Developing Innovative Marker Systems for Garlic Post-Harvest Quality Assessment

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

By

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Synthesis

Work presented here furthers current understanding of the postharvest biology of garlic and identifies volatile organic compounds (VOCs) as markers of quality for culinary and biotechnological uses of garlic. Quality markers were developed for gauging storage time, storage temperature, harvest year, cold stress, incidence of disease and alliinase activity. This work also furthers understanding of interspecific and interseasonal variance in postharvest quality traits of garlic. Significant headway was made in translating the VOC quality markers into techniques for industrial application.

Chapter 3 focusses on a single garlic cultivar and discovered improved maintenance of alliinase activity in ambient storage, compared to the industry standard of -2 °C but also found that alliinase activity loss did not correlate with alliinase gene expression. The water content of cloves was stable over time and weight loss in garlic occurred at a 1:2 ratio of DW to FW. VOC profiles varied significantly between storage time and temperature; compounds were identified as putative markers for storage conditions.

Chapter 4 explores the interseasonal and interspecific variation in quality and alliinase activity throughout storage, and whether VOC based quality assessment is reliable across years and cultivars. A complex relationship was identified, with quality traits of cultivars changing differently according to season, time and temperature of storage. VOC marker systems correlated with alliinase activity, disease rate and discriminated between seasons.

Chapter 5 applies the findings from lab work to the industry. A new thermal desorption sampling method allows passive sampling of VOCs from warehouse air and was trialled over a 6 month period in industry. Cold stress did not affect alliinase activity,

but caused significant changes to quality parameters and the VOC profile. Finally, a pulsed flame photometric detector was trialled as an alternative tool for VOC assessment.

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1. Chapter 1: Introduction

1.1. Garlic Background

Garlic (*Allium sativum* L.) is a monocotyledonous bulb forming plant in the family Alliiaceae, order Asparagales. It is the world's second most widely consumed *Allium*, after onions, with a global production of 28 million tons (FAOSTAT, 2017). There are two main subspecies of garlic, *A. sativum var. ophioscorodon*, commonly known as hard-necked garlic, and *A. sativum var. sativum*, soft-necked garlic. Garlic is believed to have originated from Central Asia, where potential wild ancestors of *A. sativum* can be found growing, however it is likely that the *A. sativum* group was domesticated and then bred in the Mediterranean for thousands of years before being spread more widely across the world in the last 500 years (Maaβ & Klaas, 1995). Genetic taxonomy has suggested *A. tuncelianum* to be the wild progenitor species of garlic, based on amplified fragment length polymorphisms and nucleotide sequence analysis of the internal transcribed spacer (ITS) region (Ipek, 2008). Garlic has been cultivated for thousands of years as a flavouring and spice across a broad range of countries and cultures (Rivlin, 2001). There are hundreds of garlic cultivars grown worldwide, with significant levels of variation in agronomically important traits (Pooler & Simon, 1993a). The convoluted and long history of garlic cultivation has contributed to the difficulty in identifying its ancestry.

Garlic is an important commercial food crop, harvested for its subterranean storage bulbs, and a plant of considerable interest in the biotech industry. A garlic clove primarily consists of water (~ 65 %) and of the dry weight; the largest fraction is fructose-containing carbohydrates, followed by organosulphur compounds, protein, fibre and amino acids (Lawson, 1996). A garlic bulb typically contains around 20 cloves, however clove number per bulb is very variable between cultivars, ranging from < 5 to > 50 (Sharma *et al*., 2004; Panthee *et al.*, 2006).

There are two main classes of garlic; hard-neck and soft-neck also known as bolting and non-bolting cultivars respectively. Hard-neck cultivars are termed so as they produce a scape, whereas soft-necked varieties do not (Hahn, 1996). A scape is a leafless stem rising directly from the bulb, upon which the inflorescence develops. Scape formation in garlic is also controlled in part by environmental conditions, with cool temperatures and long photoperiods being conducive to scape formation (Kamenetsky *et al.*, 2004).

In this work, we examine the storage characteristics of 4 cultivars of garlic, three hard-necks and one soft-neck. Scape removal prior to flowering increases bulb yield significantly, by between 5 % and 15 %, with the greatest increases seen on less fertile soils (Rosen & Tongn, 2001). This improvement was attributed to a higher dry matter partitioning to the bulb and had no effect on total above ground dry weight of the plant. Soft-necked varieties are preferred by growers as they are higher yielding and easier to harvest, a result of their inability to produce a scape.

1.2. Garlic Agronomy

Garlic as a wild plant will begin its growth cycle in autumn (Fig. 1.1). Each clove forms a separate plant to its sister cloves within the bulb, with each plant establishing a network of roots and a shoot, before becoming dormant over winter. It is during winter than new clove primordia form in the axils of leaf sheaths near the basal plate (Jones & Mann, 1963). When conditions warm in Spring, the plant undergoes a second growth stage, where the shoot develops and the leaf canopy expands throughout late spring and early summer, lasting until ~140 days post sowing. The final growth stage is bulbing, which lasts for approximately 50 days, to a total of ~195 days, when bulb maturity is reached (Ledesma *et al.*, 1994). At this point, the leaves senesce and the new bulb lies dormant in the soil, until autumn, when the cloves of the newly formed bulb sprout and form plants of their own, repeating the cycle.

Farmed garlic has a different yearly growth cycle and can either be autumn or spring sown. In this thesis, all garlic studied was autumn sown and this process is outlined in Fig. 1.2. As in the natural growth cycle of garlic, a clove of garlic grows and develops to form a new bulb over the course of one growing season. The growth cycle of farmed garlic predominantly differs between senescence in summer and replanting in autumn. Bulbs are lifted from the ground in late summer, once half the leaves have senesced, where they are taken to a warehouse for cleaning, processing and grading. After this, they are stored in a warehouse until they are broken into cloves and sown in autumn. The bulbs which are not destined to be replanted, but instead sold, are kept in the warehouse for longer periods of time, typically not exceeding 9 months.

