a result of the ionization process itself (Strupat 2005). Unassigned peaks that are 1 mass unit higher than those detailed are smaller fragments of the same molecule containing C^{13} rather than C^{12}, which accounts for approximately 1.1% of the carbon content. Furthermore, the peaks that are 2 mass units higher than M + $^{39}K^+$ and/or M + $^{39}K^+$ - H_2O are smaller fragments containing $^{41}K^+$ ions rather than $^{39}K^+$, accounting for approximately 6.7% of the potassium content. Peaks at 550.7 m/z (Figure 7.06) and 737.5 m/z (Figure 7.07) are unknown. For inulin mass list please see Appendix 7.1.

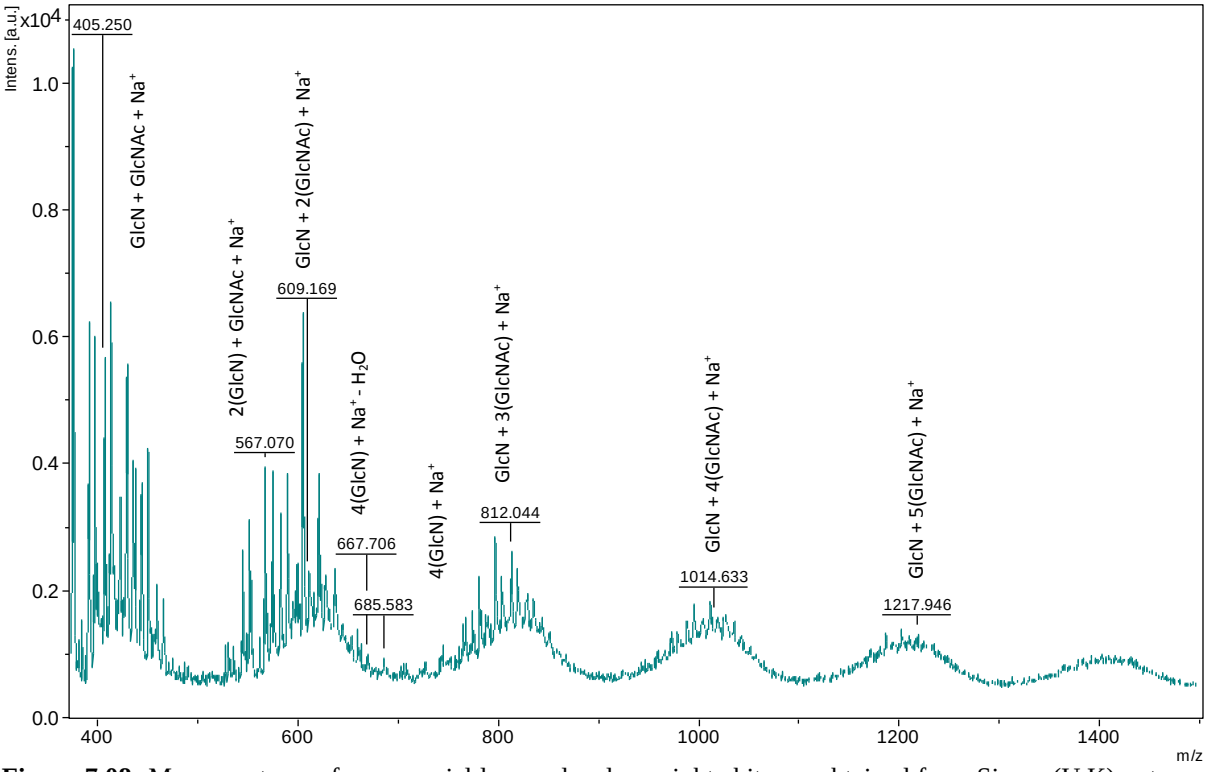

Figure 7.08: Mass spectrum of commercial low molecular weight chitosan obtained from Sigma (U.K) not subjected to hydrolysis. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units can be seen. All peaks are M + Na^+ with or without the loss of a H_2O molecule.

Figure 7.08 shows the MALDI-TOF-MS spectrum obtained when commercial low molecular weight chitosan was analysed. The peaks were difficult to identify, although those that could be show a high degree of acetylation (DA) in the compound, particularly at the high end of the spectrum. The peaks at the low end of the spectrum, below 500 m/z, come predominantly from interference of the matrix signals. The 'mounds' in the peak patterns are an anomaly that at first we believed indicated the different homologous series' members of the compound – the trimer, tetramer etc. - but, analysis of the mass values (see Appendix 7.02) in the 'mounds' revealed that they did not correspond to the DPs at all. Comparing this spectrum to those obtained from inulin does, however, illustrate the complexity of chitosan as a polysaccharide as well as show how differing preparation of the compound for mass spectrometry analysis alters the observed ions. The fact that chitosan has to be dissolved in acid and then neutralised with NaOH in order for it to be suitable for experimentation or analysis means that Na^+ ions are observed in the MALDI spectrum whilst K^+ ions are absent.

7.3.4.2: Chitosan Oligosaccharides Produced by Acid Hyrolysis

Analyses of the chitosan oligosaccharide samples obtained by acid hydrolysis were made using MALDI-TOF MS, and the full spectra of the three hydrolysates are shown in Figures 7.09, 7.10 and 7.11. Figure 7.09 shows the spectrum of OT52, which was created without precipitation in methanol. It contained oligomers from DP 4 up to approximately DP 7, and it was mainly composed of DP 3-4. The major peak was at 667.3 m/z, corresponding to 4(GlcN) + Na^+ - H_2O (see Appendix 7.3 for mass list). Of the two fractions of OT53, the first (Figure 7.10) was produced in a very similar way to OT52, the only difference being that a greater volume of HCl was added per gram of chitosan and it was incubated for 3.5 hours as opposed to just 3 hours. The second fraction (Figure 7.11) involved the additional step of precipitation in methanol. Figure 7.10 gives the spectrum of OT53 1st fraction and it can be seen that it is comparable to OT52 with a DP of up to 7, although it mainly consisited of DP 4. The major peak was a 667.4 m/z, which corresponds to 4(GlcN) + Na^+ - H_2O (see Appendix 7.4 for mass list), the same as in OT52. However, there are differences between the products of these two acid hydrolysis methods which can be seen by comparing the quantity of peaks as well as their intensity

values. There are fewer unknown peaks in OT53 1st fraction compared to OT52, whilst the intensity values of those of interest are comparatively much higher. For example, the major peak at 667 m/z has an intensity value of 3881 in Figure 7.10 and 1127 in Figure 7.9. This indicates that the acid hydrolysis of method b produced a better quality chitooligosaccharide than method a.

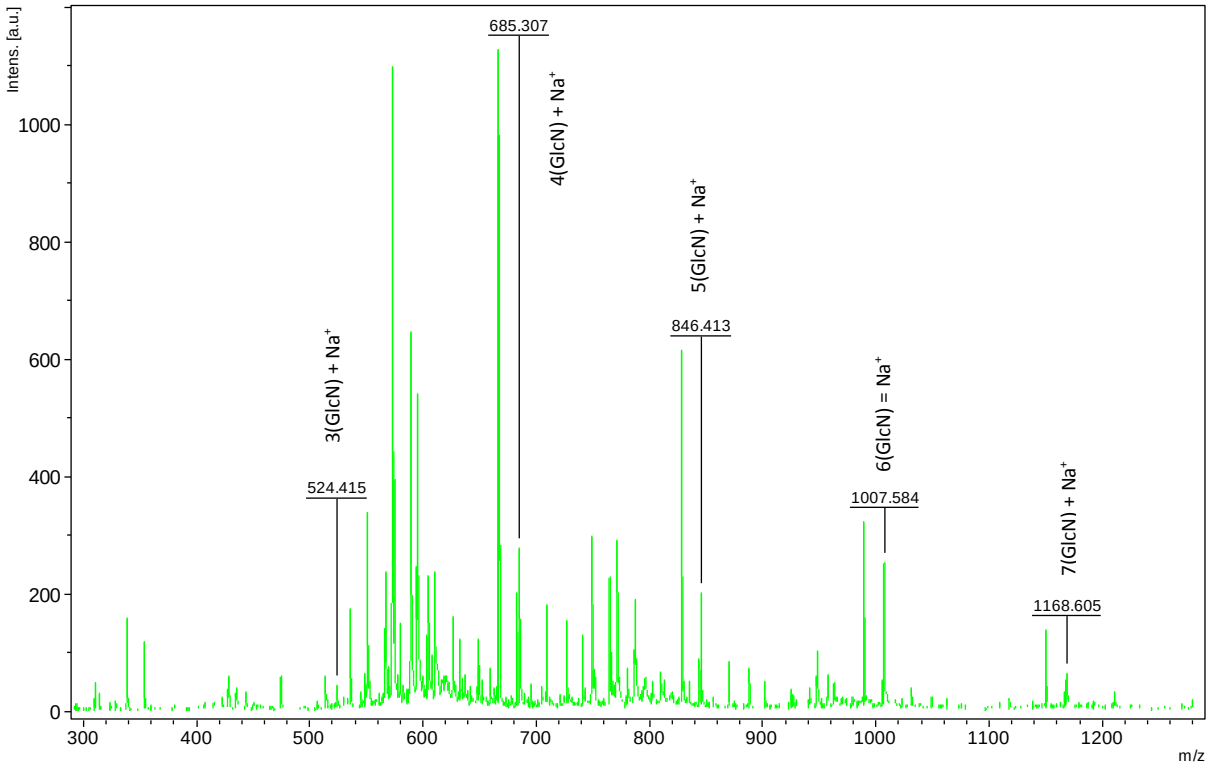

Figure 7.09: Mass spectrum of OT52 obtained by acid hydrolysis method a. Oligomers of DP3 to DP7 can be seen and all assigned peaks are M + Na^+.

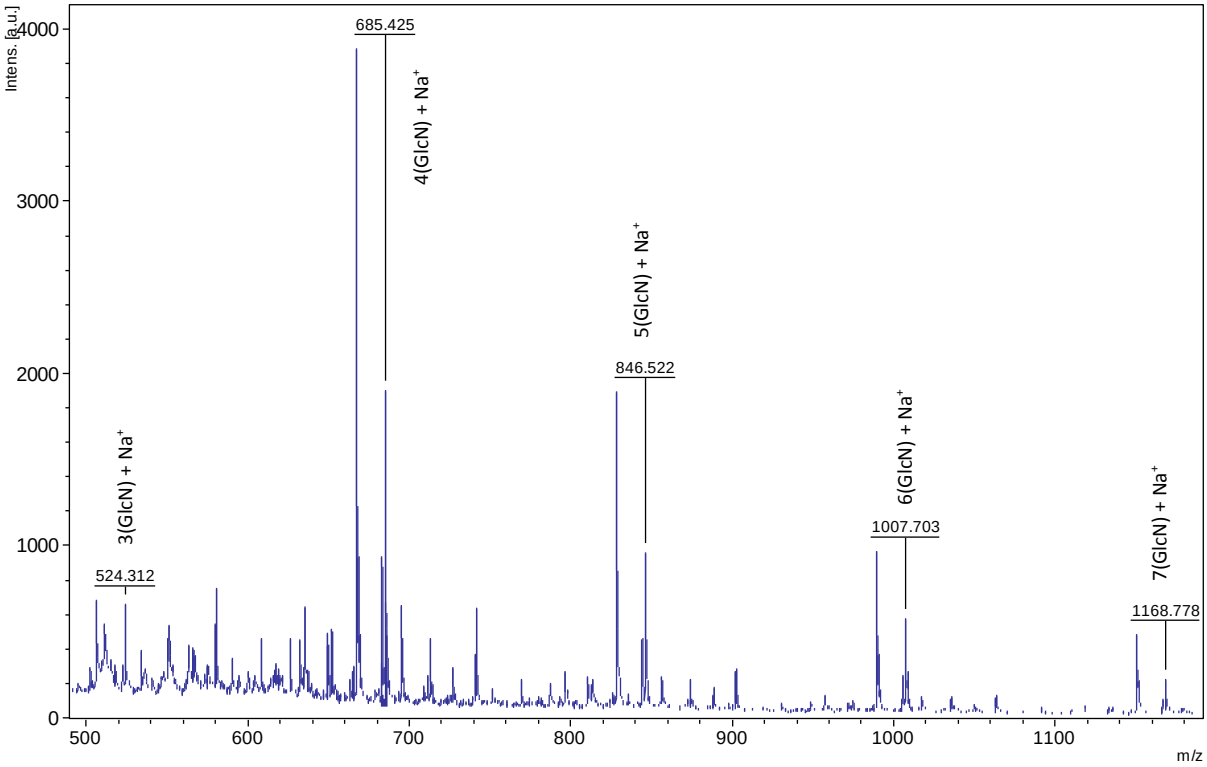

Figure 7.10: Mass spectrum of OT53 1st fraction obtained by acid hydrolysis method b. Oligomers of DP3 to DP7 can be seen and all assigned peaks are M + Na^+.

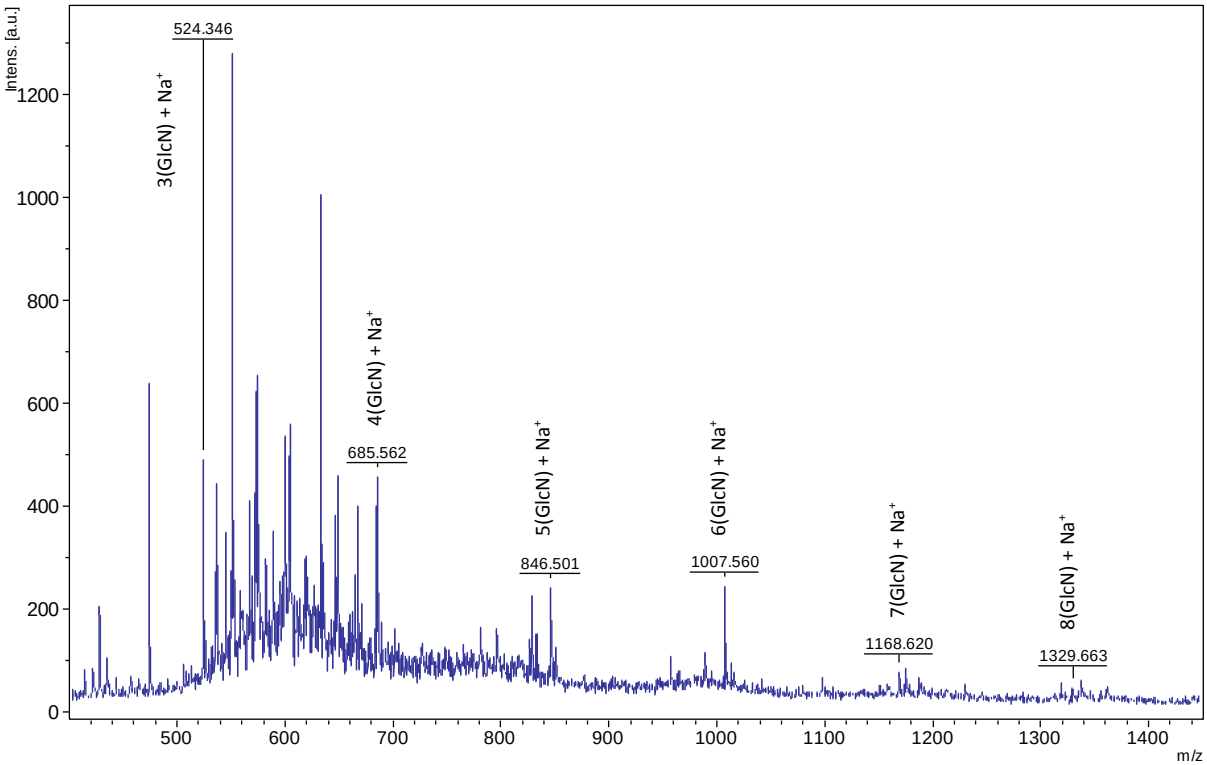

Figure 7.11: Mass spectrum of OT53 2nd fraction sample obtained via acid hydrolysis method b. Oligomers of DP3 to DP8 can be seen and all assigned peaks are M + Na^+.

The 2nd fraction of OT53 (Figure 7.11) gave chitosan oligomers of up to DP 8 and was mainly composed of DP 3-4. The major peak here was at 551 m/z, which we have not been able to identify, but the peak with the second highest intensity is at 633.3 m/z and corresponds to 3(GlcNAc) + Na^+ - H_2O (see Appendix 7.5 for mass list). This is unlike the first fraction and OT52, and overall this spectrum is less clear than that of both the previous hydrolysates, indicating a compound that is much more fragmented.