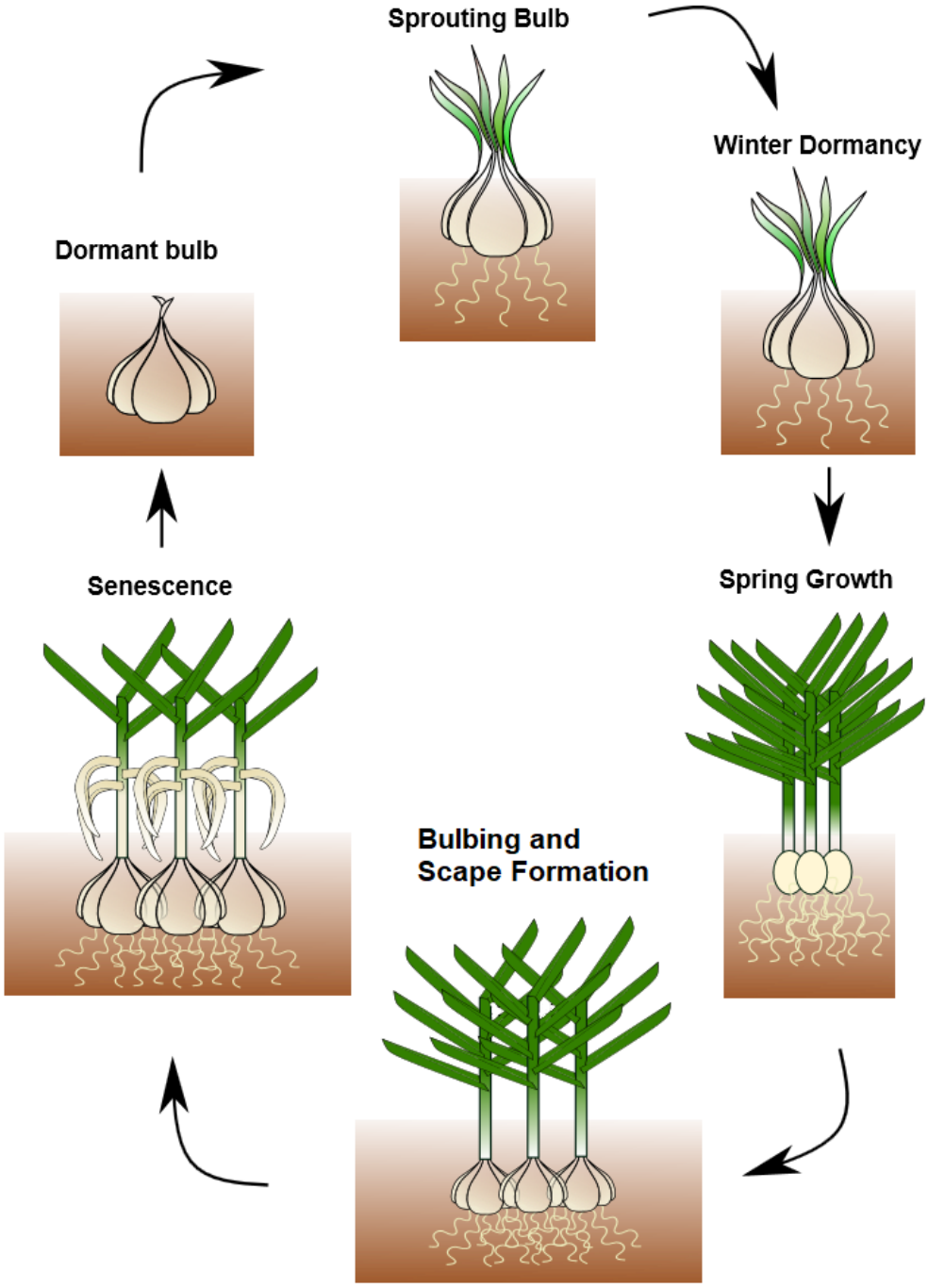


Figure 1.1: Yearly growth and development cycle of garlic without human intervention.

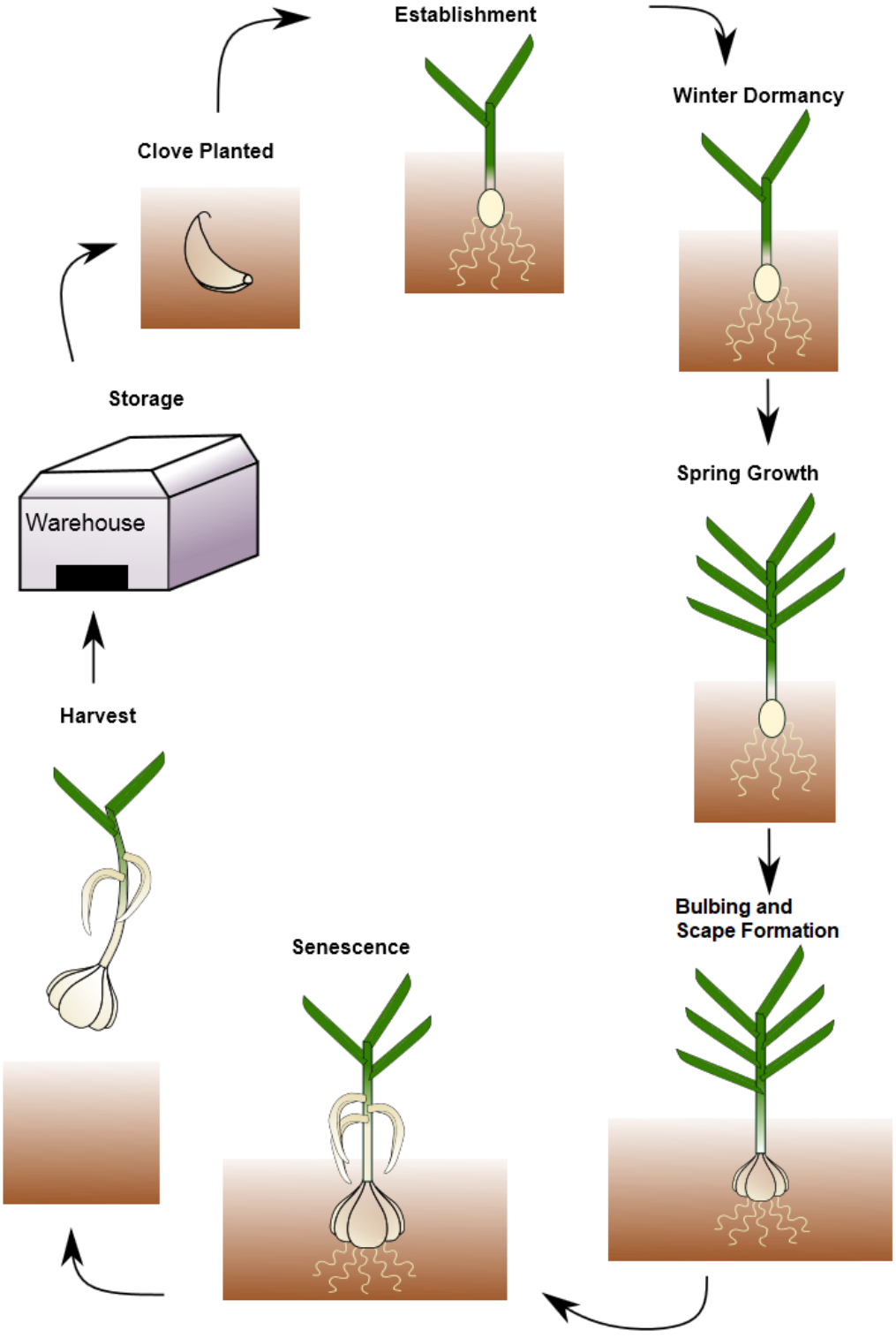


Figure 1.2: Yearly growth and development cycle of garlic in farmed garlic.

Over the course of a growing season, a seed clove is planted and it grows and differentiates into a bulb, consisting of multiple cloves, in a process referred to as bulbing. It is essential that seed cloves experience a period of cold over winter to facilitate bulbing later in the growing season, as exposure to cold induces the development of new clove primordia (Jones & Mann, 1963). Optimal temperatures to induce bulbing are 0 to 10 °C, whilst temperatures over 15 °C are not effective (Mann & Minges, 1958; Takagi & Qu, 1994). Cloves can experience these temperatures either in the field if autumn planted, or in chilled storage if spring-planted. Bulbing occurs through the development of clove primordia which form in the axils of leaves, on the basal plate of the bulb. The rate of bulbing is primarily driven by light intensity, as photosynthetic assimilates from the leaves are required for clove primordial growth (Rahim & Fordham, 1990; Rahim & Fordham, 1988).

Regarding plant nutrition, nitrogen is in highest demand in the early phases of growth, up until the beginning of senescence, where high N levels are deleterious to bulb yield (-10%) and quality (Bertoni *et al.,* 1992). A key determinant of yield and quality is sulphur, with garlic having one of the highest sulphur demands of any bulbous crop (Hlusek *et al.,* 2002). Significant positive correlations have been found between soil sulphur content and bulb sulphur content (Bloem *et al.,* 2011; Bloem *et al.*, 2005; Volk &Stern, 2009). However, growth and nutrient uptake in response to fertiliser applications to garlic is influenced by soil type and environmental conditions during crop growth (Diriba-Shiferaw *et al.*, 2013).

Autumn sown garlic typically has a higher yield per hectare than spring sown garlic (Mann & Minges, 1958). The optimal date for planting varies between location, depending on factors such as longitude, latitude and altitude (Volk & Stern, 2009; Adekpe *et al.*, 2007). Furthermore, there is a degree of variance between the optimal planting date

for different cultivars, specifically with some cultivars adapting poorly to spring planting (Volk *et al.*, 2004). A confounding factor which is not always considered in studies is the storage temperature of garlic prior to planting. Cold storing the garlic seed cloves prior to planting at 5 to 10 °C leads to increased vigour and can negate reductions in bulb weight normally associated with later sowing (Mann & Minges, 1958; Siddique & Rabbani, 1985). Storing seed cloves at 15 to 20 °C and then planting late, in February, was associated with a reduction in percent dry weight of cloves at harvest, a lower yield and delayed date of maturity. It was hypothesised that the lower dry weight was caused by delayed maturity, as nutrient accumulation in the bulb may not have been complete (Mann & Minges, 1958). Furthermore, storing at the optimal temperature for most of the storage season and then transferring to 20 °C for six weeks or more before planting reduced the effectiveness of 5 °C storage for priming the bulbs for growth (Mann & Minges, 1958).

Garlic is harvested when the lower ½ to ¾ of leaves have senesced. They are first pulled from the ground before being laid out in bundles in the field to begin drying. The standard practice is that after 2-3 days, the garlic is taken to a storage facility where the scape, roots and loose/dirty outer sheath are removed (personal communication, Coopaman, 2016). Garlic is transported to the storage facility promptly after harvest as the strength of the outer bulb sheath decreases as it dries (Bayat & Rezvani, 2012). Therefore, by transporting and processing the garlic when it is fresh from the field, damage and the associated rise in postharvest disease risk are mitigated. The bulbs are then stored in ventilated pallets where they dry further for 10-20 days, typically losing 18-20% of the original water content (Block, 2010). Once fully dried after processing, the bulbs can be moved into long term storage at low temperatures. Without proper drying beforehand, there is increased risk of sprouting and disease.

Propagation is traditionally achieved clonally, with farmers breaking bulbs into their constituent cloves and planting last year's cloves to start the following year's crop. Propagation can also be achieved by planting the top-sets which form on the scape, however it normally takes a bulbil two years to form a whole bulb of garlic. Modern methods include excision of meristems from the clove and propagation by tissue culture (Koch & Salomon, 1993). This is especially useful when bulking up stocks of a new variety, as traditional methods of propagation would take too many seasons to be viable. However, due to the complexity of the procedure and the need for specialist personnel and equipment, only the larger growing cooperatives and seed farms can afford to attempt this process, further hampering the development of improved garlic genotypes.

Some phenotypic traits of garlic vary between cultivar, whilst others are influenced by growth location and conditions. The number of cloves per bulb, clove skin colouration and the number of top-sets produced vary significantly amongst cultivars but are stable amongst growing locations, whereas bulb outer sheath colour, bulb size and elemental composition of the bulb vary amongst growing sites (Volk & Stern, 2009). Cooler growing temperatures are associated with higher rates of scape formation, which is deleterious to bulb yield (Pooler & Simon, 1993b). Bulbing is initiated earlier and for a longer duration in response to an application of organic matter (vermi-compost) (Argüello *et al.*, 2006). However, the higher nitrogen content of soils that have been fertilised in such a manner have been associated with lower alliin content (Bloem *et al.*, 2011)

Despite *A. sativum* being principally seed sterile, it has been noted that the species displays considerable variability, differing in in morphological characteristics such as bulb size and colour and in physiological characteristics such as maturation date and storability (Maaβ & Klaas, 1995). It is generally accepted that garlic lost its ability to produce seed through the process of domestication, with increases in bulb yield associated

with reductions in scape formation, however some research suggests that garlic may have been in the process of losing the ability to produce seed before humans intervened (Etoh, 1985). Most wild ancestors of garlic, such as *A. longicuspis*, from which *A. sativum* is thought by taxonomists to have evolved, are also predominantly sterile. However, fertility has persisted in a small number of accessions of both species (Etoh, 1985; Zizina, 1956). Some such fertile clones of garlic have been studied more extensively and found to have large variation in vegetative and reproductive characters, including bulbing ability, bulb colour and size, clove number, and response to environmental conditions, which were compared to that of the genepool variance of vegetatively propagated garlic clones (Shemesh *et al.*, 2008). The production of fertile garlic lines has been a goal of modern horticultural breeding for some time, owing to the benefits that could come from traditional crop breeding techniques. Not only would sexual reproduction create novel forms of genetic diversity through genetic recombination events, but seeds could be used to speed up multiplication of new varieties and to assist in the maintenance of disease free seed cloves (Walkey *et al.* 1987; Walkey & Antill, 1989). The sexual sterility of commercial garlic lines has hampered efforts to develop new cultivars with agronomically important traits such as higher yield, disease resistance and quality (Kamenetsky & Rabinowitch, 2000).

Despite fertile clones of garlic being reported in the literature for over 60 years, little progress has been made in recent years to develop fertile commercial cultivars of garlic (Kononkow, 1953; Hong & Etoh, 1996; Jenderek & Hannan, 2004). Efforts to restore fertility have so far focussed on methods such as manual removal of topsets from inflorescences, as topsets compete strongly with developing seeds (Etoh *et al.*, 1988; Kamenetsky & Rabinowitch, 2001). Recent research has identified metabolic restrictions and/or non-regulated programmed cell death of the tapetum, a specialised layer of nutritive cells found within the anther, which has a crucial role in pollen nutrition and

development, as contributing to garlic sterility (Shemesh-Mayer *et al.,* 2015). An analysis of sexually derived progenies of garlic found significant variability in agronomically important traits, including: bulb and clove weight, number of cloves per bulb, scape height, number of leaves, plant height and days to bulb maturity (Jenderek & Zewdie, 2005). The application of these findings was hampered by poor seedling germination and survival rates. The lack of viable seed for nearly all cultivated forms of *A. sativum* poses challenges for preservation of genetic resources in genebanks, with field culture and cryopreservation being the only two options available which work well with garlic (Keller *et al.,* 2013). The lack of fertility in garlic has, in part, contributed to a culture of replanting cloves from the previous growing season. This has resulted in various ecotypes consisting of heterogeneous clone populations being cultivated in certain areas for many decades, raising quality issues in regard to both uniformity and the content of bioactive compounds (Figliuolo *et al*, 2001). As genotype affects the suitability of cultivars to long-term storage, it is possible that heterogeneous clone populations also vary in their storage traits (Fanaei *et al.*, 2014; Martins *et al.*, 2016). It is possible that garlic has lost fertility over years of successive breeding efforts to select for larger bulbs; infertile lines will inherently have more carbon available to partition to the bulb if seeds or scapes are not produced.

Garlic crops are not only variable in terms of yield and morphology between cultivars, but also across planting location and between years. Overall, an analysis of eight garlic cultivars grown in Saskatchewan over two years found a significant increase in average bulb weight of autumn vs spring planted cloves (Waterer & Schmitz, 1994). However, the Mexican and Jamaican cultivars of garlic reversed this trend in the second planting year, with significantly heavier bulbs when Spring planted, suggesting a high degree of seasonal variance between some cultivars. Garlic is highly plastic in its response to the environment it is grown in and evidence suggests higher yield and better quality is

obtained from seed stock grown in the area it is going to be planted in the following year (Waterer & Schmitz, 1994; Engeland, 1991). Seed cloves used to grow garlic studied in this thesis were raised from fields at the Coopaman growing cooperative in Spain.

1.3. Garlic Dormancy and Storage

In order to maintain a year-round supply of garlic, bulbs are stored long term, for up to 9 months. Current farming and storage practices aim to minimise disease, sprouting and damage in bulbs. Bulbs are harvested in late summer and typically stored long term in cold rooms at approximately -2 °C for up to 9 months to induce dormancy and prolong shelf life (Coopaman, personal communication, 2016). Dormancy in garlic starts when the bulb is lifted from the soil for harvest and lasts for approximately 90 days (Pellegrini *et al.,* 1988). During this period, the cloves have very low responsiveness to sprouting cues and are reluctant to sprout even if planted in the field. Cell elongation may still occur, but meristematic activity and cell division is minimal. In onion, postharvest respiration rates were found to be much lower than in crops which did not have a dormant period, with the rate of respiration increasing shortly before sprouting (Ward & Tucker, 1976). However, it is not known if this is the case in garlic.