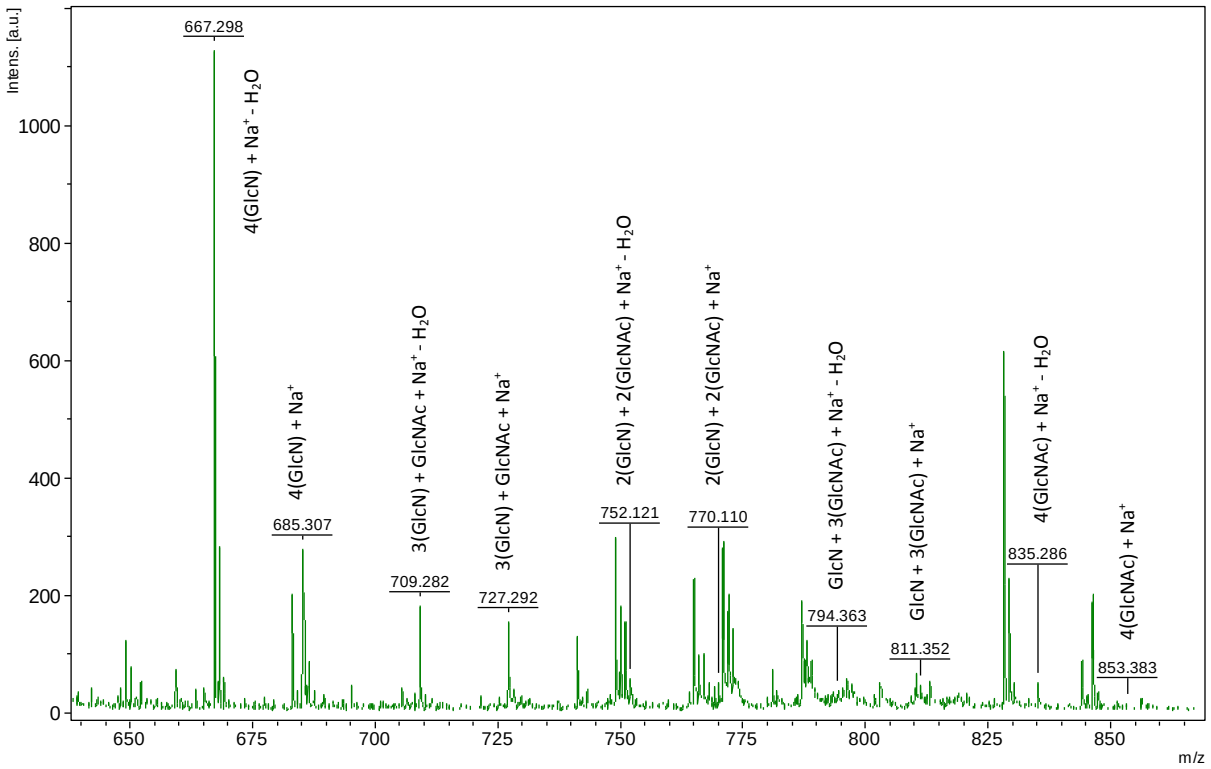

Figure 7.12: Mass spectrum illustrating the tetramer of OT52 sample obtained by acid hydrolysis method a. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

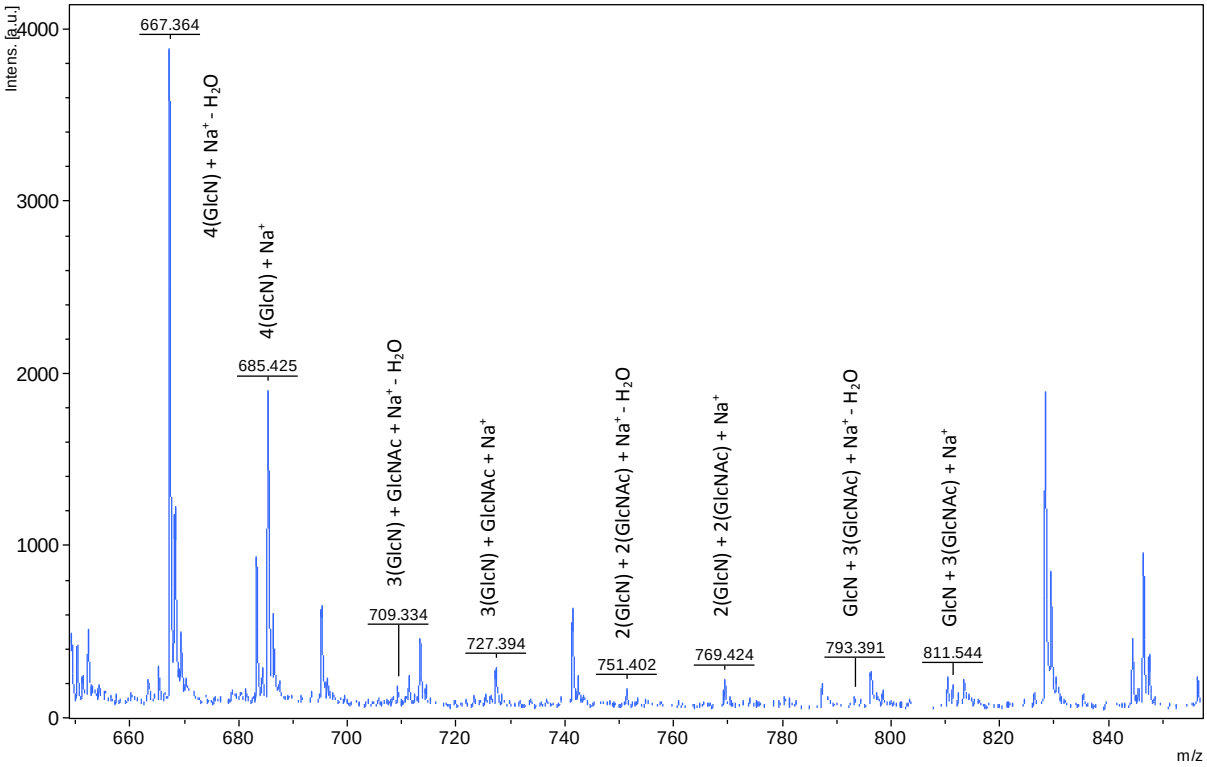

Figure 7.13: Mass spectrum illustrating the tetramer of OT53 1st fraction sample obtained via acid hydrolysis method b. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

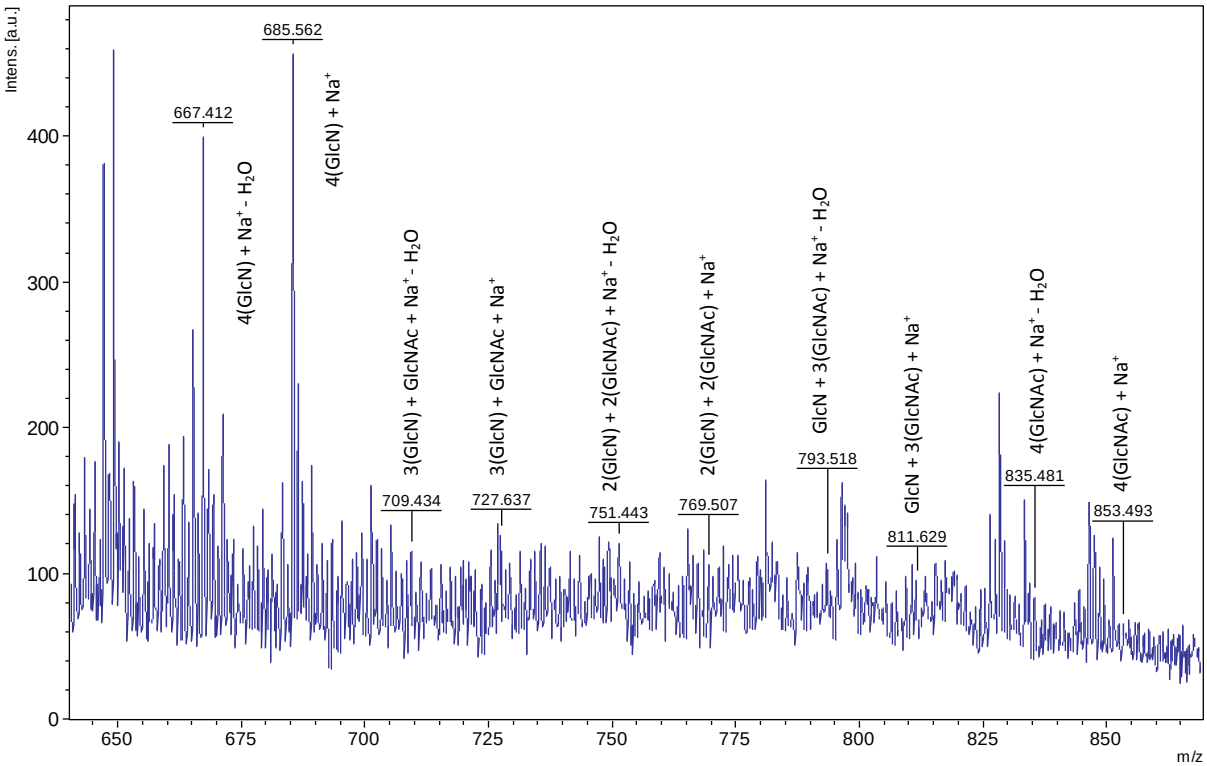

Figure 7.14: Mass spectrum illustrating the tetramer of OT53 2nd fraction sample obtained via acid hydrolysis method b. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

Figures 7.12, 7.13 and 7.14 illustrate the tetramers of the three acid hydrolysis chitooligosaccharides. Figure 7.12 shows the chitosan tetramer of OT52, with the different combinations of GlcN and GlcNAc fragments detected as sodium (Na^+) ions, with or without the loss of a water (H_2O) molecule. Examination of the tetramer of OT53 1st fraction (Figure 7.13) reveals that it contained no fully acetylated fragments of DP 4, unlike OT52 which had all possible combinations of the two different glucosamine units. Calculations made using the relative intensity values of the peaks revealed that the tetramer of OT52 had a DA of 13.4% whereas the DA of OT53 1st fraction was 7.9%. Analysis of the tetramer of OT53 2nd fraction (Figure 7.14) was difficult as the peaks were not very clear or well-defined, but it is believed that all possible GlcN and GlcNAc combinations were found.

The DA of this fraction was 29.9%, so it was more than twice as acetylated as OT52 and almost four times as acetylated as the first fraction of OT53. The percentage of dehydrated molecules was also different in the three acid hydrolysates, with the tetramer of OT52 being 72.7% dehydrated compared to 62.7% for OT53 1st fraction and 52% for the second fraction. As with the spectra of inulin, there were also peaks with 1 mass unit higher than those assigned - molecules containing C^{13} rather than C^{12}. There were no potassium peaks, which was to be expected as the sample was prepared with NaOH.

Figures 7.15, 7.16 and 7.17 illustrate the trimers of the three acid hydrolysis chitooligosaccharides. All possible combinations of the two different glucosamine units were found in all three, although there were a much higher percentage of DP3 molecules in the two OT53 fractions, as can be seen by comparing the intensity values. The highest peak of interest (3(GlcN) + Na^+) was at 661 intensity units in the first fraction of OT53, whilst 883 intensity units matched 3(GlcNAc) + Na^+ - H_2O in the second fraction. The highest peak in the trimer of OT52 was 2(GlcN) + GlcNAc + Na^+ with an intensity value of 237.

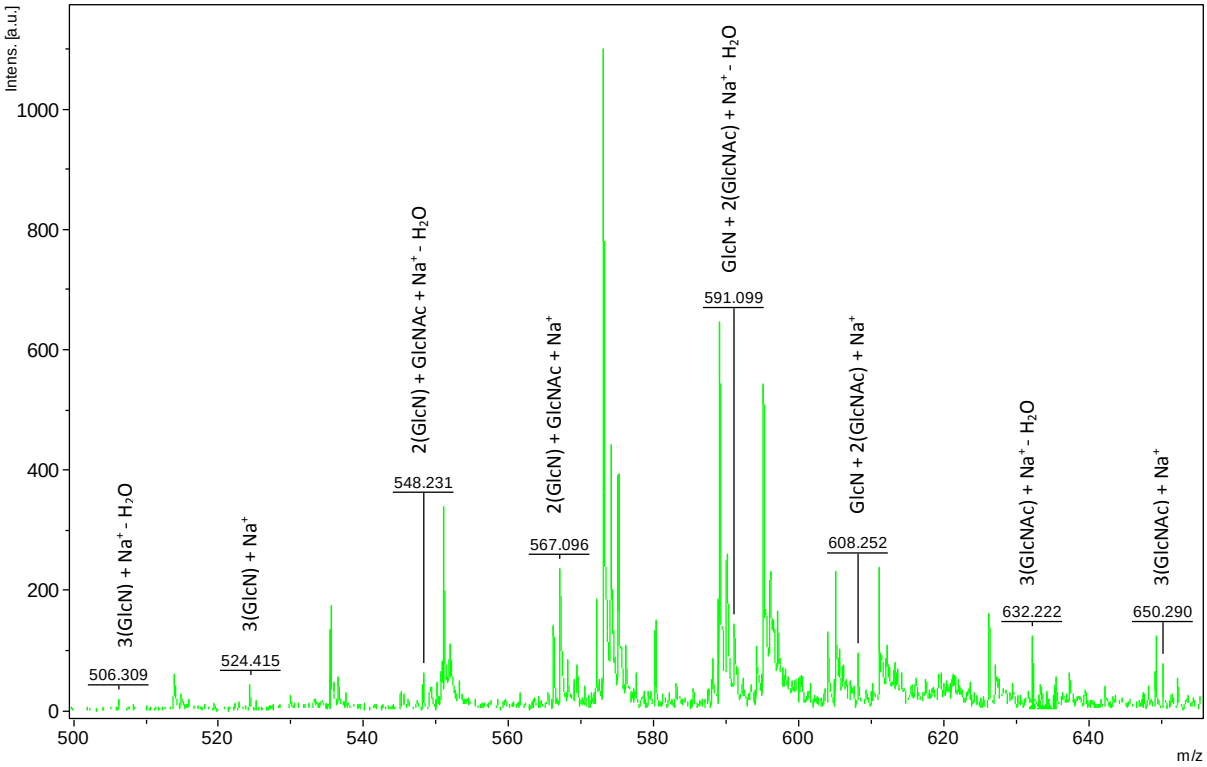

Figure 7.15: Mass spectrum illustrating the trimer of OT52 sample obtained via acid hydrolysis method a. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

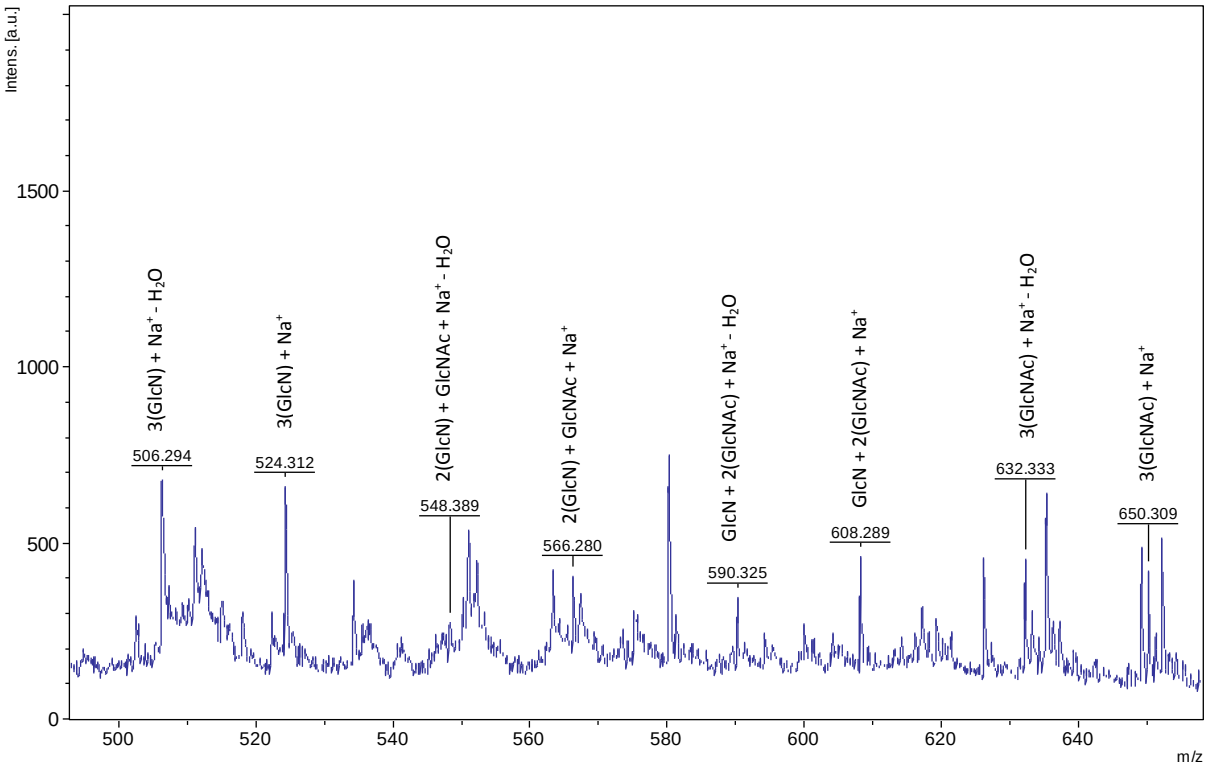

Figure 7.16: Mass spectrum illustrating the trimer of OT53 1st fraction sample obtained via acid hydrolysis method b. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