The key objectives of garlic storage are to delay sprouting, reduce disease and maintain fresh weight, as once sprouted or withered, bulbs lose marketability and their storage life is ended. Fresh weight loss in stored vegetables occurs due to respiration and transpiration (Mathur, 1963; Platenius, 1942). Respiration occurs faster at higher temperatures, however a confounding factor may exist in garlic and onion, as higher temperatures promote dormancy and dormant garlic has a hormonally controlled low rate of respiration (Stow, 1975b). Garlic requires a period of low temperatures for the induction of flowering and bulb development and cold-priming cloves leads to a more rapid sprouting and development once planted (Rahim & Fordham, 1988). Storing garlic at 5 °C for 5 weeks

shortened the cropping cycle by 32-42 days and increased growth rate by nearly 50 % compared to garlic stored at 20 °C, however, 20 °C stored garlic had an overall higher yield (Guevara-Figueroa *et al.,* 2015). Note also that responses to cold-storage treatments are cultivar-dependant (Volk *et al.*, 2004).

Fresh weight can be maintained for longer with increased relative humidity, by reducing transpiration. However, if the relative humidity is too high, there is an increased risk of fungal spoilage, which can cause losses of 50-60 % over a 5 month period (Musa, 1972). Furthermore, at high relative humidity (75 – 100% RH), there is an increased incidence of sprouting (Guoyi, 1992). However, it was noted that temperature was a stronger determinant of sprouting and that high humidity predominantly exacerbated sprouting at temperatures which would typically already induce sprouting (5 °C) (Guoyi, 1992). Root initiation is dependent on vernalisation and the subsequent growth rate is closely associated with humidity levels (Hwang & Lee, 1990). The optimal humidity for garlic storage is 60-70% RH and the optimal temperature is between 0 and -4 °C (Hong *et al.,* 2001; Bertolini,& Tian,1996)

The control of sprouting is temperature dependent in *Alliums*, with higher storage temperatures (>25 °C) and low temperatures (< 0 °C) exhibiting low rates of sprouting over long term storage by maintaining dormancy in garlic and onion (Cantwell, 2004; Ichikawa *et al.*, 2006; Stow *et al.*, 1973). The highest incidence of sprouting occurs at 4-15 °C, (Atashi *et al.*, 2011; Stow *et al.,* 1973). Moreover, storing at these high temperatures has significant deleterious impacts on fresh weight loss and is not recommended for periods beyond 2 months (Cantwell, 2004). Sprouting can be controlled equally well by cold storing garlic at sub-zero temperatures with greatly reduced fresh weight loss (Volk *et al.*, 2004). Cold storing garlic at -3 °C for 8 months produced no noticeable differences in quality as compared to pre-storage scores by a qualitative taste

panel, whereas the same cultivar remained edible for only 2 months at room temperature storage (Volk *et al.*, 2004). The downside to cold storage is that once taken out of cold storage, the bulb has been cold-primed and therefore lost dormancy and will grow rapidly if environmental conditions suit. Plants increase their freezing tolerance upon exposure to low non-freezing temperatures through cold acclimation, however little research has been done on the effects of abrupt cooling that garlic is exposed to when plunged into cold storage (Thomashow, 1999). Garlic cultivars also vary significantly in their ability to cope with cold stress, with northern cultivars tolerating and indeed requiring freezing temperatures, whilst southern varieties being able to remain dormant at 5-10 °C over winter (Son *et al.*, 2012).

Other than cold-storing at sub-zero temperatures, there has been considerable research into developing treatments aimed at reducing sprouting whilst maintaining FW in stored garlic. Some commercial storage units also keep the bulbs under a high nitrogen atmosphere, which further inhibits sprouting with no deleterious effects on FW loss (Block, 2010). Gamma irradiation has been proven to reduce and delay sprouting in garlic without adversely affecting organoleptic quality of the bulbs (Croci & Curzio, 1983). A reduction in the rate of weight loss over time in storage was noted in this study, with garlic being stored for 300 days in a warehouse without climate control where the temperature fluctuated from 6-32 °C, suggesting that shoot development may be implicated in weight loss throughout storage. Gamma irradiation arrests the development of leaf primordia in garlic shoots through disruption of meristematic cells and can halt shoot development when applied at any point post-harvest with doses over 60 Gy when bulbs are dormant and over 10 Gy once dormancy has broken (Pellegrini *et al*, 2000).

Disease control in stored garlic is primarily achieved by managing temperature and humidity. Fungal spoilage organisms such as *Aspergillus niger*, *A. alliaceus* and *Pantoea*

ananatis, the causative agents of black-rot and centre-rot can be highly prevalent on seed cloves and in the soil of fields where *Alliums* have been grown (Varga *et al.,* 2004; Tyson & Fullerton, 2004; Abd-Alla *et al.,* 2006; Walcott *et al.,* 2002). Whilst soil-borne spores are the principal source of inoculum, black mould is predominantly a post-harvest pathogen and disease only progresses at higher temperatures and humidity (30 °C and 90 % RH), so control measures focus on cold storage and low humidity (Tyson & Fullerton, 2004). A relative humidity of 60 - 70 % RH is considered optimal for garlic storage (Shiina *et al.,* 2004; Brewester, 1994). Accurate storage temperature can be extremely important in managing disease in garlic, with control of blue-mould (*Penicillium hirsutum*) being achieved at -4 °C but not -2 °C (Tian & Bertolini, 1996).

Chemical treatments for inhibiting sprouting in garlic include maleic hydrazide (1,2-dihydropyridazine-3,6-dione; MH) (Moreno *et al.,* 2012). MH, a common plant growth regulator, is applied to garlic two weeks before harvest (Pellegrini *et al.,* 1988). MH acts as an antagonist of pyrimidine bases and has the effect of lengthening dormancy by affecting hormonal balance, suppressing meristematic activity and respiration rate in onion (Appleton *et al.,* 1981; Isenberg *et al.,* 1974; Ward & Tucker, 1976). Low respiration rate is associated with dormancy and increasing respiration rate in stored *Alliums* is associated with dormancy break and the onset of sprouting. Respiration rates were held at levels equal to those of the first 3 months of dormancy of control samples for 5 months in samples treated with MH and in subsequent months, respiration rate increase occurred significantly slower in MH treated onion bulbs (Ward & Tucker, 1976).

1.4. Organosulphur Biochemistry

The organosulphur biochemistry which defines garlic centres on the reaction of alliin through the enzyme alliinase to yield allicin (Fig. 1.3). Allicin is regarded as the principal component of garlic and responsible for much of the biologically active properties of garlic (Borlinghaus *et al.*, 2014; Block *et al.*, 2010).

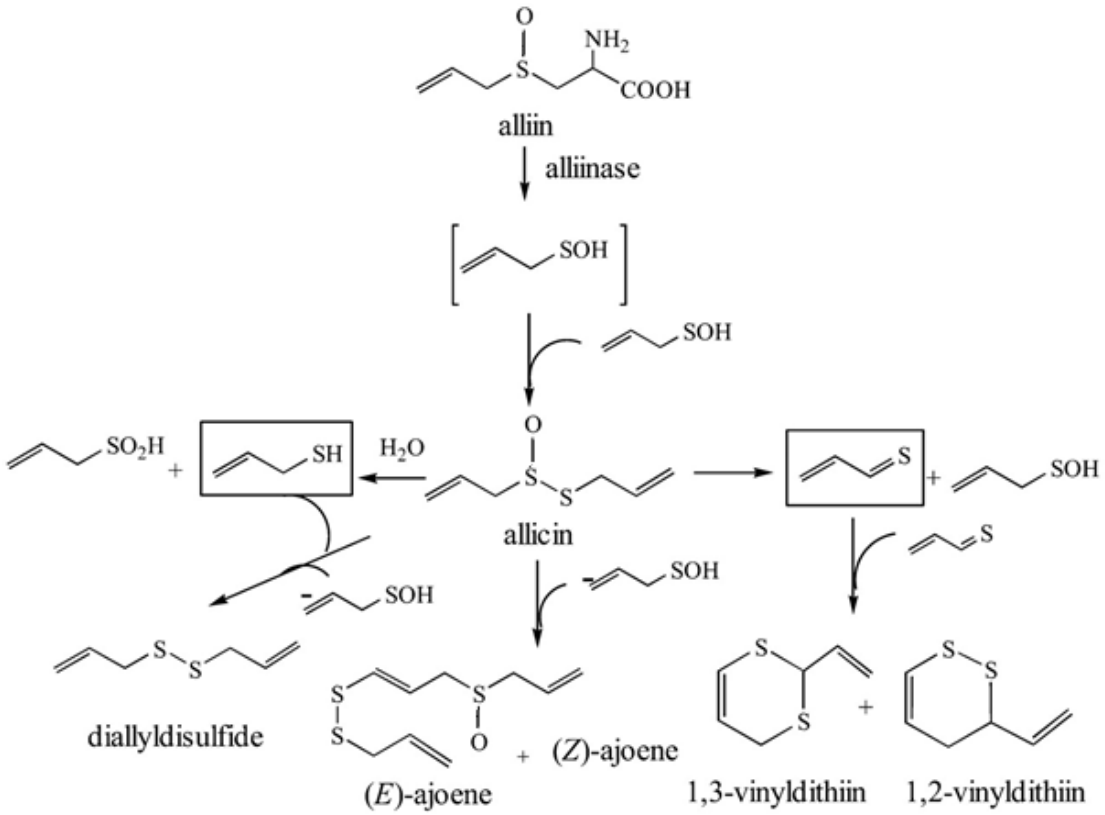


Figure 1.3: Biosynthesis pathway of allicin from alliin via highly unstable sulphur-substituted sulphenic acid intermediaries and allicin's subsequent breakdown products. Source: Amagase, 2006.

1.4.1. Alliin

Alliin (S-Allyl-L-cysteine sulfoxide) is a stable, non-volatile sulphoxide, derived from the amino acid cysteine. It is the precursor of allicin, the principal component of garlic, which is synthesised from alliin through the enzyme alliinase (Block, 2010) (Fig. 1.3). It was the first natural compound to be identified to have both carbon- and sulphur- centred

stereochemistry (Block, 2010). Alliin is synthesised in the leaves of garlic during the growing period and translocated to the bulb where it is concentrated in vesicles within the cytoplasm of mesophyll cells in the cloves (Jones *et al.* 2007; Bloem *et al.,* 2005; Lawson, 1996). Alliin is also synthesised in the bulbs of garlic cloves from sulphur containing precursors throughout storage (Ichikawa *et al.,* 2006). Alliin biosynthesis occurs through one of two reported pathways in garlic. One involves synthesis from serine and another from glutathione (Lawson 1996). Central to both pathways, is cysteine, an essential precursor for not only alliin but all biological compounds that contain reduced sulphur (Höfgen *et al*., 2001). Garlic contains between 6 and 14 mg g^{-1} alliin (Lawson, 1998). Alliin content has been shown to be negatively impacted by high nitrogen levels in the soil (Bloem *et al.*, 2011). A strong genotypic effect and a broad range of environmental conditions including sulphur fertilisation, light spectral quality and temperature were found to affect alliin synthesis and accumulation in the bulb (Huchette *et al.*, 2004).

Alliin concentration was studied in exterior and interior cloves from bulbs stored for 0-180 days at 4 °C, where no statistically significant changes in concentration were detected (Hughes *et al.*, 2006). During storage, alliin levels have been shown to remain relatively stable at 0-1 °C over a 6 month period, except for storage in high CO_2 controlled atmospheres, which resulted in a modest but significant decrease in alliin concentration (Hong *et al.*, 2001). Ichikawa, *et al.*, (2006) however, reported that γ-glutamyl peptides undergo conversion to sulfoxides, including alliin, when garlic cloves are stored at 4 °C. There, alliin concentrations increased significantly above pre-storage levels after 90 days and remained at such levels for the remainder of the 150 day study at 4 °C, however this change was not seen when garlic was stored at -3 °C or 23 °C for the same duration, suggesting a temperature dependent response. Changes were noticed in other sulphoxides at these temperatures, such as γ-glutamyl-S-alkylcysteine, one of the proposed precursors of alliin (Lawson *et al.*, 1991b; Lancaster & Shaw, 1989). However, another report

showed that storage at ambient conditions (20 °C) resulted in an increase in alliin concentration over a 12 week storage period with an average of 9.2 mg g^{-1} dry weight at harvest and 21.4 mg g^{-1} dry weight after storage (Bloem *et al.*, 2011).

Alliin is not only the precursor for allicin, but itself has biotechnological applications. Alliin has been discovered to be a health-promoting compound, with hydroxyl- and superoxide scavenging abilities as an antioxidant (Chung, 2006). There is also evidence that alliin exerts immunomodulatory effects *in vitro*, stimulating certain peripheral blood cell immune functions and improving the engulfing capacity of phagocytic cells (Salman *et al.*, 1999).

1.4.2. Alliinase

Alliinase is the most abundant protein in garlic and is the enzyme that facilitates the synthesis of thiosulphinates from cysteine sulphoxides, most notably, alliin. It is a dimeric glycoprotein, first purified in 1949, which cleaves carbon-sulphur bonds and has a MW of 51.5 kDa per subunit and contains 6 % carbohydrate (Stoll & Seebeck, 1949; Rabinkov *et al.*, 1994). When alliinase acts upon alliin, it causes the molecule to decompose into 2-propenesulphenic acid, which in turn dimerises with a second molecule of 2-propenesulphenic acid to form allicin (Fig. 1.3). It employs a single pyridoxal-5-phosphate (P5P) molecule as a co-factor and has a pH optimum of 6.5 and temperature optimum of 33 °C (Mazelis & Crews, 1968; Ilić *et al.*, 2011).

Alliinase has a pivotal role in the organosulphur chemistry of garlic and many garlic-related products and chemicals used in the biotech industry. However, the enzyme itself is thermolabile and also denatured by its product (allicin) or a pH below 3.0 (Jansen *et al.*, 1989; Blania & Spangenberg, 1991). Alliinase is unstable in an aqueous solution, even when frozen, but can be stabilised with 10 % v/v glycerol to a point where no activity

is lost over a 1 month period and the enzyme was also stable over multiple freeze-thaw cycles (Mazelis & Crews, 1968). The lack of allinase stability poses significant challenges for the biotech industry if developing products which in some way rely on the activity of alliinase.

Alliinase is highly abundant within garlic tissues and constitutes approximately 10 % of the total cell protein (Ellmore & Feldberg, 1994). This allows the conversion of alliin to allicin to be nearly complete (> 97 %) in 30 s (Block, 2010). In garlic bulbs, alliinase is localised in the vacuoles of bundle sheath cells, surrounding the phloem vessels (Ellmore & Feldberg 1994). As alliin is localised in vesicles in the cytoplasm, alliinase cannot interact with alliin until there is mechanical damage to the bulb (Yamazaki *et al.*, 2002). Alliinase is most abundant in the bulb, but can be found to a lesser degree in leaves and roots, however root alliinase has a low amino acid sequence homology to leaf or bulb alliinase (Rabinkov *et al.*, 1994). Furthermore, alliinase mRNA is present in both leaves and bulbs at all growth stages and persists for as long as there are living cells, suggesting constitutive expression (Smeets *et al.*, 1997). Alliinase gene expression was found not to change significantly over a ~6 month storage experiment in stored bulbs (Cho *et al.*, 2011). Alliinase is encoded by a multigene family that has not been fully characterised, however, when 45 full-length alliinase amplicons from one cultivar of garlic were cloned, 9 sequence variants were identified (Cavagnaro *et* al., 2003; Ovesná *et al.*, 2015). Furthermore, the variability in the alliinase gene family indicated the presence of up to 14 sequence variants.