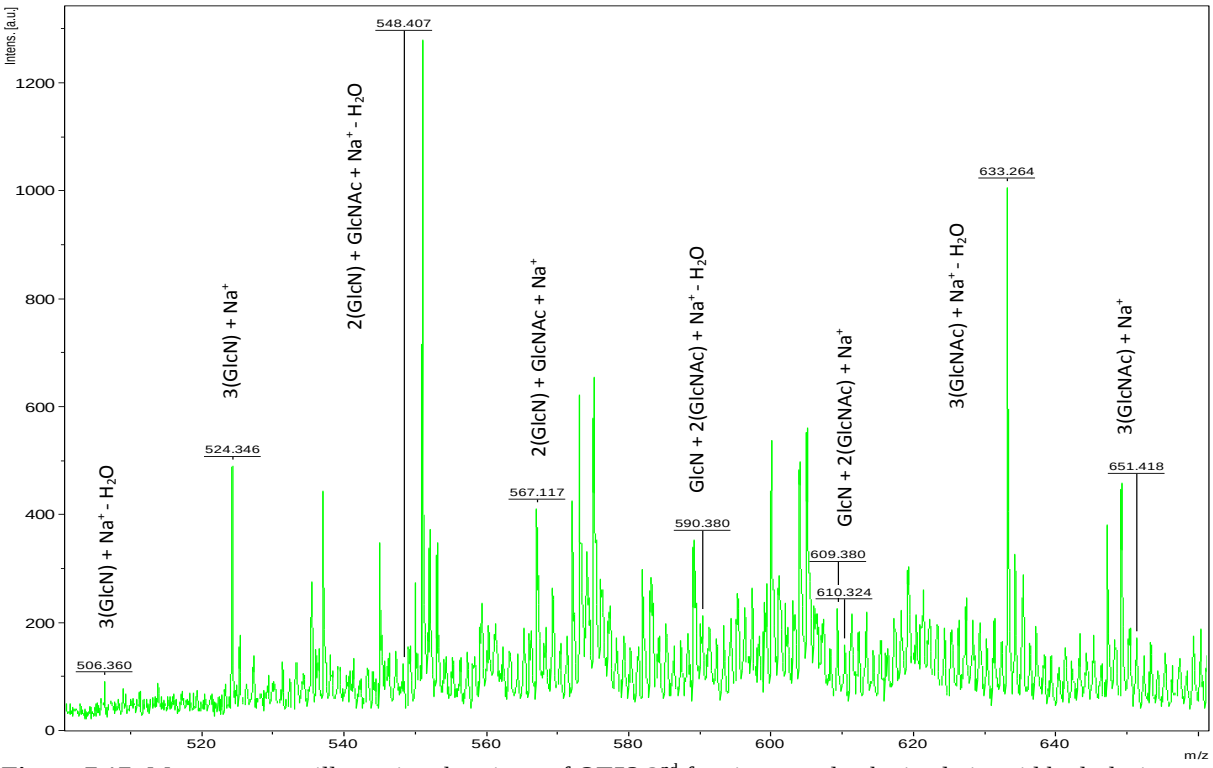

Figure 7.17: Mass spectrum illustrating the trimer of OT53 2nd fraction sample obtained via acid hydrolysis method b. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

Calculations made using the relative intensity values of the peaks revealed that all three trimers had a much higher DA than the tetramers, with that of OT52 being 59% and the first and second fractions of OT53 being 45.4% and 58.8%, respectively. This did mean, however, that the trimers of the three hydrolysates were much more similar to each other in regards to DA than the tetramers were. The percentage of dehydrated molecules was also more similar in the trimers - 48.9% for OT52, and 46% and 50.9% for OT53 1st and 2nd fractions, respectively.

7.3.4.3: Chitosan Oligosaccharides Produced by Enzyme Hydrolysis

Analyses of the four chitosan oligosaccharide samples obtained via enzyme hydrolysis with the three enzymes reveals some major differences between the hydrolysates produced (Figures 7.18, 7.19, 7.20 & 7.21).

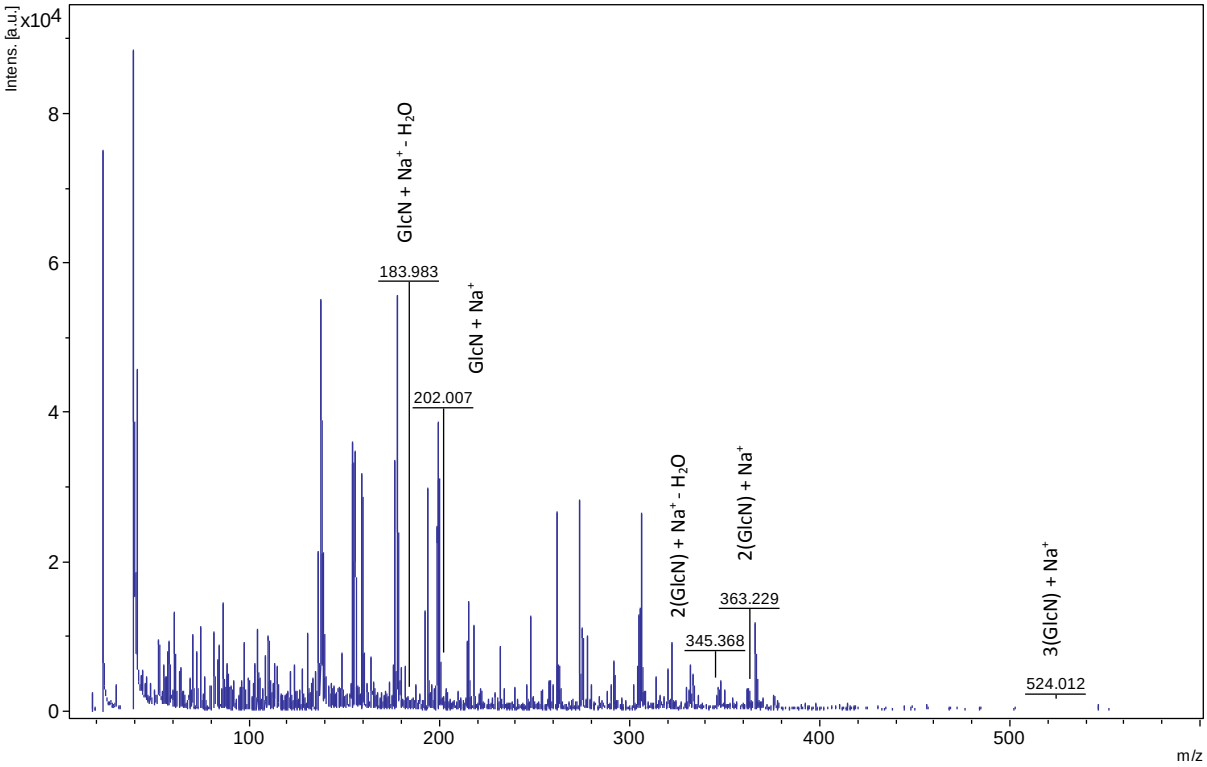

Figure 7.18: Mass spectrum of OT54 sample produced by enzyme hydrolysis with Pepsin from porcine gastric mucosa. Oligomers of DP1 to DP3 can be detected, although there were very few identifiable peaks of interest and two M + Na^+ - H_2O have been assigned to add to the M + Na^+ that could be found.

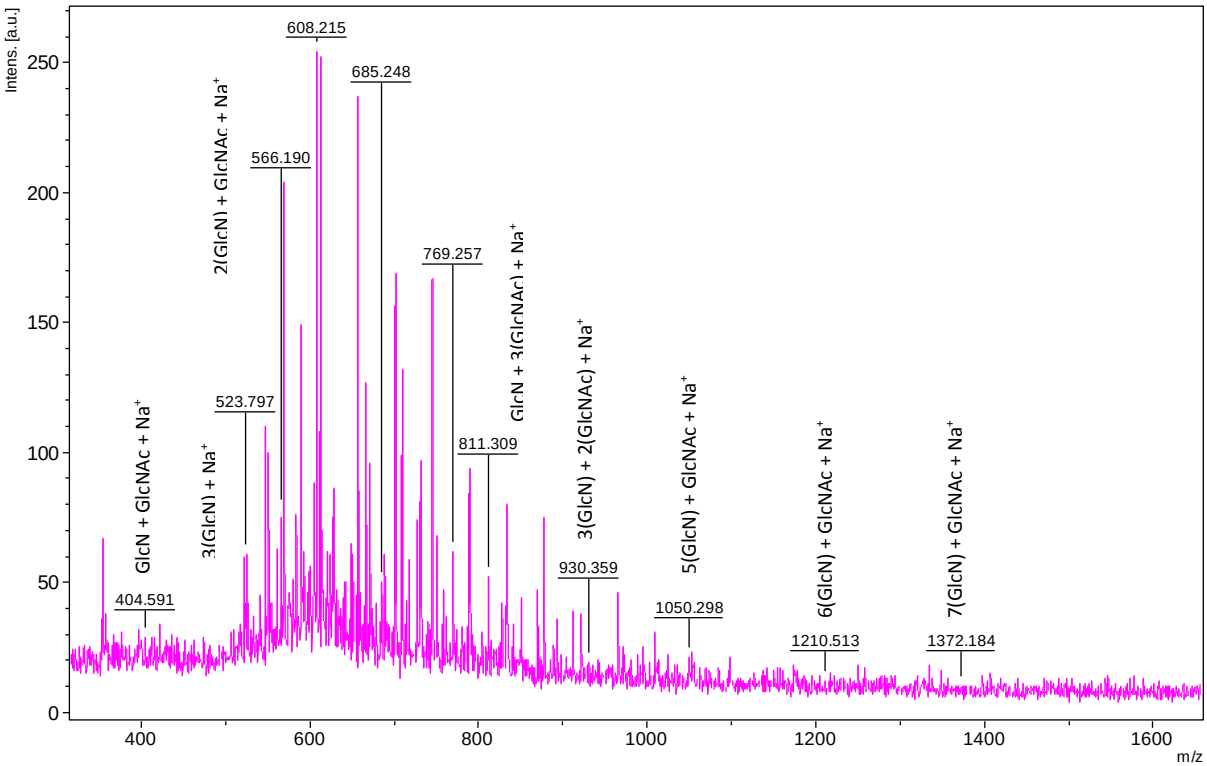

Figure 7.19: Mass spectrum of OT55 sample produced by enzyme hydrolysis with Amano Lipase A from *Aspergillus niger*. Oligomers of DP2 to DP8 can be seen, and all assigned peaks are M + Na^+.

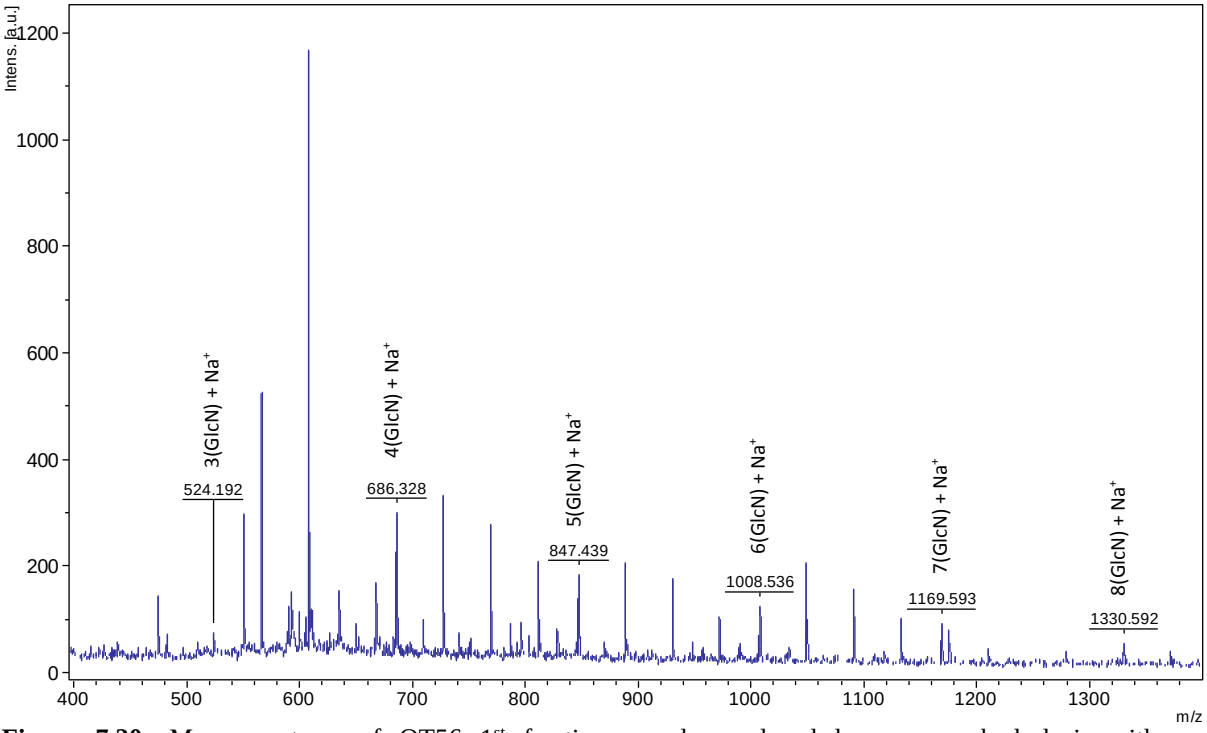

Figure 7.20: Mass spectrum of OT56 1st fraction sample produced by enzyme hydrolysis with Laminarinase from *Trichoderma* sp. Oligomers of DP3 to DP8 can be seen, and all assigned peaks are M + Na^+.

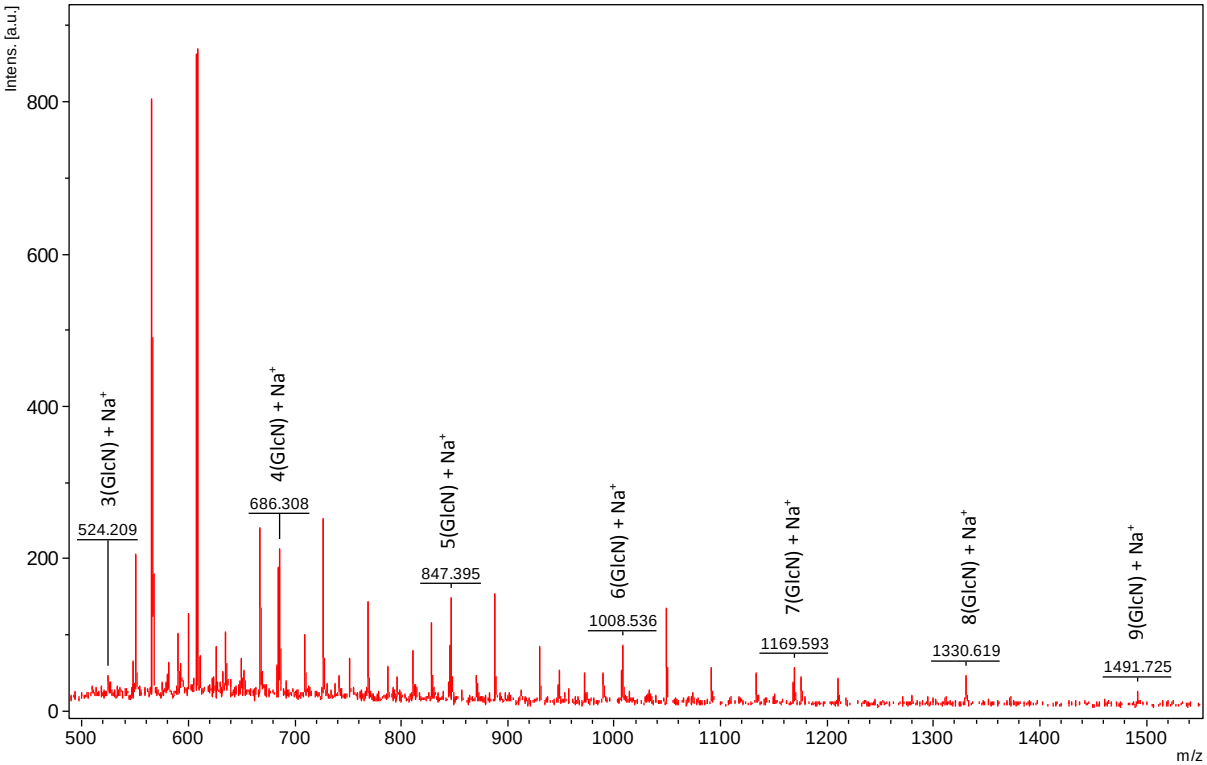

Figure 7.21: Mass spectrum of OT56 2nd fraction sample produced by enzyme hydrolysis with Laminarinase from *Trichoderma* sp. Oligomers of DP3 to DP9 can be seen, and all assigned peaks are M + Na^+.