1.5. Allicin and derivatives

Allicin is the principal component of garlic, responsible for the characteristic, pungent odour of the bulb when mechanically damaged (Block, 2010). It is rapidly synthesised from alliin in response to injury and believed to exist in garlic as a defence against herbivory by insects and animals alike, but also has strong antimicrobial properties (Fig. 1.3). Allicin is classed as a thiosulphinate, a class of reactive sulphur species and its structure was first described in 1947 (Stoll & Seebeck, 1947). The direct antimicrobial effects of allicin are attributed to its ability to undergo redox-reactions with thiol -SH groups such as those in glutathione and proteins, through its activated disulphide bond -S(O)-S- (Cavallito & Bailey, 1944; Prager-Khoutorsky *et al.*, 2007).

Allicin (2-propenyl-2-propenethiosulphate) is produced from the enzymatic reaction of alliin and alliinase (*Fig*. 1.3). It is a reactive, unstable molecule which rapidly undergoes a complex series of chemical reactions, yielding a broad range of volatile organosulphur compounds which vary in composition according to temperature, time and reaction medium (Block, 1992). The compounds continue to change over time and many have reported bioactivity (Amagase, 2006). Allicin held at 20 °C for 20 hours decomposed to diallyl disulphide (DADS) (66%), diallyl sulphide (DAS) (14%), diallyl trisulfide (9%), and sulphur dioxide (Brodnitz *et al.,* 1971). Other breakdown pathways include three allicin molecules combining to form two molecules of ajoene, and allicin self-decomposing to form two isomeric compounds: 2-vinyl-4H-1,3dithiin and 3-vinyl-4H-1,3-dithiin (1,2 vinyldithiin and 1,3 vinyldithiin in Fig. 1.3) under higher temperatures (Block, 1985, Lawson & Gardner, 2005). These breakdown products show promise for developing novel garlic based therapies and as such, the synthesis of allicin and exerting control over its subsequent breakdown is of commercial interest and value (Ilić *et al.*, 2011).

During storage, biochemical quality is known to change over time, however there is little information on the variations of pharmaceutical compounds throughout storage in garlic (Lawson, 1996; Hughes *et al.,* 2006). Post-harvest storage conditions have been shown to affect alliinase activity in onion, where activity increased after 9 weeks storage under regular atmosphere but decreased when stored under elevated CO_2 (Uddin & MacTavish, 2003). Allicin levels can be roughly predicted by the visual index of dormancy (VID) scale throughout storage at 0 and 20 °C, whereby dormancy is measured as the % length of the growing shoot compared to the length of the total clove (González *et al.* 2012). Dormancy broke faster at higher storage temperatures; after 90 days at 20 °C, as opposed to after 120 days at 0 °C, corresponding with more rapid decreases in allicin and total pyruvic acid levels. Vitamin C content and titratable acidity decrease along with allicin content throughout time in storage at both 0 and 5 °C (Akan *et al.*, 2019). The application of methyl jasmonate has also been shown to improve physiochemical quality maintenance in cold stored garlic, but did not affect the rate of decline in allicin potential (Akan *et al.*, 2019).

1.6. Garlic in medicine

Besides our culinary interest in garlic, it has been used for thousands of years as a medicinal plant for a range of ailments. Some of the earliest references are found in the Codex Ebers (circa1550 BC), the Bible and garlic was frequently referenced by Pliny the Elder in *Historia Naturalis*, where 61 remedies involving garlic are described (Block, 1985; Rivlin, 2001; Block, 2010). Garlic is well known to have antimicrobial properties, with efficacy against a wide spectrum of microbes; it is antibacterial, antiviral, antifungal and antiprotozoal (Harris et al., 2001).

More recently, benefits of garlic on the cardiovascular system (Banerjee & Maulik, 2002; Ackermann *et al.* 2001; Gebhardt, 1993), *in vitro* antimicrobial properties (Harris *et al.*, 2001; Reiter *et al.,* 2017) and potential to reduce the risk of, or inhibit, cancer have been reported (Santhosha *et al.,* 2013; Luo *et al.* 2016; Dorant *et al.,* 1993). These properties have been researched for decades and are predominantly attributed to organosulphur compounds derived from the enzymatic action of alliinase on the cysteine sulphoxide alliin, producing allicin (Cavallito & Bailey, 1944; Ankri & Mirelman, 1999).

Garlic has been shown to benefit the wider cardiovascular system by lowering cholesterol levels. Epidemiological studies have shown an inverse correlation between garlic consumption and the progression of heart disease (Turner, 1990; Rahman & Lowe, 2006). Garlic and its preparations lower cholesterol levels by inhibiting human squalene monooxygenase, an enzyme involved in cholesterol biosynthesis, at levels approximate to normal consumption levels (Gupta & Porter, 2001;). Total sterol biosynthesis was reduced by 23% in HepG2 human liver carcinoma cell lines by a garlic powder extract, with the action predominantly attributed to HMG-CoA reductase inhibition (Gabhardt, 1993). This study was repeated with an ajoene extract which corroborated inhibition of HMG-CoA reductase and reported a reduction in aspartate transaminase (AST) activity, an enzyme which when present at high levels of activity is an indicator of poor liver or heart health (Augusti *et al.,* 2005). Moreover, aged garlic supplementation was shown to reduce total cholesterol by 7% in hypercholesterolemic men and this activity was attributed to S-allylcysteine (Yeh & Liu, 2001).

Several studies have reported a significant reduction in blood pressure from garlic consumption in double-blind, randomized trials, but the magnitude of reduction is relatively small, making garlic potentially suitable for treating mild hypertensive patients or used as an adjunct to other therapies (Harenberg *et al.,* 1988; Silagy *et al.,* 1994;

Banerjee & Maulik, 2002). However, questions have been raised regarding the reliability of blood pressure measurements in such studies, with significant variability between measurement methods, and the potential for false-positive results (Musini & Wright, 2009). Compounds from garlic have been shown to reduce platelet aggregation, with adenosine having the highest anti-aggregatory activity of garlic homogenates in platelet-rich-plasma, but allicin and other garlic thiosulphinates had higher anti-aggregatory activity in whole blood (Lawson *et al.*, 1992; Banerjee & Maulik, 2002).

There is epidemiological evidence to suggest consumption of raw and cooked garlic offer a degree of protection against stomach and colorectal cancers (Fleischauer *et al.*, 2000). However, it is unclear at what minimum level of consumption the protective effects are conferred. It was also reported that garlic supplements did not confer such benefits. In many of the papers reviewed, confounding factors including broader diet were not controlled, however across all studies the negative correlation between cancer risk and garlic consumption was consistent.

Whilst these benefits are broadly attributed to organosulphur compounds, garlic is also rich in polyphenols which have also been shown to display antimicrobial effects, as well as being potent antioxidants and inhibitory to cancer cell proliferation (Del Rio *et al.*, 2010; Lanzotti, 2006; Bozin *et al*., *2008*). The most abundant phenolic compounds in *A. sativum cv.* 'Rosato' and 'Caposele' were reported to be gallic acid, hyperoside and allyl isothiocyanate (Fratianni *et al.*, 2016). However, it was noted that the concentration and ratios of the various phenolic compounds in garlic varied significantly between cultivars of the same endemic area. Polyphenol concentration is higher in some garlic products such as aged garlic extract and black garlic than raw garlic (Nencini *et al.*, 2011; Imai *et al.*, 1994). Both the products have a thermal processing step, and it is this which is responsible for increasing polyphenol concentration.