Figure 7.18 shows OT54, produced via hydrolysis with Pepsin from porcine gastric mucosa, and it can be seen that this sample was so degraded that even fragments of DP 3 were almost impossible to detect (see Appendix 7.6 for mass list). Sample OT55 (Figure 7.19), obtained via hydrolysis with Amano Lipase A from *Aspergillus niger*, contained oligomers of up to DP 8 with the majority of them being DP 3-4 (see Appendix 7.7 for mass list). The major peak was at 608.2 m/z, corresponding to GlcN-2(GlcNAc) + Na^+, the same as those seen in Figures 7.20 and 7.21 which show the spectra of the two fractions produced via enzyme hydrolysis with Laminarinase from *Trichoderma* sp. These two spectra are the clearest and simplest out of all those produced from the different hydrolysis methods utilised. Figure 7.20 gives the full spectra of OT56 1st fraction, showing it had a DP of up to 8 and mainly consisted of DP 3 (see Appendix 7.8 for mass list). All GlcN and GlcNAc combinations were detected up to DP 7 meaning that the sample contained even and well-distributed acetyl groups. The 2nd fraction of OT56 (Figure 7.21) had a DP of up to 9 (one higher than the 1st fraction) and again mainly consisted of DP 3 (see Appendix 7.9 for mass list). However, unlike the 1st fraction all the GlcN and GlcNAc combinations could only be detected up to DP 5 and larger fragments did not exhibit full acetylation. Comparing the products of the enzyme hydrolysis with Laminarinase with that with Amano Lipase A, the distribution of acetyl groups across the compounds was one of the things that distinguished them from each other, as OT55 (Amano Lipase A) contained mainly acetylated units and non-acetylated fragments could not be detected above DP 4. This suggests that the compound was more similar to native chitosan. The other overall difference is the size of the peaks, which are higher in the fractions of OT56 compared to OT55, indicating a poorer yield in the chitooligosaccharide produced using Amano Lipase A. For example, the major peaks mentioned previously are at 1168 and 869 intensity units for the first and second fractions of OT56, respectively, and only 254 intensity units for OT55. These comparisons also indicate that the first fraction produced with Laminarinase is of higher quality than the second fraction where an additional step of acetone precipitation was incorporated. However, both are better than the chitooligosaccharide produced with Amano Lipase A and all three are superior to OT54 made with Pepsin.

As the enzyme hydrolysis with Pepsin was unsuccessful, figures 7.22, 7.23 and 7.24 illustrate the tetramers of the three other enzyme hydrolysis chitooligosaccharides. All

possible combinations of the two different glucosamine units were found in all three, although there were a much higher percentage of DP4 molecules in the two OT56 fractions produced via hydrolysis with Laminarinase, as can be seen by comparing the intensity values. The highest peak of interest in the first OT56 fraction tetramer (Figure 7.23) was 3(GlcN) + GlcNAc + Na^+ at 333 intensity units whilst that of the second fraction (Figure 7.24) was the same but with an intensity value of 252. The highest peak of interest in the OT55 tetramer which was produced via hydrolysis with Amano Lipase A (Figure 7.22) was 3(GlcN) + GlcNAc + Na^+ - H_2O at 132 intensity units. Calculations to discover the percentage dehydration of the compounds also showed that OT55 was a more dehydrated compound than the OT56 fractions, as that of OT55 was 59.5%, whilst those of the two OT56 fractions were 34.7% and 39.5%, respectively. The DAs of the three hydrolysates were slightly more similar, however, with that of OT55 being 34.5%, whilst those of the first and second OT56 fractions were 26.8% and 27%, respectively. Again, close examination of the peaks revealed peaks with 1 mass unit higher than those assigned - molecules containing C^{13} rather than C^{12} – and no potassium peaks, which was to be expected.

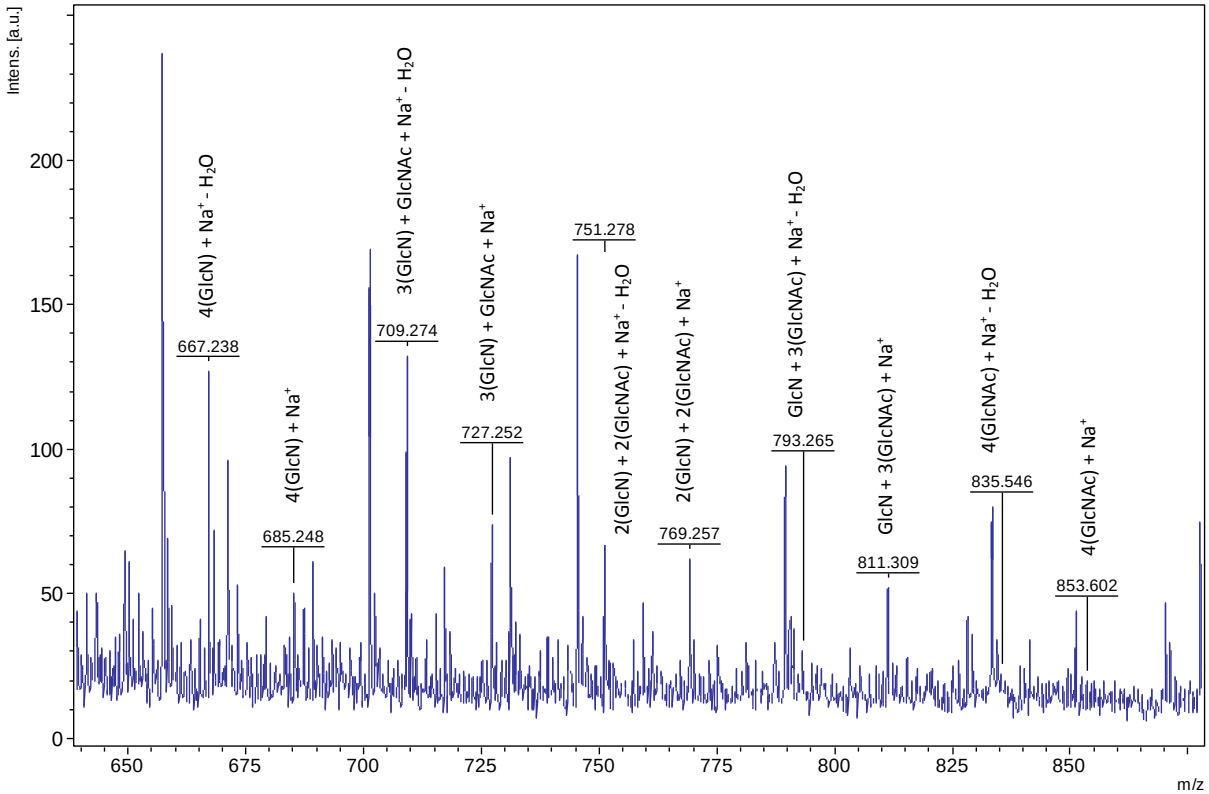

Figure 7.22: Mass spectrum illustrating the tetramer of OT55 produced via enzyme hydrolysis with Amano Lipase A from *Aspergillus niger*. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

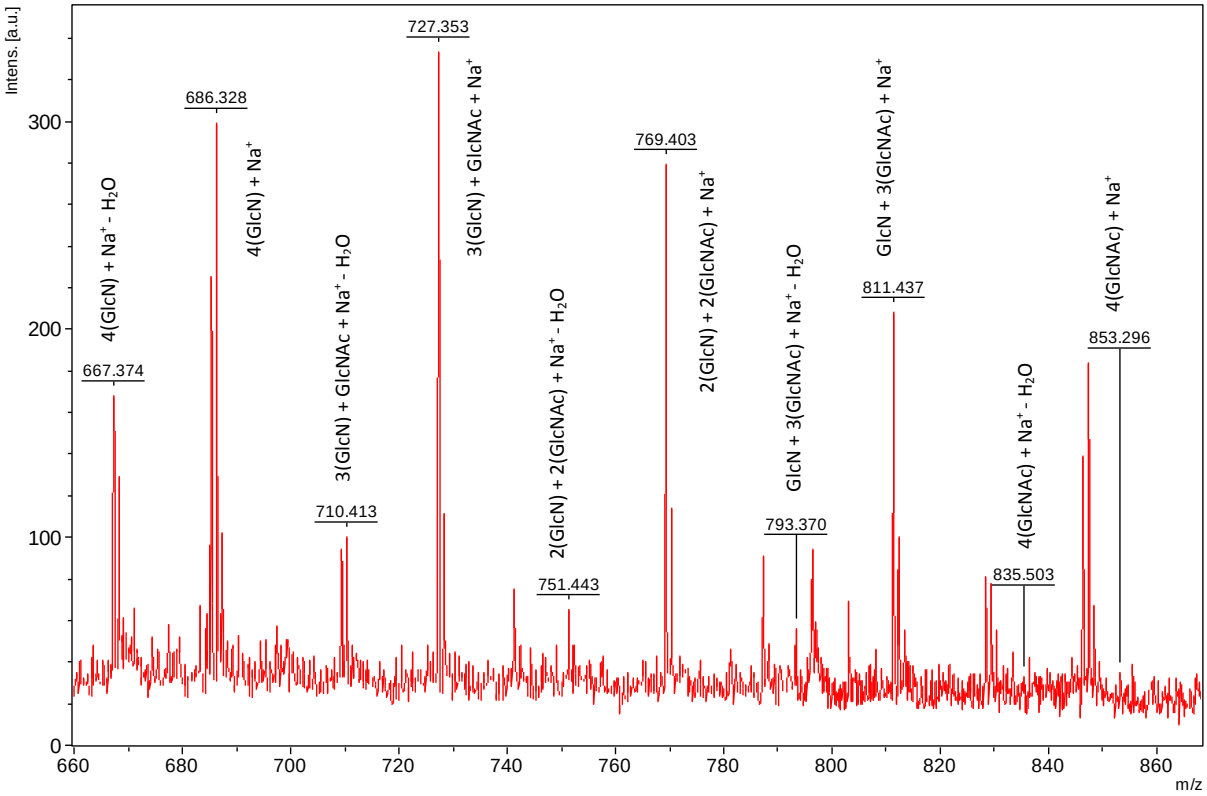

Figure 7.23: Mass spectrum illustrating the tetramer of OT56 1st fraction produced via enzyme hydrolysis with Laminarinase from *Trichoderma* sp. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

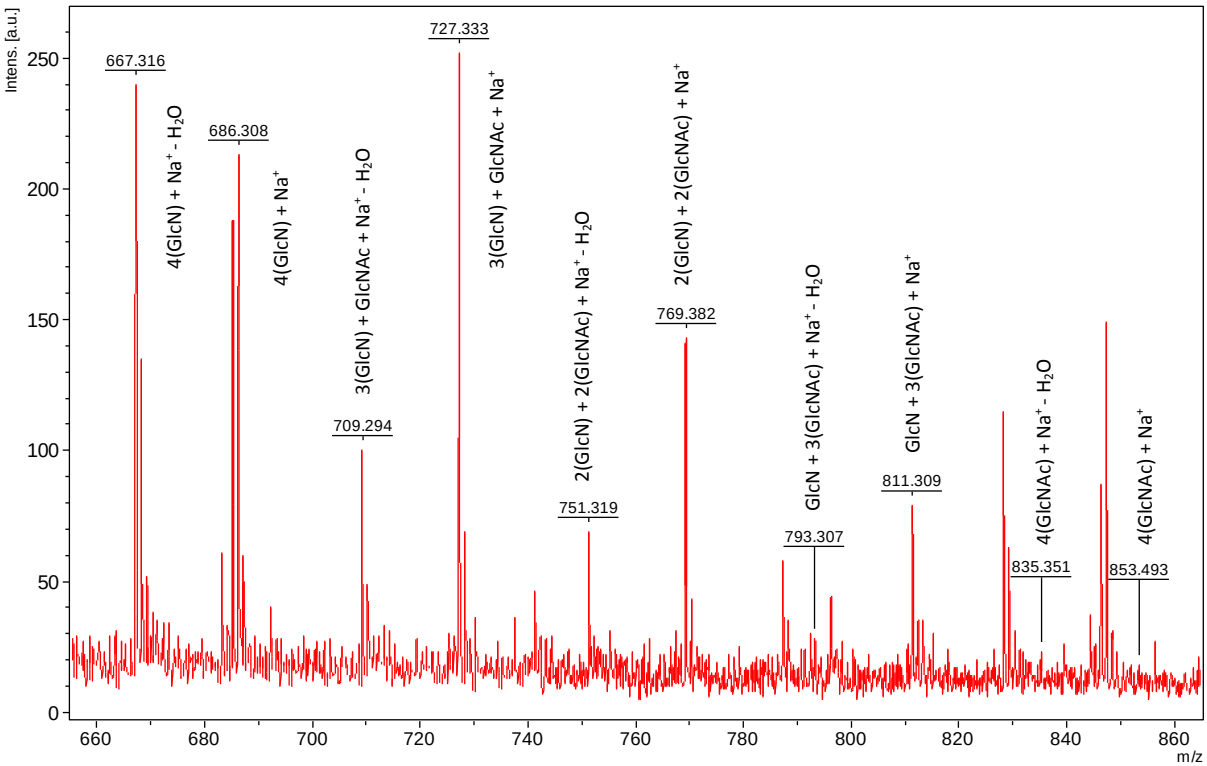

Figure 7.24: Mass spectrum illustrating the tetramer of OT56 2nd fraction produced via enzyme hydrolysis with Laminarinase from *Trichoderma* sp. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan tetramer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

Figures 7.25, 7.26 and 7.27 illustrate the trimers of OT55, OT56 1st fraction and OT56 2nd fraction, respectively. As stated previously, the highest peak of interest in all three was at 608 m/z corresponding to GlcN-2(GlcNAc) + Na+, although there were some differences in the intensity values of these peaks. In OT55, the hydrolysate produced by Amano Lipase A, it was 254, whilst those for the two Laminarinase fractions were 1168 for the first and 869 for the second. This illustrates again how hydrolysis with Laminarinase produced much higher quality oligosaccharides than that with Amano Lipase A. Calculations made using the relative intensity values of the peaks revealed that all three trimers had similar DAs to each other, but that these were almost twice as high as the tetramers, with that of OT55 being 57.3% and the first and second fractions of OT56 being 56.5% and 51.4%, respectively. However, the percentage of dehydrated molecules was very dissimilar in the trimers, both in comparison to each other and in comparison to the tetramers – 43.5% for OT55, and 12.8% and 12.7% for OT53 1st and 2nd fractions, respectively.

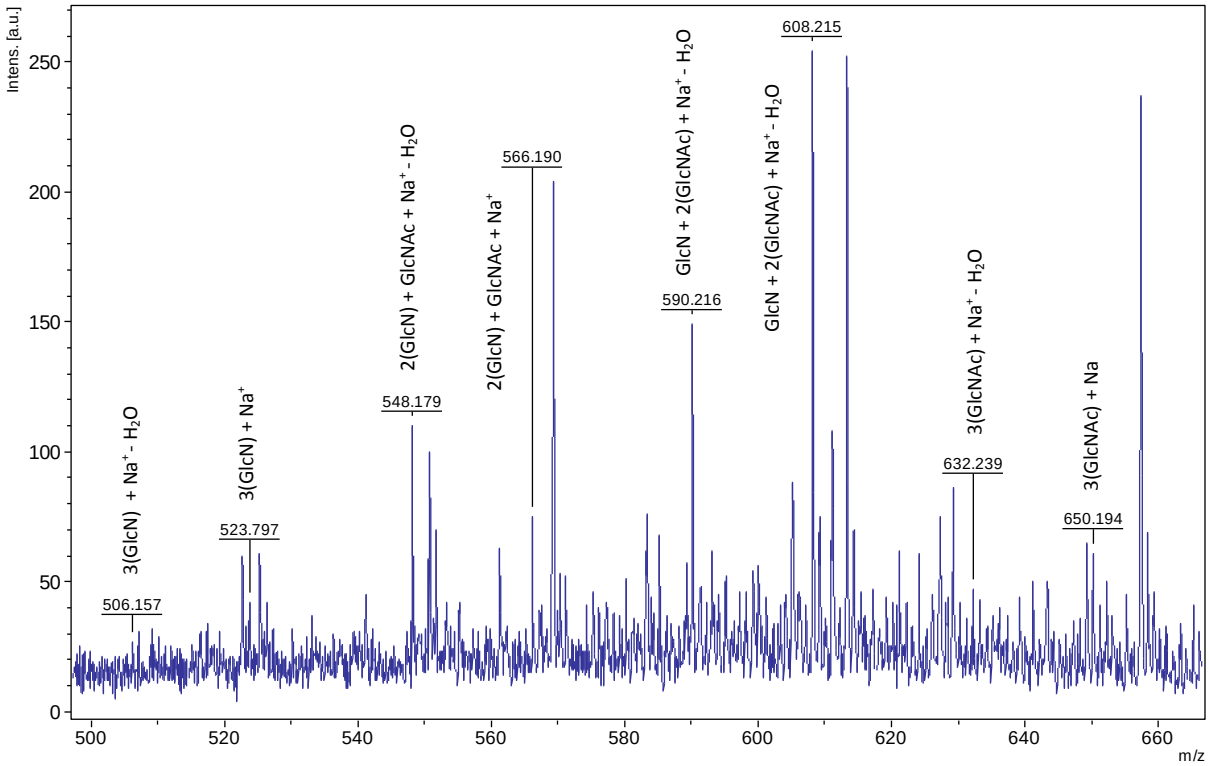

Figure 7.25: Mass spectrum illustrating the trimer of OT55 produced via enzyme hydrolysis with Amano Lipase A from *Aspergillus niger*. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

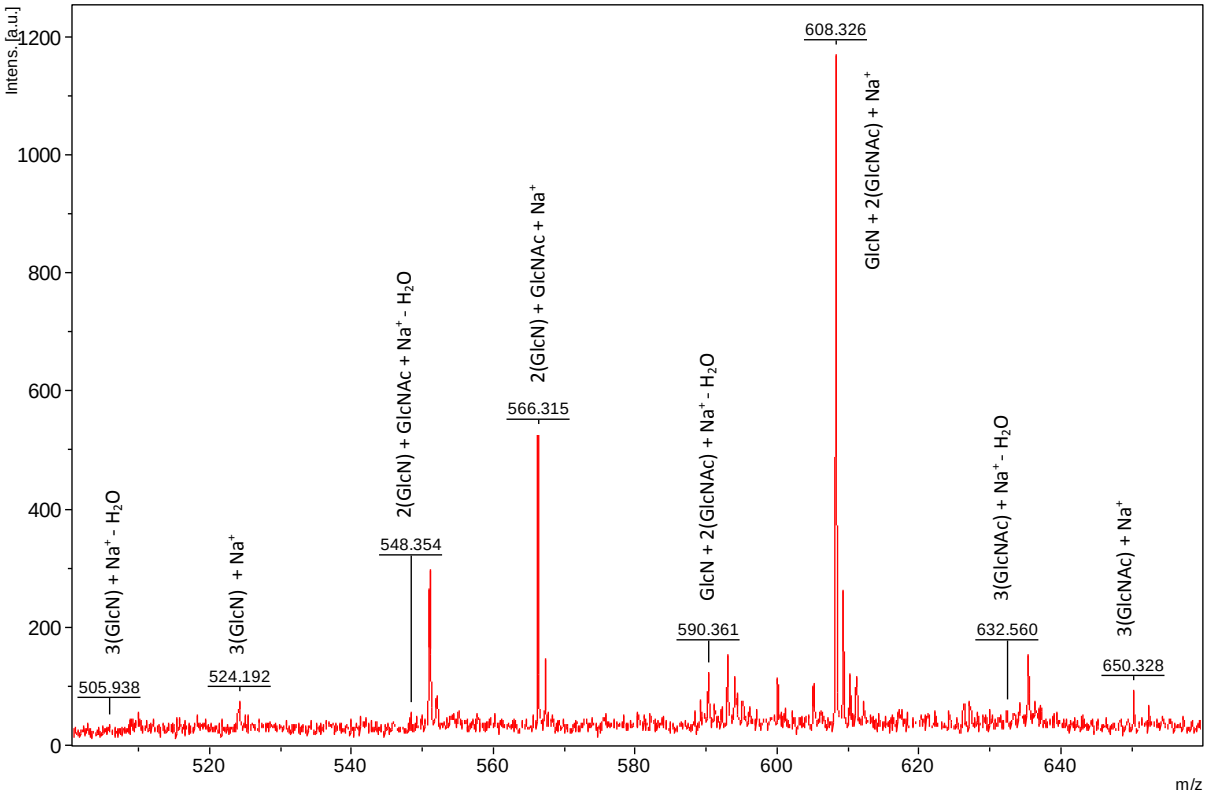

Figure 7.26: Mass spectrum illustrating the trimer of OT56 1st fraction produced via enzyme hydrolysis with Laminarinase from *Trichoderma* sp. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

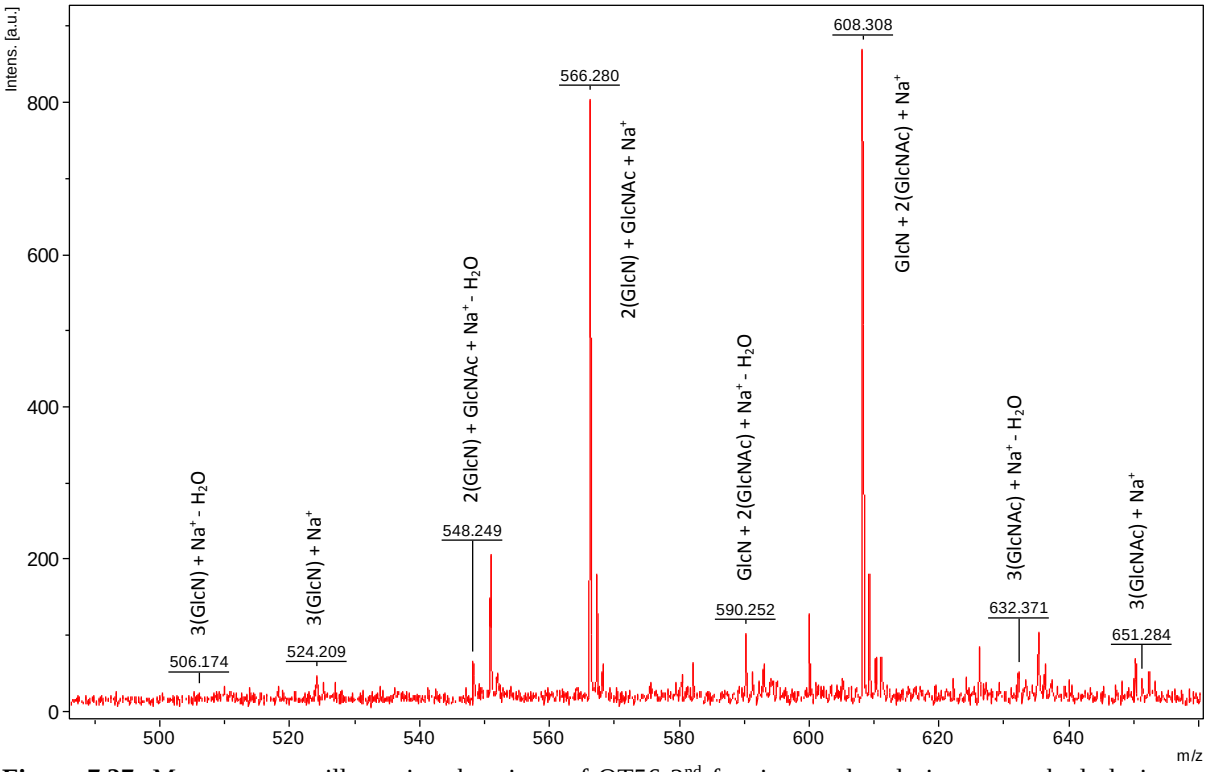

Figure 7.27: Mass spectrum illustrating the trimer of OT56 2nd fraction produced via enzyme hydrolysis with Laminarinase from *Trichoderma* sp. The different combinations of glucosamine (GlcN) and acetylated glucosamine (GlcNAc) units of the chitosan trimer can be seen, and all peaks are M + Na^+ with or without the loss of a H_2O molecule.

7.4: Discussion

Overall, our work with chitosan as a protective coating for harvested fruit produced rather incomplete results. In the first two studies (Figures 7.02 and 7.03) in which weight/water loss was the only focus of the investigations, it was found that 2% chitosan protected the oranges whilst lower concentrations induced higher rates of weight/water loss than no coating at all. Neither chitosan nor pectin were used at 2% concentrations in the subsequent experiment where there were the additions of volatile treatments (Figures 7.04 and 7.05) so these observations were not replicated. However, the fact that the lower concentrations again did not provide protection from water/weight loss for the fruit was confirmation as to the lack of efficacy of these coatings utilised at less than 2% (Figure 7.05). Although it increased water/weight loss when acting alone, 0.1% chitosan reduced this factor compared to the controls with the addition of all the different volatile compounds, in the majority of cases with greater efficacy than the volatiles only. This was most marked in regards to valencene, which only induced a 2.2% reduction when acting alone, whilst in combination with a 0.1% chitosan coating this reduction rose to 44.5%. Regarding pathogenicity, it was found that chitosan at 0.1 and 1% discouraged *Penicillium* growth on the inoculated fruit by 47.8 and 31.7% respectively when acting in isolation, but was less effective with the addition of volatile compounds. This is in marked contrast to the results regarding water/weight loss where it appeared that the chitosan was working in an additive manner with the majority of the volatiles, thus adding further frustration to the limited nature of the work undertaken.

A number of studies have previously looked at the effects of chitosan on the post-harvest quality of citrus fruit. In an investigation conducted by Chien and Chou (2006), the antifungal activity of 0.05, 0.1 and 0.2% chitosan solutions were examined for their ability to control post-harvest infections and weight loss in Tankan citrus fruit. The higher the concentration the lower the percentage decay and weight loss was observed, which somewhat corresponds to our results in regards to weight loss, although we did not find that chitosan at lower concentrations than 2% was effective. Chien conducted another investigation into chitosan as a protectant of harvested citrus fruit which confirmed the results of the first (Chien *et al.* 2007), whilst another Chinese group (Deng *et al.* 2008) found that 2% chitosan solutions reduced disease incidence and weight loss in Navel oranges. This does agree with the results of our work, particularly in regards to weight

loss, although other authors have found that chitosan coatings do not reduce weight loss in mandarins to any great degree compared to un-coated fruit (Contreras-Oliva *et al.* 2012). The application of 2% chitosan has also been shown to induce almost 100% reduction in decay of strawberry and orange fruit inoculated with *B. cinerea* and *Penicillium* pathogens respectively, and although we did not measure pathogen growth in the experiment where 2% chitosan was utilised (Figure 7.03) solutions of 1% and 0.1% alone did reduce *Penicillium* growth by 31.7% and 47.8% respectively (Figure 7.04). Rappussi and colleagues (2009) also tried different concentrations of chitosan (0.5, 1.0, 1.5, 2.0, and 3.0%) to evaluate its effectiveness against the black spot disease of Valencia oranges caused by *Guignardia citricarpa*. Chitosan at all concentrations except for 3% were effective in inhibiting lesion development caused by the pathogen and analysis revealed that chitinase, β-1,3-glucanase, peroxidase and polyphenoloxidase activities were increased in the fruit. It was therefore surmised that the action of chitosan could be due to a direct fungicidal effect and/or the induction of resistance in the fruit (Rappussi *et al.* 2009). Another study, conducted to evaluate how disease resistance and reactive oxygen species (ROS) metabolism in harvested Navel oranges may be affected by chitosan (2%) found that both disease incidence and lesion diameters were significantly lower in chitosan-treated fruit, and the activities of peroxidase (POD) and superoxide dismutase (SOD) were increased (Zeng *et al.* 2010). This chitosan treatment also increased the levels of glutathione (GSH) and hydrogen peroxide (H_2O_2), inhibited the activities of catalase (CAT) and the decreases of ascorbate (AsA) content, and induced ascorbate peroxidase (APX) activity in the stored oranges. These observations inferred that chitosan treatment may induce disease resistance by regulating H_2O_2 levels, antioxidant enzyme and the ascorbate–glutathione cycle of the fruit. The antifungal activity of chitosan has also been proposed to be related to its ability to interfere with fungal plasma membrane functions as well as interacting with fungal DNA and RNA (Allan and Hadwiger 1979; Hadwiger and Loschke 1981).

Regarding pectin as a stand-alone treatment for the protection of harvested fruit, our work did not reveal its potential to any great degree. It performed quite well when combined with some of the volatiles, particularly 1% pectin in combination with (+)-linalool (-49.8%), but 1% pectin alone actually increased infection compared to the control fruit by 8.4%. Previously, it has been found to be inhibitory to various bacteria (Yamashita *et al.*

2001), but work on any antifungal potential does not seem to appear in the literature except for when it has been combined with chitosan in biofilms. When incorporated into polypropylene to produce a multi-layered film for utilisation as food packaging, these films have proved to be both stable and have excellent antifungal and antibacterial properties (Elsabee *et al.* 2008). Similarly, a multi-layered edible coating made of chitosan and pectin was modified by the addition of a microencapsulated β-cyclodextrin and trans-cinnamaldehyde complex (2%) and investigated for its ability to extend the shelf-life and quality of fresh-cut papaya fruit (Brasil *et al.* 2012). The coating improved fruit firmness and colour and were preferred over uncoated fruit by a team of panellists. Pectin, therefore, appears to have other properties rather than direct antifungal activity that adds to the efficacy of chitosan so that the two combined are better than either alone.

Proposed interactions with essential oils and chitosan have also been investigated. For instance, Wang and colleagues (2011) showed that the antimicrobial activity of cinnamon oil against *E. coli, S. aureus, A. oryzae,* and *P digitatum* was enhanced when it was incorporated into chitosan film, whilst Chafer and others (2012) combined chitosan (1%, high molecular weight, with a DA of 82.7%) with the essential oils of bergamot, thymol or tea tree oil and applied them to Navel oranges either prior to or after inoculation with *P. italicum*. The antifungal effects of the coatings as well their effects on fruit quality (including weight loss) were evaluated and it was found that the preventative (i.e. prior to inoculation) treatment with chitosan coating containing tea tree oil was the most effective in reducing pathogen growth, whilst that with bergamot oil slightly reduced weight loss compared to the uncoated controls (Chafer *et al.* 2012). Limonene and the essential oil of peppermint were also incorporated into chitosan solutions to create edible coatings for strawberries, and that containing limonene was found to be the most effective at extending the shelf life of the fruit (Vu *et al.* 2011). These findings add weight to the results we obtained with volatile compounds in combination with chitosan, although the applications utilised were very different. In the previous studies, the essential oils and/or volatile compounds were incorporated into the chitosan formulations, whereas our investigations utilised the volatiles separately as fumigants for the chitosan-coated fruits.

Turning to the work on hydrolysing chitosan, we succeeded in produced hydrolysates by using both acid and enzyme hydrolysis methods of similar DPs that previous authors found to be useful in inhibiting pathogens. Of the acid hydrolysis methods utilised, our

method b using HCl at 4.6 mL per gram of chitosan and without the addition of methanol precipitation (OT53 1st fraction, Figure 7.10) yielded high quality chitooligosaccharides with a DP of 3-7, similar to those found to inhibit *F. solani* (Kendra and Hadwiger 1984) *B. subtilis* (Wu 2012), *E. coli* and *B. cereus* (Jeon and Kim 2000; Kittur *et al.* 2005). Enzyme hydrolysis using Laminarinase (Figures 7.20 and 7.21) was also extremely successful in producing high quality chitosans of this size. In some cases, chitooligosaccharides with a slightly higher DP have been found to have the greatest activity against pathogens. For example, Kulikov and colleagues (2006) found that chitosan with a DP of 6-11 had the highest antiviral activity in plants, and Kittur and others stated that it was the hexamer in particular that had the greatest antibacterial properties in their studies. Although, none of our chitooligosaccharides had high concentrations of hexamers or above, those produced with Laminarinase contained oligomers of up to DP 9 and it would presumably be possible to produce compounds with a greater concentration of these larger oligomers by reducing the incubation time or the concentration of the Laminarinase enzyme in the mix. Regarding DA, it has been stated that acetylated oligomers greater than trimers have the capacity to induce phytoalexin formation in plant cells (Yamada *et al.* 1993). Of the methods utilised that gave hydrolysates with the closest desired DP, that with Laminarinase yielded acetylated oligomers of DP 4 and above, which may have shown this activity had we extended the research into this area.

No previous work on chitosan has looked into percentage dehydration of the oligosaccharides as having potential influence on pathogen inhibition to our knowledge, so this factor is something else that could be investigated in the future. It is however probably just an artefact of the ionization process in the MALDI-TOF-MS technique, so there may not be any evidence of it in the hydrolysates themselves. The fact that our own project did not continue researching chitosan hydrolysates and their possible effects on our pathogens (*P. digitatum*, *P. italicum* and *B. cinerea*) and plants of interest (namely, harvested oranges and strawberries) is unfortunate, as our results with commercial chitosan were rather disappointing and incomplete. Considering the amount of research that is continuing into natural compounds as plant protectants and the observations made regarding the potential of chitosan in this respect, it would be worthwhile experimenting and studying it in greater detail. Hydrolysates of chitosan with different DPs and DAs

could be utilised as coatings of differing concentrations, with or without the addition of volatiles, to ascertain which, if any, could be potentially used as postharvest treatments. This could then aid in reducing our reliance on chemicals in agriculture – something both the general public and governments around the world are growing increasingly concerned about.

Chapter 8: General Discussion and Conclusions

We developed a facility to assay different treatment methods to extend the post-harvest life-span of fresh produce. These large chambers allowed the testing of a wide range of application devices (dipping, volatile, slow release etc.) under different environmental conditions. As respiration is low in harvested fruits (Saltveit 1991), we have also shown that measuring weight loss as a proxy for water loss can be a valuable way of ascertaining a commodity's freshness. Temperature was shown to be the most important factor, as revealed by the significant differences observed in the weight/water loss of oranges even when RH levels were relatively similar (Figure. 2.04, Phase A). This is in agreement with previous authors (Paull 1999). However, it was also evident that maintaining fresh produce at low temperatures and high RH levels is the most effective way of reducing weight loss (Figure 2.04, Treatment 5), as higher temperatures and low RH increases water deficits (Sharkey and Peggie 1984; Paull 1999).