A limiting factor of garlic preparations is that excessive consumption of garlic and garlic preparations have been shown to exhibit toxicity and cause irritation (Banerjee, 2001). When 10-25 mL of garlic extract was given as part of a study to five volunteers, side effects were reported including burning sensations in the mouth, oesophagus and stomach with the 25 mL dose eliciting additional symptoms of nausea, diaphoresis and light-headedness (Caporaso *et al.,* 1983). It was hypothesised that by isolating compounds with the most bio-active properties, therapeutic doses may be tolerated and this is one of the key areas of biotechnological research in garlic.

1.7. Garlic Preparations

There are a wide range of garlic supplements available, in part a result of a long-held belief by the public that garlic is beneficial to one's health. The demand for these products is fuelling constant development by industry to find methods of manufacturing garlic extracts with higher efficacies. These supplements broadly fall into four main categories: dried garlic powders, garlic oils, garlic oil macerates and aged garlic extract (Amagase *et al.,* 2001). Garlic preparations such as dried powders, steam distillates and aged garlic extracts vary significantly in their chemical makeup, making comparisons of efficacy difficult (Banerjee et al., 2003; Lawson *et al.,* 1993). Increasingly, biotech companies and research groups are focussing on studying the efficacy of single compounds found in garlic against specific diseases (Ledezma & Aptiz-Castro, 2006; Singh *et al.,* 1990).

Raw garlic homogenate, an aqueous extract of garlic not subjected to heat treatment, drying or purification by distillation etc is the most commonly studied form of garlic as it is considered a common method of garlic consumption (Banerjee & Maulik, 2002). One may question the logic of this, as in many countries, garlic is rarely consumed raw and is

typically cooked thoroughly. However, there is little information in the literature about the typical mode of garlic consumption by country. By crushing garlic into a homogenate, alliin is completely and rapidly converted to allicin and other dialkyl thiosulphinates by alliinase and free adenosine is slowly released (Stoll & Seebeck, 1951; Lawson *et al.*, 1991a). Allicin is considered to be the principal active compound in raw garlic homogenate and much of the biological activity is attributed to it. However, allicin has never been detected in human blood, urine or stool after consuming large amounts of raw garlic or garlic extracts (Nian *et al.*, 2008; Miron *et al.*, 2010). The half-life of allicin in blood was reported to be less than one minute, rapidly reacting to form allyl mercaptan, however allicin breakdown compounds containing a dithioallyl group yielded S-allylmercaptocysteine in the intestine before also forming allyl mercaptan shortly after entering the blood (Lawson & Wang, 1993). However, anticandidal and anticryptococcal activity was detected in serum after the ingestion of 25 mL raw garlic homogenate 30 and 60 minutes after consumption (Caporaso *et al.*, 1983). Allicin has been shown to rapidly break down in simulated gastric juices, primarily to diallyl disulphide (DADS), diallyl sulphide (DAS), allyl methyl sulphide (AMS) and allyl methyl disulphide (AMDS) (Rosen *et al.*, 2001). It is therefore widely accepted that allicin itself does not have any health promoting effects when ingested, but it is instead the breakdown products of allicin and other unrelated compounds in garlic which are bioactive.

Powdered garlic is a widely available garlic supplement, typically taken in tablet form for their perceived health benefits, commonly believed to promote cardiovascular health. Indeed, some studies show garlic tablets to have significant beneficial effects on cardiovascular risk in patients with coronary artery disease, with a 1.3 to 1.5 fold reduction over a 10 year period (Sobenin *et al.*, 2010). However, the broader literature paints a more complex picture, with other studies not showing the same benefit to clinical outcomes such as myocardial infarction or claudication (Mulrow *et al.*, 2000). This may

be due to the high variability between the chemical composition of garlic tablets. The suggested mechanisms behind a potential cardiovascular benefit are broad, with reported reduction of patient blood pressure, inhibition of platelet aggregation, normalisation of blood lipids, increases antioxidant status and enhances fibrinolytic activity (Agarwal, 1996; Rahman & Lowe, 2006). Besides cardiovascular patients, garlic tablets have been shown to significantly reduce LDL cholesterol in type 2 diabetics suffering from dyslipidaemia in the short term (Hosseini & Hosseinzadeh, 2015; Ashraf *et al.*, 2005). However, no benefit has been noted in blood glucose or insulin levels in such patients. There is also limited evidence, mostly from case-control studies, to suggest that garlic tablets may have anti-cancer properties, with studies focussing mostly on cancers of the digestive tract (Mulrow *et al.*, 2000).

Aged garlic extract (AGE) is extracted over a prolonged period of up to 20 months and produces a near odourless product containing stable sulphur compounds (Nencini *et al.*, 2011). Typically, cloves are sliced and added to a solution of water and ethanol and left for > 10 months at room temperature (Harauma & Moriguchi, 2006; Ide & Lau, 1999; Moriguchi *et al.,* 1994). The markedly different mix of sulphur compounds in AGE compared to raw garlic, the result of enhanced chemical breakdown, has the potential for novel applications in medicine. A major difference between AGE and raw garlic is the higher polyphenolic content and the associated increase in antioxidant potential (Kim *et al.,* 2013; Choi *et al.,* 2008; Imai *et al.*, 1994). Furthermore, higher polyphenolic content was detected in AGE produced from leaves and flowers than in AGE from bulbs (Nencini *et al.,* 2011). One of the major constituents of AGE is S-allylcysteine, which has a number of health benefits and is not present at such high levels in raw garlic (Rosen *et al.,* 2001). This may have potential for medicinal extracts to be created synergistically with crops such as wet garlic. Whilst manufactured under similar conditions to AGE, the principle compounds responsible for the high anti-oxidant properties of black garlic extract were

different; these were identified as adenosine, uridine, 2-acetylpyrrole, 5-hydroxymethylfurfural, (1S, 3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, and (1R, 3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (Lu *et al.*, 2017).

1.8. Volatile Organic Compounds in Plants:

Plants ubiquitously release a broad range of volatile organic compounds (VOCs), some of which are common to many plant families, and others which are unique to individual genera or species (Baldwin, 2010). VOCs are as broad in their chemical make up as they are in their function, with volatiles having roles within and amongst plant, plant-animal and plant-microbe signalling (Baldwin *et al.,* 2006). VOCs are used by plants to attract pollinators to their flowers and to attract herbivores to their ripening fruit to aid seed dispersal (Brodmann *et al.,* 2008). They play a role in attracting beneficial microbes to plant roots within the soil environment and can improve abiotic stress tolerance (Wenke *et al.,* 2010; Vickers *et al.,* 2009). VOCs may attract or repel herbivores and may also attract predators/parasitoids of these herbivores (Dicke & Baldwin, 2010; Halitschke *et al.*, 2008; Unsicker *et al.,* 2009). They can also be directly antimicrobial to pathogens or exhibit allelopathic effects on neighbouring plants to reduce competition for resources (Dorman & Deans, 2000).

Improvements in analytical techniques have helped identify over 1,000 plant volatiles, however less is known about their synthesis (Pichersky & Gershenzon, 2002). The synthesis of plant volatiles has been shown to have been derived from primary metabolism through the emergence of enzymes with new substrate specificities, however a relatively small proportion of plant volatiles have had their biosynthetic pathways characterised (Pichersky *et al.,* 2006) (Fig. 1.4). Furthermore, where the pathways are

described, even less information is available regarding the regulatory processes of these volatiles (Wang *et al.*, 2016). The largest class of plant volatiles are those derived from the isoprenoid pathways (Buchanan *et al.*, 2015). Terpenes are a common and diverse group of plant volatiles, with hemi-, mono-, sesqui-, and diterpenes deriving from products of the isoprenoid pathway and are involved in stress acclimation (Starks *et al.*, 1997; Vickers *et al.*, 2009). The second largest class of plant volatiles are the aromatics, and these are typically derived from phenylalanine or phenylalanine precursors (Hayashi *et al.*, 2004; Boatright *et al.*, 2004). The final major classes of plant volatiles are fatty acid derivatives and those derived from amino acids other than phenylalanine (Baldwin, 2010).