Water loss was also shown to vary quite considerably in different produce (Figure 2.05). This is due to evaporation rates being dependent both upon the area of exposed surface and the nature of that surface – i.e. loose leafy vegetables like lettuce have a large exposed surface and a thin cuticle that makes them highly susceptible to loss (Aguero *et al.* 2011). Therefore, the "biology" of different commodities will ultimately dictate the design of any post-harvest procedures, as it is qualitatively different between produce.

Regarding the use of volatile compounds to reduce water/weight loss in harvested fruit and diminish the effects of pathogen infection, the results were complex and in some cases inconsistent or incomplete. Tables summarising the effects are shown in Tables 8.01 and 8.02. Concerning *in vivo* experiments with citrus fruit, there were only two volatiles that consistently reduced *Penicillium* growth in both organic and non-organic fruit – orange oil and neryl acetate (Figures 3.01 & 3.03). These two treatments also reduced weight loss in every experiment with non-organic citrus fruit (Figure 3.04), but only orange oil displayed 100% reliability when it came to organic oranges (Figure 3.02). This can be seen clearly in Table 8.01, where the row specifying orange oil has a set of

Volatile Compound	Reduction of *Penicillium sp.in vitro*	Reduction of *Penicillium sp. in vitro* when combined with fungicides	Reduction of *Penicillium sp. in vivo* (organic fruit)	Reduction of *Penicillium sp. in vivo* (non-organic fruit)	Reduction of water loss in organic oranges	Reduction of water loss in non-organic mandarins
1-octen-3-ol						
(-)-linalool (licareol)						
(+)-linalool (coriandrol)						
(-)-terpinen-4-ol						
(E)-2-hexenal						
octanal						
nonanal						
decanal						
citral						
methyl salicylate						
geranyl acetate						
neryl acetate						
methyl jasmonate						
(R)-carvone						
(S)-carvone						
myrcene						
γ-terpinene						
(-)-α-pinene						
(+)-α-pinene						
(-)-β-pinene						
limonene						
valencene						
β-caryophyllene						
Orange oil						
Lemon oil						

Table 8.01: Summary of the effects of volatile compounds on *Penicillium sp. in vitro* and *in vivo*, plus water/weight loss in oranges and mandarins. Outcomes are colour-coded for pathogen inhibition and reduction in water/weight loss in the fruit: Green = 100% positive; Yellow = > 50% positive; Red = < 50% positive. Blank cells = no info.

four green cells indicating 100% reliability in reducing both fungal growth and water/weight loss in the inoculated fruit. Most of the other volatile compounds have at least one red cell indicating a negative result or less than 50% reliability, but there were a number of other compounds that had generally positive effects on fruit health. Besides neryl acetate, these were (E)-2-hexenal and both enantiomers of linalool. The aldehyde has been found to be effective by previous authors (Neri *et al.* 2007), but to our knowledge this is the first time the others have been reported to beneficial to harvested produce *in vivo*. Methyl salicylate (MeS) was also a notable compound, in that it reduced water/weight in the fruit on the whole and also inhibited the *Penicillium* pathogens by 100% in organic fruit. It did not display this efficacy in the non-organic fruit, however, perhaps due to the effects of the fungicides or pesticides sprayed on these conventionally grown mandarins. There were also a number of compounds that were successful at inhibiting the fungal pathogens in either organic or non-organic produce, but not both. For example, γ-terpinene, (-)-α-pinene, limonene and lemon oil were all consistently effective in organic oranges (Figure 3.01), whilst nonanal, (S)-carvone, myrcene, (-)-β-pinene, valencene and β-caryophyllene displayed 100% efficacy in non-organic fruit (Figure 3.03). One explanation for this has to be the fact that the non-organic crops have been treated with both pre- and post-harvest synthetic fungicides (as well as other agricultural chemicals) and the volatiles are interacting with these in either a positive of negative way.

It is unfortunate that the scant availability of slow-release sachets meant that we did not obtain a full set of data regarding the volatile compounds and their effects on the two *Penicillium* pathogens *in vitro*, as these may have given us a bit more insight as to their interactions with the commercial treatments. However, there a number of compounds already mentioned for their efficacy *in vivo* which were also inhibitory *in vitro*, both when working alone and in combination with the fungicide-amended agar. Aldehydes have been previously reported to be active *in vitro* against these pathogens (Moleyar and Narasimham 1986; Wuryatmo *et al.* 2003), and in our work (E)-2-hexenal particularly stood out. Although the aldehydes were the most effective overall *in vitro* (see Chapter 4), it can be seen in Table 8.1 that it is the only one of this group that had consistently good (either green or yellow cells) across all experiments involving *P. digitatum* and *P. italicum*. The two linalool enantiomers also performed very well across the board and

there were a number of others (particularly the two carvone enantiomers) that inhibited the pathogens both *in vivo* and *in vitro*, although they did not achieve the desired effect when it came to reducing water/weight loss in the fruit. Carvone, particularly the (R)-enantiomer, has been found to be effective against *P. digitatum* by du Plooy *et al*. (2009) and Combrinck and colleagues (2011), so this corroborates our findings. It is regrettable that orange oil was not used in the *in vitro* studies given that it had the greatest efficacy *in vivo*, but as stated in Chapter 3 it is clear that it must be a combination of two or more of its components that provide it with its activity. As all the compounds used in our studies are citrus volatiles found in orange oil, the observations examined thus far perhaps provide indications as to which of its individual components are in fact responsible.

The ketone, (R)-carvone was probably the most effective volatile compound in our *in vivo* work with strawberries and *B. cinerea.* It reduced pathogen growth in both organic and non-organic fruit (Figures 5.01 & 5.03) and it was also effective in reducing water/weight loss in the non-organic fruit, although it was not 100% reliable (Figure 5.04). These effects have not been observed by previous authors to our knowledge. Table 8.02 summarizes the effects of the volatile treatments on *B. cinerea in vitro* and *in vivo*, as well as on water/weight loss in the strawberries. If we disregard the lack of data for organic strawberries (particularly in reference to water/weight loss), there were a number of compounds that had positive effects at least 75% of the time across both experiments (i.e. have green and yellow-coloured cells only). These were (-)-linalool, terpinen-4-ol, (E)-2-hexenal, geranyl acetate, neryl acetate (-)-β-pinene, limonene, valencene, β-caryophyllene and orange oil.

Across the results of all *in vitro* studies conducted regarding the effects of citrus volatiles on the pathogen *B. cinerea*, the principal conclusion was that the aldehydes exhibited very high overall efficacy, whilst MeS stood out as the most effective ester. The two enantiomers of carvone also exhibited high activity. Of all these, MeS was probably the most effective *in vitro* (Figures 6.01 and 6.03), with 100% inhibition both when acting alone and when in combination with the fludioxonil-based fungicide. This has not been observed by previous authors, but there have been reports showing that aldehydes display high efficacy against *B. cinerea in vitro* (Hamilton-Kemp *et al.* 1992; Vaughn *et al.* 1993; Kishimoto *et al.* 2005; Myung *et al.* 2007). In our work, the aldehydes were very

effective in reducing the growth of the pathogen, but there are some gaps where they were not utilised in certain experiments (Table 8.2). However, if their activity patterns are similar to that of the other aldehydes, plus the ketones and methyl salicylate, it would be plausible to say that those blank cells should also be green.

If we look at the results concerning the volatiles and *B. cinerea* as a whole, it would seem that (R)-carvone was the most effective compound overall. This has not been observed by previous authors to our knowledge. If we had obtained a complete set of data it is quite probable that some of the others would be potentially very good too, as there are a number with no negative results (i.e. red cells) and indeed have proved to be more effective at reducing water/weight loss in the fruit than the ketone. Unlike the results with *Penicillium* species and citrus, there were not many compounds that only performed well on either organic or non-organic fruit but not both. The main exception to this was (+)-linalool, but if we examine its *in vitro* effects we can see that it had higher efficacy when combined with fungicide-amended agar, thus offering a possible explanation as to its improved effects on non-organic fruit.

If we examine the results obtained regarding all the volatile compounds and their effects on the post-harvest pathogens of citrus fruit and strawberries plus water/weight loss in the produce, it appears we found a small number that may be potentially very good at maintaining freshness and extending shelf-life. One of these is (E)-2-hexenal, which was highly inhibitory to both *Penicillium* species and *B. cinerea* and did not produce any negative effects in the fruit either. This compound has been examined as a potential post-harvest treatment by many previous authors and has been found to be effective both *in vitro* and *in vivo* (Vaughn *et al.* 1993; Archbold *et al.* 1997; Gardini *et al.* 2001; Kishimoto *et al.* 2006; Neri *et al.* 2006c) and our results confirm this. Linalool, particularly the (-)- enantiomer, is another compound that delivered beneficial results across all the experiments it was included in, along with neryl acetate and orange oil. Methyl salicylate and (R)-carvone have been also been highlighted as having high efficacy in most aspects of the two main studies, but are weak in just one area or another. It would be worth including these in any future work, as there are a number of avenues of investigation which could be attempted in which they may prove useful.

Volatile Compound	Reduction of *B. cinerea in vitro*	Reduction of *B. cinerea in vitro* in combination with FLU	Reduction of *B. cinerea in vivo* (organic fruit)	Reduction of *B. cinerea in vivo* (non-organic fruit)	Reduction of water loss in organic strawberries	Reduction of water loss in non-organic strawberries
1-octen-3-ol						
(-)-linalool (licareol)						
(+)-linalool (coriandrol)						
(-)-terpinen-4-ol						
(E)-2-hexenal						
octanal						
nonanal						
decanal						
citral						
methyl salicylate						
geranyl acetate						
neryl acetate						
methyl jasmonate						
(R)-carvone						
(S)-carvone						
myrcene						
γ-terpinene						
(-)-α-pinene						
(+)-α-pinene						
(-)-β-pinene						
limonene						
valencene						
β-caryophyllene						
Orange oil						
Lemon oil						

Table 8.02: Summary of the effects of volatile compounds on *B. cinerea in vitro* and *in vivo*, plus water/weight loss in strawberries. Outcomes are colour-coded for pathogen inhibition and reduction in water/weight loss in the fruit: Green = 100% positive; Yellow = > 50% positive; Red = < 50% positive. Blank cells = no info.

The combination of two or more of the compounds in various concentrations would be a good approach to start with. Phytotoxic effects (i.e. increased water/weight loss) have to be considered, so any undesirable influences on the fruit could be diminished further by reductions in concentration, as long as the inhibitory effects on the pathogens were not negatively affected. It is also clear from our studies that the integration of volatile treatments with commercial products like fludioxonil and/or imazalil could provide a means for reducing our reliance on synthetic fungicides for the management of postharvest decay. Our *in vitro* work with amended agar suggests that some of these volatiles may work in parallel to the fungicides, in which case the volume and concentrations of these synthetic chemicals could perhaps be lessened by combining them with these natural treatments. The establishment of alternative decay control programs would help minimize the risks of resistance development, residues on food and environmental pollution. Many studies besides ours have reported the fungicidal activity of volatiles, but to our knowledge the subsequent step of semi-commercial trials has never been instigated. This is probably due to problems with inconsistency and low efficacy in many of those previously examined. Further research is required to reveal the mode of action and cost-efficacy of the most active compounds, as well as the method and timing of application. However, delivery method via a slow-release system like the sachets used in our studies may be one aspect that has been resolved enough to promote the utilisation of volatiles to the next phase of development. The efficacy of the most active compounds in our studies is highly convincing and their potential as natural biocontrol agents is worthy of further investigation.

Regarding our work with chitosan as a protective coating for harvested fruit, the main conclusion has to be that it requires further work. At 2% concentration, it certainly had a protective effect on the oranges by reducing water/weight loss (Figures 7.02 and 7.03), but this concentration was not used in the subsequent experiment where there were the additions of volatile treatments (Figures 7.04 and 7.05) so these observations were not replicated. With the volatile compound additions was 0.1% chitosan that reduced this factor compared to the controls, in the majority of cases with greater efficacy than the volatiles only. In regards to pathogen inhibition, it was found that chitosan at 0.1 and 1% discouraged *Penicillium* growth on the inoculated fruit by 47.8 and 31.7% respectively when acting in isolation, but was less effective with the addition of volatile compounds. If

we disregard the fact that the data is somewhat limited, these results suggest that lower concentrations (0.1%) of chitosan are more successful when it comes to protecting the produce from water/weight loss. Pectin was ineffective in the brief study we conducted, but further work looking at chitosan concentration is undeniably required to obtain more detailed information on the observations noted so far. This could perhaps include the incorporation of the volatiles into the chitosan formulations prior to coating the fruit, similar to investigations conducted by previous authors (Vu *et al.* 2011; Wang *et al.* 2011; Chafer *et al.* 2012). There is also the evidence that chitosan may stimulate plants' natural defence mechanisms (Rappussi *et al.* 2009; Zeng *et al.* 2010), so perhaps timing of application could be a focus of any future work also.

Another aspect of future work involving chitosan has to involve the use of chitooligosaccharides, such as those we obtained via our hydrolysis work. We successfully produced hydrolysates of similar DPs to those found to possess antimicrobial activity by previous authors (Kendra and Hadwiger 1984; Jeon and Kim 2000; Kittur *et al.* 2005; Kulikov *et al.* 2006; Wu 2012) via both an acid hydrolysis method (OT53 1st fraction, Figure 7.10) and enzyme hydrolysis using Laminarinase (Figures 7.20 and 7.21). It is unfortunate that time constraints meant that we did not extend our own investigations into examining these chitosan hydrolysates and their possible effects on our pathogens (*P. digitatum*, *P. italicum* and *B. cinerea*) and plants of interest (namely, harvested oranges and strawberries), particularly as our results with commercial chitosan were somewhat inconclusive. There is so much more research, both on-going and required, into natural compounds as plant protectants that the potential of chitosan would be worthwhile studying in greater detail.

In conclusion, this work has revealed the potential of some alternative methods for maintain the freshness and extending the storage and shelf-life of fresh fruits, with citrus (oranges and mandarins) and strawberries as examples. A number of naturally-occurring citrus volatiles, particularly (E)-2-hexenal, neryl acetate, linalool and perhaps a couple of others, have been found to be effective at reducing the growth of the major pathogens of these produce both *in vitro* and *in vivo*, as well as protecting the fruit from water/weight loss. They have also been found to operate synergistically with synthetic fungicides in current commercial use, thus implying the possibility that the domination of these

chemicals in the post-harvest industry could be diminished by combined applications. Chitosan as an edible coating for fresh produce has also been shown to have potential, although additional work is required to elucidate this further. However, it perhaps could be utilised in conjunction with volatile applications to provide completely natural post-harvest treatments, which would both aid the organic market as well as the agricultural industry in general. Reducing the world's reliance on synthetic chemicals is something both the general public and governments around the world are growing increasingly concerned about, so any techniques which may help achieve this aim have got to be worthwhile investigating further.

Chapter 9

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Appendices

7.1.1 Mass List of Inulin

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
383.8374	26370.3	388.009	31.65033	2512.019	349.2242	609.7312	0.248904	1.099115	660.9373
401.0552	26950.13	95.11774	7.139035	65.92501	5480.895	11.57706	0.061017	7.32E-02	279.4824
509.2318	30338.78	211.38	12.6306	696.4548	2454.87	69.1108	0.135598	0.207437	243.1355
510.2545	30369	120					7.70E-02		
522.654	30733	634					0.406704		
522.6545	30733.01	562.4664	33.59947	4491.922	3474.707	128.2721	0.360816	0.150417	833.3282
523.6483	30762	236					0.151392		
525.1628	30806.12	113.7464	6.77598	114.0308	3699.983	25.577	7.30E-02	0.141937	333.2911
525.193	30807	134					8.60E-02		
527.208	30865.6	689.0224	41.09501	11362.02	3180.226	180.272	0.442001	0.165777	734.6247
528.1686	30893.5	154					9.88E-02		
531.2218	30982	86					5.52E-02		
539.4935	31220.5	133					8.53E-02		
543.1508	31325.37	249.1679	14.75751	743.5008	3361.619	68.6014	0.159839	0.161574	590.6772
544.169	31354.5	123					7.89E-02		
550.6838	31540.26	534.5006	31.509	2052.756	3236.201	133.74	0.342877	0.170164	655.1797
551.66	31568	238					0.152675		
671.2023	34797.15	1090.108	64.9381	9643.099	4161.275	292.9053	0.699293	0.161297	2651.07
672.2259	34823.5	287					0.184108		
673.2367	34849.5	118					7.57E-02		
687.1686	35205.89	278.9053	16.90577	157.2018	4536.565	71.43918	0.178915	0.151473	2863.152
687.1729	35206	318					0.203994		
688.1752	35231.5	94					6.03E-02		
689.1852	35257.18	1558.872	94.12314	13548.47	5157.219	354.2999	1	0.133635	3950.59
690.2017	35283	380					0.243766		
691.2259	35309	90					0.057734		
705.1655	35660.96	347.1409	21.63607	1267.004	5606.386	72.40057	0.222687	0.125779	479.66
706.1823	35686.5	92					5.90E-02		
707.1187	35710	70					4.49E-02		
737.515	36464.6	93.95907	6.234901	207.9865	6617.019	18.35247	6.03E-02	0.111457	96.2597
738.5289	36489.5	82					5.26E-02		
833.2212	38744.14	821.9689	67.39678	6184.585	6093.079	204.1645	0.527285	0.136749	2243.966
851.2216	39157.97	1362.898	115.9022	6959.017	6455.639	326.1102	0.874285	0.131857	5762.713
867.2043	39521.77	282.227	24.2855	628.8166	6436.476	68.05231	0.181046	0.134733	683.0216
995.2654	42323.36	541.2305	61.61583	3366.678	7043.257	148.396	0.347194	0.141308	1515.727
1011.223	42659.51	176.2779	20.8194	95.90413	7218.734	49.25025	0.11308	0.140083	2075.833
1013.254	42702.11	832.8452	98.27734	8396.483	6707.621	258.897	0.534262	0.15106	2324.786
1029.231	43035.68	203.6301	24.64669	1822.74	7578.994	55.85918	0.130627	0.1358	223.7898
1157.269	45620.57	295.3462	42.08331	1223.922	7522.314	96.70693	0.189462	0.153845	1058.019
1175.268	45972.24	540.865	76.9703	4519.905	7391.614	190.5409	0.346959	0.159	1932.138
1191.229	46281.84	128.7513	18.78713	1341.823	9025.681	39.43062	8.26E-02	0.131982	139.7978
1319.27	48694.07	205.9603	32.90773	2130.055	8550.573	77.82384	0.132121	0.15429	412.8711
1337.272	49023.67	479.815	76.41197	4707.907	9323.613	170.0783	0.307796	0.143429	1707.027
1353.248	49314.33	117.957	18.93494	1019.957	9314.389	43.98642	7.57E-02	0.145286	190.6957
1481.295	51584.51	185.4861	31.2119	3070.122	10364.36	72.85668	0.118987	0.142922	284.1694

1499.305	51895.81	352.0191	58.4368	4604.006	9212.223	159.9199	0.225817	0.162752	1072.393
1515.289	52170.55	58.55401	9.837279	304.5061	7792.64	31.38683	0.037562	0.194451	91.662
1643.304	54320.56	113.8772	19.454	1365.792	11636.19	51.76229	7.31E-02	0.141223	222.3789
1661.342	54616.69	266.525	45.06908	6846.097	9057.441	154.1648	0.170973	0.183423	458.3905
1677.323	54877.7	49.75534	8.37415	555.5367	10339.68	24.63989	3.19E-02	0.162222	35.6418
1805.379	56925.86	46.6136	7.980446	535.2106	9635.911	29.43216	2.99E-02	0.187359	42.43502
1823.375	57207.79	190.0815	32.39578	4790.299	8566.89	140.5243	0.121935	0.21284	350.952
1985.432	59686.57	136.5245	25.41208	2813.627	8358.785	113.1163	8.76E-02	0.237526	257.1451
2147.449	62065.57	93.71432	18.94366	1302.689	8019.219	87.41219	6.01E-02	0.267788	141.5279
2309.458	64356.34	65.19443	14.04711	761.4657	7573.873	71.18426	4.18E-02	0.304924	90.30844
2471.427	66567.59	51.53547	12.13537	603.4435	7283.246	67.04314	3.31E-02	0.33933	89.52671

7.1.2 Mass List of Commercial Chitosan

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
338.2185	24780.9	188.3938	10.06911	3708.825	1252.792	70.59342	9.63E-02	0.269972	93.07987
354.1263	25350.97	1631.003	84.7198	101186.9	1189.31	693.1092	0.833685	0.297758	918.8453
443.6088	28343.11	264.7783	8.972528	1737.067	577.4046	277.2884	0.135341	0.768281	232.4962
504.972	30223	71					0.036292		
506.034	30254.5	50					2.56E-02		
509.0238	30343	99					5.06E-02		
523.1094	30756.5	118					6.03E-02		
535.1706	31106.17	332.541	6.061568	394.1582	2543.96	101.7255	0.169978	0.210369	975.8211
544.7277	31380.46	698.3596	12.21778	1419.921	3655.188	162.8677	0.356965	0.149029	3181.179
547.971	31473	98					5.01E-02		
550.6983	31550.6	1096.054	18.8133	1181.448	3642.599	279.342	0.560247	0.151183	12136.44
565.7438	31975.3	376.6938	6.299028	104.7065	2724.84	130.3321	0.192546	0.207625	5352.203
566.725	32002.8	1279.242	21.43015	2415.093	2467.524	513.607	0.653882	0.229674	12303.96
572.7404	32170.88	759.1353	12.71741	1221.306	2697.942	274.1441	0.388031	0.212288	5597.35
574.7258	32226.15	1340.762	22.46249	2906.381	2798.187	477.8604	0.685328	0.205392	9970.406
581.6674	32418.69	466.0033	7.817573	451.8508	3526.377	131.6327	0.238197	0.164948	2994.881
582.7241	32447.89	590.1537	9.975993	1165.337	2364.477	241.7104	0.301656	0.246449	3546.331
588.7547	32614.08	1654.837	27.82814	2089.645	2129.573	797.5713	0.845868	0.276466	30973.58
590.6999	32667.5	650					0.332246		
594.7505	32778.46	465.0487	7.819401	125.3629	2704.102	177.2137	0.237709	0.219944	9680.485
598.6971	32886.21	548.8786	9.182242	1257.449	2565.016	229.6432	0.280558	0.233409	1437.478
599.7214	32914.12	470.2299	7.888541	861.2469	4041.331	109.0693	0.240357	0.148397	807.7233
603.6731	33021.57	1801.561	30.21553	1714.008	3365.392	566.564	0.920865	0.179377	30540.51
604.7226	33050.04	1956.379	32.83802	1946.46	2068.955	968.3169	1	0.292284	55756.9
608.7651	33159.5	221					0.112964		
619.6461	33452.33	463.9474	7.881211	144.3041	3184.52	156.2059	0.237146	0.194581	8865.214
620.6887	33480.25	850.1734	14.47574	1167.983	1968.248	468.0106	0.434565	0.315351	10904.95
626.7032	33640.88	368.7189	6.35598	72.6052	2108.668	192.3086	0.18847	0.297203	6258.508
633.2559	33815	173					8.84E-02		
650.8642	34278.5	86					4.40E-02		
668.1515	34727.5	67					3.42E-02		
685.2137	35165	100					0.051115		
709.7611	35785	41					2.10E-02		
727.2818	36221	61					3.12E-02		
751.5977	36817.5	77					3.94E-02		
769.7274	37256	93					4.75E-02		
780.6574	37517.87	420.4618	10.96792	1561.524	3150.725	205.9314	0.214918	0.247771	1535.483
786.6827	37661.45	239.1526	6.294528	89.96748	2946.5	124.2696	0.122242	0.266989	2610.505
793.6797	37827.5	137					0.070027		
795.632	37873.7	249.9635	6.657731	101.7249	3451.779	110.9731	0.127768	0.230499	2512.169
796.6484	37897.73	462.1157	12.34032	1736.165	2370.023	304.8642	0.23621	0.336135	2724.966
802.6802	38040.02	254.7247	6.892589	858.3035	1078.276	398.0121	0.130202	0.744411	876.1174
811.6442	38250.5	248					0.126765		
812.6053	38273	442					0.225928		
828.5791	38645	186					9.51E-02		
835.6056	38807.5	127					6.49E-02		
847.3241	39077	81					4.14E-02		

853.8817	39227	66					3.37E-02		
1008.146	42603	117					5.98E-02		

7.1.3 Mass List of OT52 Chitosan Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
506.2754	30255.5	18					1.60E-02		
507.3048	30286	6					5.32E-03		
524.415	30788.5	16					1.42E-02		
525.2736	30813.5	17					1.51E-02		
548.2312	31474.5	64					5.68E-02		
549.2847	31504.5	38					3.37E-02		
567.1004	32007.5	237					0.210293		
568.1005	32035.5	84					7.45E-02		
591.09	32672.5	144					0.127773		
592.4029	32708.5	54					4.79E-02		
609.2324	33166.5	35					3.11E-02		
609.2509	33167	27					2.40E-02		
610.1393	33191	45					3.99E-02		
632.2391	33782.5	124					0.110027		
633.2572	33809.5	44					3.90E-02		
650.2897	34258	78					6.92E-02		
651.3031	34284.5	27					0.023957		
667.2963	34700	1127					1		
668.2841	34725.5	283					0.251109		
685.3068	35162	279					0.24756		
685.3488	35163.07	222.0258	15.10754	450.4181	2165.813	121.5804	0.197006	0.31644	938.6638
686.3275	35188	46					4.08E-02		
709.2944	35768	182					0.161491		
710.3128	35793.5	31					2.75E-02		
727.2929	36216	155					0.137533		
728.2836	36240.5	40					3.55E-02		
751.114	36800.5	154					0.136646		
752.1208	36825	58					5.15E-02		
769.2783	37240	45					3.99E-02		
770.11	37260	51					4.53E-02		
793.3701	37815	35					3.11E-02		
794.5105	37842	38					3.37E-02		
811.3516	38238.5	47					4.17E-02		
812.398	38263	26					0.02307		
835.2863	38795	51					4.53E-02		
836.088	38813.5	16					1.42E-02		
846.4134	39051	202					0.179237		
853.3833	39210.5	16					1.42E-02		
854.3908	39233.5	11					9.76E-03		
1007.584	42586	253					0.22449		
1168.605	45845.22	51.32269	7.556517	583.6722	3532.035	40.81618	4.55E-02	0.330859	32.13259

7.1.4 Mass List of OT53 First Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
506.2939	30256.05	584.9327	6.675309	253.3664	1053.478	495.0259	0.150717	0.480593	7231.587
507.2035	30283	345					8.89E-02		
507.3385	30287	377					9.71E-02		
524.312	30785.5	661					0.170317		
525.308	30814.5	248					6.39E-02		
548.3891	31479	272					7.01E-02		
566.2796	31984.5	403					0.103839		
567.4932	32018.5	357					9.20E-02		
590.3249	32651.5	345					8.89E-02		
591.4181	32681.5	218					5.62E-02		
608.2892	33141	460					0.118526		
609.2509	33167	206					5.31E-02		
632.3333	33785	455					0.117238		
633.295	33810.5	309					7.96E-02		
650.3088	34258.5	421					0.108477		
651.3605	34286	244					6.29E-02		
667.3544	34701.5	3881					1		
668.3422	34727	1227					0.316156		
683.3801	35112.87	796.7049	13.70907	1940.03	2324.348	407.7627	0.205283	0.294009	3040.919
685.4096	35164.62	1729.208	29.7997	28097.23	2054.952	1017.174	0.445557	0.33354	2809.13
685.4246	35165	1897					0.488792		
686.4257	35190.5	605					0.155888		
709.3343	35769	184					4.74E-02		
727.4141	36219	288					7.42E-02		
728.4455	36244.5	136					3.50E-02		
751.4016	36807.5	169					4.35E-02		
752.3675	36831	100					0.025767		
769.3822	37242.5	220					5.67E-02		
770.4844	37269	123					3.17E-02		
793.3912	37815.5	124					3.20E-02		
794.426	37840	111					2.86E-02		
811.5438	38243	189					4.87E-02		
812.5049	38265.5	143					3.68E-02		
846.5225	39053.5	958					0.246844		
846.5241	39053.54	815.2174	22.41997	12776.58	2431.129	541.0815	0.210053	0.348202	1174.814
972.5677	41843.5	87					2.24E-02		
1007.703	42588.5	578					0.148931		
1168.778	45848.6	157.6871	7.864759	1275.224	2170.968	185.5832	0.040631	0.538368	193.8024

7.1.5 Mass List of OT53 Second Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
474.6628	29303.28	532.2596	13.51187	14010.73	1591.371	237.5928	0.679942	0.298273	514.3652
506.3597	30258	91					0.116249		
507.1866	30282.5	51					6.52E-02		
524.3289	30785.99	439.9215	9.453834	4610.143	2858.711	118.614	0.561983	0.183414	254.888
524.3463	30786.5	490					0.625957		
525.3595	30816	177					0.226111		
548.4067	31479.5	123					0.157128		
567.117	32007.96	332.4094	6.465138	152.1341	1975.347	165.0735	0.424641	0.287097	3272.705
568.4221	32044.5	191					0.243995		
590.3795	32653	213					0.2721		
591.3087	32678.5	191					0.243995		
609.3804	33170.5	225					0.287429		
610.3244	33196	158					0.201839		
633.2642	33809.68	782.8016	14.81656	6679.854	3399.512	256.4923	1	0.186281	2013.16
634.2951	33837	326					0.416453		
651.4179	34287.5	172					0.219724		
652.3939	34313	138					0.17629		
667.4124	34703	399					0.509708		
668.4585	34730	171					0.218446		
685.5109	35167.2	367.568	7.514096	948.6163	1827.079	220.1925	0.469555	0.375195	633.362
686.6221	35195.5	230					0.293816		
709.4341	35771.5	115					0.146908		
710.5326	35799	87					0.111139		
727.3939	36218.5	126					0.16096		
728.5062	36246	102					0.130301		
751.4427	36808.5	120					0.153296		
752.4497	36833	96					0.122636		
769.5069	37245.5	106					0.135411		
770.4012	37267	98					0.125191		
793.5179	37818.5	107					0.136689		
794.3626	37838.5	88					0.112417		
811.6292	38245	95					0.121359		
812.6758	38269.5	76					9.71E-02		
835.4813	38799.5	83					0.106029		
836.6298	38826	63					8.05E-02		
846.5007	39053	240					0.306591		
853.4928	39213	65					8.30E-02		
854.5441	39237	68					8.69E-02		
1007.596	42586.26	193.0043	7.908432	2077.033	2894.151	144.8926	0.246556	0.348149	154.606
1168.62	45845.5	76					9.71E-02		
1329.663	48887.5	45					5.75E-02		

7.1.6 Mass List of OT54 Chitosan Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
18.14758	5863.108	2395.231	7.383919	24041	1487.829	34.86895	2.47E-02	1.22E-02	985.2318
23.14604	6599.09	75028.98	162.1418	3581.247	1082.555	2050.377	0.774476	2.14E-02	14603074
23.32411	6623.76	11388.25	24.63895	449.3848	64.6811	4964.391	0.117554	0.360602	2811869
23.69018	6674.183	3827.757	8.21115	141.1679	290.7026	381.4154	3.95E-02	8.15E-02	1202344
30.24834	7519.082	3280.698	6.399336	652.3603	1285.318	98.82428	3.39E-02	2.35E-02	47395.44
39.23877	8539.788	96877.05	158.616	14930.73	767.3095	6472.166	1	5.11E-02	11553508
40.2355	8645.379	30786.05	49.13035	2153.141	647.2466	2522.742	0.317785	0.062164	9065220
40.67925	8691.967	6251.424	9.818175	550.0741	22.39013	14673.22	6.45E-02	1.816839	2253160
41.24716	8751.222	30259.33	46.82897	1281.101	941.9112	1761.739	0.312348	4.38E-02	12508273
44.35748	9068.747	4252.907	6.596319	100.7578	835.0654	295.4019	4.39E-02	5.31E-02	1155981
52.30741	9833.011	8606.987	12.63207	1218.717	940.8001	613.339	8.88E-02	5.56E-02	712254.5
52.40233	9841.771	4988.358	7.22351	322.7865	1159.006	294.1619	5.15E-02	0.045213	353777.5
53.40777	9934.079	7091.55	10.45096	348.8665	898.8192	476.2039	7.32E-02	5.94E-02	600865.9
55.41297	10115.61	5199.83	7.740382	497.4504	934.4363	362.5707	5.37E-02	5.93E-02	226949.5
57.34229	10287.2	6227.819	9.29491	165.0408	742.491	653.1918	6.43E-02	7.72E-02	1340140
60.45385	10557.97	12451.33	18.97624	8268.371	1248.116	740.4067	0.128527	0.048436	192479.7
61.43508	10641.9	7076.475	10.81582	2638.71	1685.278	339.4958	7.30E-02	0.036454	127150.9
63.42918	10810.43	4827	7.494691	686.3856	682.5302	494.3586	4.98E-02	9.29E-02	69327.08
64.3974	10891.3	4795.045	7.484307	228.1007	729.0186	517.6821	0.049496	8.83E-02	471794.2
70.5235	11389.48	9770.639	15.18891	877.1183	1354.336	612.5217	0.100856	5.21E-02	1046063
74.57114	11706.84	10647.87	16.50527	17441.97	1489.87	642.6309	0.109911	5.01E-02	51380.16
76.56113	11859.71	4392.458	6.770764	252.8284	1549.63	259.3434	4.53E-02	4.94E-02	151780.6
81.53719	12233.47	8218.549	12.70595	241.48	459.9193	1690.732	8.48E-02	0.177286	1326840
83.54396	12380.97	5411.417	8.578131	726.2801	576.4666	893.7271	5.59E-02	0.144924	148822.1
84.61542	12458.99	7996.016	12.90927	276.604	1139.022	682.28	8.25E-02	7.43E-02	1167952
86.63087	12604.44	13517.41	22.23355	2413.488	1295.559	1143.472	0.139532	6.69E-02	1046174
88.52199	12739.37	5082.552	8.264366	227.135	654.8857	934.2814	5.25E-02	0.135172	386965.3
89.53019	12810.72	4553.849	7.219612	317.3748	1403.992	392.0283	0.047006	0.063768	225498.3
97.50975	13361.82	8947.814	13.47049	920.5782	1369.411	836.0563	9.24E-02	7.12E-02	868261.4
102.7329	13710.4	5789.813	8.005844	459.3195	1130.779	587.8123	5.98E-02	9.09E-02	237908.9
104.7251	13841.01	9751.865	13.19423	2095.769	1385.418	891.351	0.100662	7.56E-02	349575.7
105.601	13898.04	4656.235	6.163624	175.1973	864.8779	722.4619	4.81E-02	0.122099	327532.3
108.6851	14097	7233.891	9.121693	4249.045	1214.34	785.0388	7.47E-02	8.95E-02	42422.86
109.698	14161.72	9164.372	11.47109	6146.982	1043.953	1248.037	9.46E-02	0.105079	114314
110.7091	14226.03	8473.146	10.53212	215.2435	1230.625	914.7137	8.75E-02	9.00E-02	1635096
113.5493	14405.13	5875.203	7.055708	254.5514	1355.602	646.8796	6.06E-02	8.38E-02	550140.4
130.8826	15452.69	9586.797	9.755509	851.3646	1632.621	917.3164	0.098958	8.02E-02	340090.2
138.0802	15867.13	12365.67	12.37056	103.6357	432.5834	5614.172	0.127643	0.319199	11963204
138.7782	15906.75	37300.55	37.13464	3058.718	1460.699	4562.738	0.38503	9.50E-02	8055535
139.1141	15925.77	12732.27	12.31591	940.9482	3862.561	532.418	0.131427	0.036016	305166.6
148.7169	16460.31	7670.837	7.389867	237.337	2123.475	745.0464	7.92E-02	0.070035	678195.5
154.8149	16790.83	37289.83	36.36555	11457.27	1779.99	4285.671	0.384919	8.70E-02	1941315
155.8239	16844.89	33436.29	32.56905	15736.75	1635.907	4370.252	0.345141	9.53E-02	1266185
156.837	16898.99	14595.77	14.20645	4003.576	2659.276	1096.797	0.150663	5.90E-02	336989
159.8162	17057.08	31210.73	30.49335	75417.29	1886.998	3617.724	0.322168	8.47E-02	190649.3
163.9522	17274.12	6238.381	6.253937	260.8842	1548.18	787.1235	6.44E-02	0.1059	333080.4
176.8594	17934.41	29861.96	32.81529	1462.605	1397.848	4722.472	0.308246	0.126523	8850874
177.8599	17984.57	54653.05	60.50331	7612.678	1773.571	7652.935	0.564149	0.100284	8956332

178.9886	18040.98	19372.62	21.53437	279.0087	1233.048	3298.551	0.199971	0.145159	9145852
183.9827	18288.5	1885					1.95E-02		
192.8708	18720.84	11976.82	14.68404	823.6522	2713.506	1123.53	0.123629	7.11E-02	1026875
193.8648	18768.56	27524.22	34.26727	9863.451	2118.24	3307.364	0.284115	9.15E-02	988153.9
198.9122	19009.04	24576.48	31.96012	18857.6	2171.445	3093.078	0.253687	9.16E-02	528095.1
199.9138	19056.4	37567.97	49.49735	14461.16	2051.655	5132.682	0.38779	9.74E-02	1612257
202.0074	19155	1850					1.91E-02		
214.9221	19752.27	8593.6	12.7486	1803.542	2640.381	965.7524	8.87E-02	8.14E-02	237221.5
215.9262	19797.94	12486.6	18.74538	4148.561	2392.254	1566.737	0.128891	9.03E-02	348990.6
218.0688	19895.05	10888.92	16.44407	31828.02	2217.305	1365.372	0.112399	9.83E-02	21269.51
232.0676	20518.11	6144.398	10.40407	150.2005	1129.617	1526.303	6.34E-02	0.205439	965768.4
248.1495	21211.1	11883.39	22.71124	15747.14	2099.814	1783.339	0.122665	0.118177	46977.42
258.1557	21630.99	3546.926	7.157226	3065.132	2230.309	554.6658	3.66E-02	0.115749	7754.295
262.1863	21797.82	27317.96	55.34765	15522.85	2075.978	4795.226	0.281986	0.126295	904351.4
274.1413	22285.24	29590.17	63.46635	42516.62	2128.224	5245.525	0.30544	0.128812	441015.9
276.2031	22368.22	8387.003	18.05606	7033.699	2229.311	1379.728	8.66E-02	0.123896	55719.88
278.1946	22448.07	9565.173	20.67115	13708.68	2500.729	1395.138	9.87E-02	0.111245	40394.8
280.2262	22529.24	2813.141	6.088425	897.3143	1864.095	581.5187	2.90E-02	0.150328	24059.12
290.2576	22925.79	2895.286	6.462732	891.5011	2169.556	496.4032	2.99E-02	0.133787	29039.38
292.2547	23003.91	5819.11	13.22905	2138.048	1858.036	1184.621	6.01E-02	0.157292	58997.11
302.2711	23391.77	2944.247	6.94387	2705.614	2243.107	533.2886	3.04E-02	0.134755	6884.505
304.2989	23469.51	5352.746	12.8658	1115.587	2054.072	1041.565	5.53E-02	0.148144	80922.64
305.453	23513.63	12760.1	30.7473	3510.406	2750.7	1860.408	0.131714	0.111046	357404.6
306.2982	23545.89	25730.69	62.47165	22647.16	1834.54	6146.23	0.265601	0.166962	488227
314.2077	23845.68	3902.201	9.743458	375.3313	2209.487	810.5826	4.03E-02	0.142208	178460.7
320.3407	24075.55	4958.641	12.51187	1308.291	1992.297	1065.3	5.12E-02	0.16079	63218.7
322.2879	24148.07	8517.391	21.65128	3398.629	2374.2	1561.981	8.79E-02	0.135746	108200
330.18	24439.77	2699.894	6.993955	181.835	1320.216	1019.57	2.79E-02	0.250095	149095.1
332.2307	24515	5496.959	14.36009	2671.967	2370.559	1166.076	5.67E-02	0.140149	75574.07
333.5253	24562.37	4018.859	10.54338	1988.092	2550.395	718.671	4.15E-02	0.130774	23192.14
334.2882	24590.24	2769.428	7.267056	138.5805	1646.764	823.7028	0.028587	0.202997	198402.8
345.3682	24991.5	580					5.99E-03		
346.3585	25027.05	2106.819	6.013292	242.8413	1155.007	932.1087	2.17E-02	0.299876	74835.55
347.1326	25054.8	2124.203	6.053088	242.6921	2383.356	467.4922	2.19E-02	0.145649	56178.18
348.272	25095.59	2692.412	7.740634	711.7044	1273.464	1088.028	2.78E-02	0.273484	42241.94
350.3557	25170.02	2179.261	6.357026	1082.079	1733.098	637.857	2.25E-02	0.202156	14285.06
362.2657	25591.24	2272.019	7.115457	454.1592	1685.474	754.7282	0.023453	0.214934	45972.33
363.2289	25625	2648					2.73E-02		
366.3218	25733.11	11900.27	38.58018	14870.29	2241.822	3032.064	0.122839	0.163404	133945.8
376.2468	26076.97	1906.624	6.515047	35.1003	2433.757	455.2257	1.97E-02	0.154595	267138.5
524.0119	30742	255					2.63E-03		
546.4513	31389.46	621.5236	7.832459	499.3143	4874.561	102.422	6.42E-03	0.112103	1931.586
609.7562	33147.55	781.5713	11.94556	1677.989	2849.724	292.568	8.07E-03	0.21397	1505.439

7.1.7 Mass List of OT55 Chitosan Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
404.5908	27072.5	29					0.114173		
506.1573	30252	27					0.106299		
507.1697	30282	31					0.122047		
523.7972	30770.5	42					0.165354		
525.2736	30813.5	61					0.240157		
548.1785	31473	110					0.433071		
549.0915	31499	29					0.114173		
566.1904	31982	75					0.295276		
590.2156	32648.5	149					0.586614		
591.327	32679	48					0.188976		
608.2152	33139	254					1		
609.2879	33168	75					0.295276		
632.2391	33782.5	47					0.185039		
633.295	33810.5	43					0.169291		
667.2382	34698.5	127					0.5		
668.2841	34725.5	72					0.283465		
685.248	35160.5	50					0.19685		
686.2294	35185.5	29					0.114173		
709.2744	35767.5	132					0.519685		
710.2329	35791.5	43					0.169291		
727.2524	36215	74					0.291339		
728.2027	36238.5	32					0.125984		
751.2783	36804.5	68					0.267717		
752.3264	36830	26					0.102362		
769.2575	37239.5	62					0.244094		
770.2972	37264.5	34					0.133858		
793.2646	37812.5	30					0.11811		
794.1725	37834	16					6.30E-02		
811.3089	38237.5	52					0.204724		
812.3767	38262.5	26					0.102362		
835.5463	38801	22					8.66E-02		
836.283	38818	20					7.87E-02		
846.4352	39051.5	19					7.48E-02		
853.6023	39215.5	20					7.87E-02		
854.5222	39236.5	19					7.48E-02		
930.359	40930.5	19					7.48E-02		
1050.298	43474.5	21					8.27E-02		
1210.513	46656	12					4.72E-02		
1372.184	49659.5	10					3.94E-02		

7.1.8 Mass List of OT56 First Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
505.9381	30245.5	35					3.00E-02		
524.1918	30782	74					6.34E-02		
524.2261	30783	74					6.34E-02		
524.2433	30783.5	59					5.05E-02		
548.354	31478	55					4.71E-02		
566.3153	31985.5	526					0.450342		
590.3613	32652.5	124					0.106164		
608.3261	33142	1168					1		
632.5595	33791	57					4.88E-02		
650.3279	34259	93					7.96E-02		
667.3737	34702	168					0.143836		
668.4004	34728.5	129					0.110445		
686.3275	35188	299					0.255993		
687.349	35214	102					8.73E-02		
710.4127	35796	100					8.56E-02		
711.4719	35822.5	48					4.11E-02		
727.3535	36217.5	333					0.285103		
728.3443	36242	111					9.50E-02		
751.4427	36808.5	65					5.57E-02		
752.4086	36832	48					4.11E-02		
769.403	37243	279					0.23887		
770.3804	37266.5	114					9.76E-02		
793.3701	37815	56					4.79E-02		
794.5316	37842.5	33					2.83E-02		
811.437	38240.5	208					0.178082		
812.4408	38264	100					8.56E-02		
835.5029	38800	33					2.83E-02		
853.2958	39208.5	35					3.00E-02		
870.4797	39599	56					4.79E-02		
871.4087	39620	44					3.77E-02		
888.4602	40003.5	206					0.17637		
889.421	40025	90					7.71E-02		
912.5009	40538	43					0.036815		
913.452	40559	35					3.00E-02		
930.5419	40934.5	176					0.150685		
931.4794	40955	55					4.71E-02		
954.4695	41454.5	31					2.65E-02		
955.5812	41478.5	30					2.57E-02		
972.4976	41842	104					8.90E-02		
973.456	41862.5	67					5.74E-02		
990.5054	42225.5	55					4.71E-02		
991.4963	42246.5	36					3.08E-02		
996.4816	42352	25					2.14E-02		
997.6175	42376	24					2.05E-02		
1008.536	42606	123					0.105308		
1009.559	42627.5	49					4.20E-02		
1014.566	42732.5	34					2.91E-02		
1032.451	43105.5	37					0.031678		

1033.487	43127	27					2.31E-02		
1039.543	43252.5	26					2.23E-02		
1049.521	43458.5	206					0.17637		
1057.499	43622.5	31					2.65E-02		
1073.522	43950	33					2.83E-02		
1091.595	44316.5	144					0.123288		
1115.493	44796.5	28					2.40E-02		
1133.485	45154.5	67					5.74E-02		
1150.553	45491.5	25					2.14E-02		
1169.593	45864.5	93					7.96E-02		
1175.521	45980	80					0.068493		
1193.16	46322	17					1.46E-02		
1200.444	46462.5	21					1.80E-02		
1210.695	46659.5	45					3.85E-02		
1234.591	47115.5	17					1.46E-02		
1252.586	47456	25					2.14E-02		
1252.878	47461.5	21					1.80E-02		
1260.209	47599.5	22					1.88E-02		
1277.104	47916	25					2.14E-02		
1294.623	48242	22					1.88E-02		
1319.382	48699	20					1.71E-02		
1336.722	49016.5	18					1.54E-02		
1360.358	49446	17					1.46E-02		
1378.269	49769	17					1.46E-02		
1402.913	50210	18					1.54E-02		
1420.819	50528	19					1.63E-02		
1444.927	50953	18					1.54E-02		
1462.496	51260.5	19					1.63E-02		

7.1.9 Mass List of OT56 Second Fraction

m/z	time	Intens.	SN	Quality Fac.	Res.	Area	Rel. Intens.	FWHM	Chi^2
506.1742	30252.5	24					2.76E-02		
506.9672	30276	15					1.73E-02		
524.209	30782.5	47					5.41E-02		
525.2221	30812	37					4.26E-02		
548.2487	31475	65					7.48E-02		
549.3023	31505	36					4.14E-02		
566.2796	31984.5	804					0.925201		
567.3146	32013.5	179					0.205984		
590.252	32649.5	102					0.117376		
591.2358	32676.5	52					5.98E-02		
608.3076	33141.5	869					1		
609.2694	33167.5	181					0.208285		
632.371	33786	52					5.98E-02		
633.295	33810.5	41					4.72E-02		
651.284	34284	42					4.83E-02		
652.2599	34309.5	42					4.83E-02		
667.3156	34700.5	240					0.27618		
668.3229	34726.5	135					0.155351		
686.3079	35187.5	213					0.245109		
687.3294	35213.5	60					6.90E-02		
709.2944	35768	100					0.115075		
710.2929	35793	49					5.64E-02		
727.3333	36217	252					0.289988		
728.3848	36243	69					0.079402		
751.3194	36805.5	69					7.94E-02		
752.2853	36829	29					3.34E-02		
769.3822	37242.5	143					0.164557		
770.3804	37266.5	43					0.049482		
793.3068	37813.5	28					3.22E-02		
794.3838	37839	21					0.024166		
811.3089	38237.5	79					9.09E-02		
812.4408	38264	35					4.03E-02		
835.3513	38796.5	23					2.65E-02		
836.348	38819.5	16					1.84E-02		
847.3949	39073.5	149					0.171461		
853.4928	39213	18					2.07E-02		
854.6099	39238.5	15					1.73E-02		
870.347	39596	46					5.29E-02		
888.3931	40002	154					0.177215		
913.3614	40557	28					3.22E-02		
930.4047	40931.5	84					9.67E-02		
954.5158	41455.5	24					2.76E-02		
972.4742	41841.5	49					5.64E-02		
989.4208	42202.5	49					5.64E-02		
996.3161	42348.5	14					1.61E-02		
1008.536	42606	87					0.100115		
1014.423	42729.5	26					0.029919		
1032.475	43106	27					3.11E-02		

1038.504	43231	13					1.50E-02		
1049.473	43457.5	135					0.155351		
1057.353	43619.5	16					1.84E-02		
1074.504	43970	24					2.76E-02		
1091.521	44315	56					6.44E-02		
1115.868	44804	12					1.38E-02		
1133.586	45156.5	50					0.057537		
1150.655	45493.5	18					2.07E-02		
1151.646	45513	22					0.025316		
1157.936	45636.5	11					0.012658		
1169.593	45864.5	56					6.44E-02		
1175.572	45981	44					5.06E-02		
1192.979	46318.5	11					1.27E-02		
1200.133	46456.5	10					1.15E-02		
1210.513	46656	43					4.95E-02		
1217.981	46799	16					1.84E-02		
1235.539	47133.5	14					1.61E-02		
1252.56	47455.5	14					1.61E-02		
1277.746	47928	12					1.38E-02		
1294.542	48240.5	19					2.19E-02		
1318.892	48690	11					1.27E-02		
1337.297	49027	11					0.012658		
1360.662	49451.5	13					1.50E-02		
1378.687	49776.5	11					0.012658		
1402.689	50206	11					0.012658		
1421.667	50543	10					1.15E-02		
1444.699	50949	11					1.27E-02		
1462.038	51252.5	10					1.15E-02		