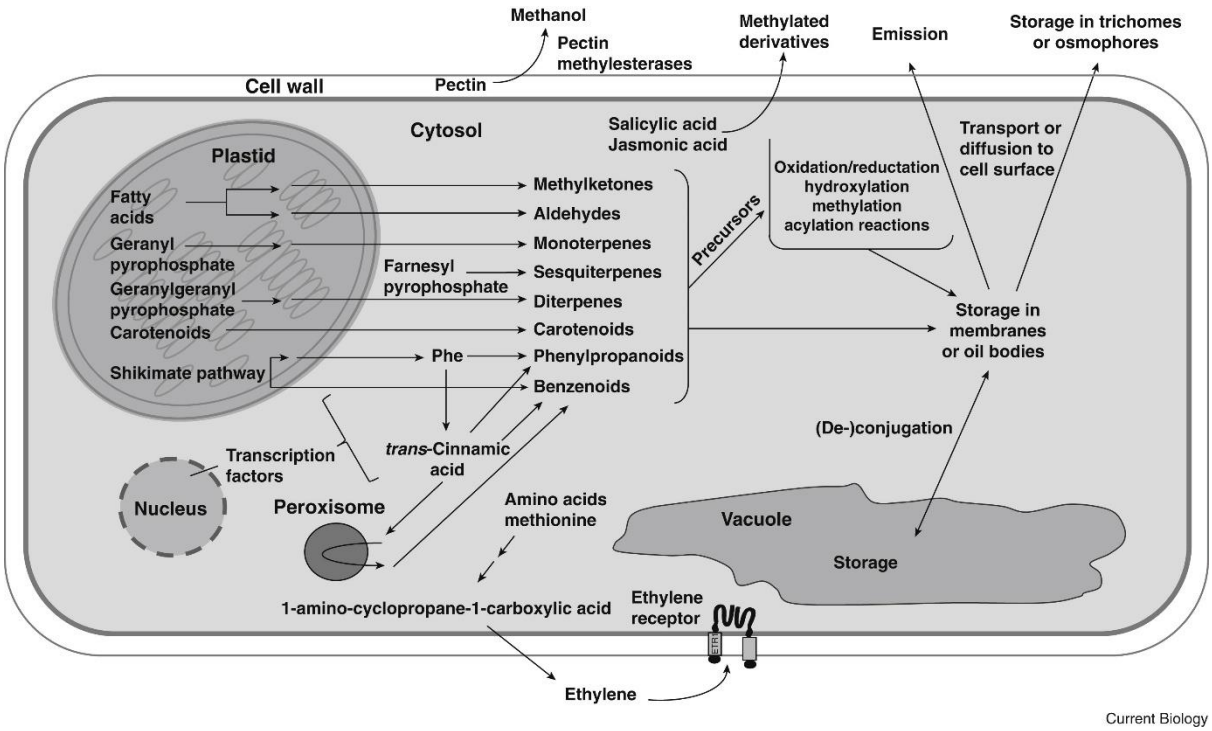


Figure 1.4: The biosynthetic origins of common plant volatiles. Source: Baldwin, 2010; modified from Pichersky et al. 2006.

1.9. Volatile Organic Compounds in Garlic

Whilst garlic produces VOCs of the four major classes, organosulphur VOCs typify garlic and other *Alliums*. The relative abundance of sulphur compounds in the garlic depends to a great extent on the preparation of the sample. In methanolic and water extracts, the VOC profile is dominated by diallyl disulphide, 3~vinyl-4H-1,2-dithiin, 2-vinyl-4H-1,3-dithiin, allyl (E)-prop-1-enyl disulphide, allyl methyl disulphide, allyl methyl trisulphide and diallyl trisulphide (Mazza *et al.*, 1992). In garlic essential oil preparations, the volatile profile is dominated by diallyl disulphide, diallyl trisulphide and allyl methyl trisulphide, with lower amounts of allyl methyl sulphide, allyl methyl disulphide, dimethyl trisulphide and diallylsulphide (Vernin *et al.*, 1986). Vernin *et al.* (1986) also reported differences in the volatile profile of extracts from garlic grown in different countries, suggesting a degree of variance between cultivars and/or growing locality. When garlic VOCs were extracted by Likens-Nickerson volatile extraction (simultaneous steam distillation and counter-current solvent extraction), a higher proportion of 2,4-dimethylfuran, 2-propen-1-o1, aniline, and 3,5-diethyl-1,2,4-trithiolane were detected from water distillation and steam distillation (Yu *et al.*, 1989). Supercritical fluid extraction and atmospheric pressure chemical ionization mass spectrometry identified 14 thiosulphinates from garlic, with the most abundant of these being allicin (Calvey *et al.*, 1997).

1.10. VOCs as quality markers:

GC-MS analysis has been proven to be a robust and reproducible tool to study VOC profiles in plant crops (Aprea *et al.*, 2009; Gaquerel *et al.*, 2009). The VOC profile of many fruit and vegetable crops is associated with quality perception amongst consumers; identifying and monitoring these sensory volatiles can help improve product quality by informing storage decisions and breeding strategies (Kader, 2008). Furthermore, many VOCs are derived from essential nutrients and health promoting compounds, evidencing

a link between VOCs and quality (Goff & Klee, 2006). VOCs are commonly emitted by fungi and bacteria, main causative agents of spoilage in garlic, with some species emitting specific volatiles and others emitting more generic volatiles (Effmert *et al.,* 2012). VOC analysis can be used to discriminate between spoilage organisms on crops throughout storage and assess the safety of food products (Vikram *et al.,* 2004; Moalemiyan *et al.,* 2007; Oms-Oliu *et al.*, 2013; Keshri *et al.*, 1998). Volatiles may offer an earlier indicator of spoilage in long-term stored crops such as grains, through the monitoring of key volatile groups indicative of spoilage and are non-destructive in their nature (Magan & Evans, 2000; Costa *et al.,* 2007).

In onion, volatiles can discriminate between healthy, blue-rot (*Penicillium sp.*) and soft-rot (*Burkholderia cepacia* and *Fusarium sp.*) infected bulbs (Wang *et al.,* 2015). Ten compounds were selected as markers, soft-rot onions had higher levels of ethanol, 1-propanol, 1-propanethiol, acetic acid and methyl propyl disulphide compared to healthy or *Penicillium* infected bulbs. 3-methyl-1-butanol was unique to soft-rot bulbs and styrene was specific to both diseased sample sets and absent in healthy bulbs. Styrene was previously reported as a VOC indicative of infection in *Alliums*, with high emission from *F. oxysporum*-infected onions and lower emission was seen from onions infected with *Aspergillus. Niger*, *Erwinia. carotovora subsp*. *carotovora* and *Penicillium sp.* (Vikram *et al.,* 2005; Fischer *et al.,* 1999). *Fusarium* oxysporum and *Botrytis allii* also produced unique VOCs on onion (Prithiviraj *et al.*, 2004). The authors also noted that the highest proportion of organosulphur VOCs was found in diseased bulbs, whereas terpenes, aromatic and aliphatic hydrocarbons were found at higher relative concentrations in healthy bulbs. It is not clear if these pathogens would emit the same VOCs if infecting garlic instead of onion, as the substrate of a microorganism has been shown to significantly alter the VOC profile (Wheatley *et al.,* 1997). Fungal volatiles also vary

significantly during interspecific interactions, with variance also between substrate and interaction status (El Ariebi *et al.*, 2016).

1.11. Thesis Aims and Objectives

The experiments performed in this thesis set out to understand how and detect when the quality of garlic changes over time in storage and whether storage can be modified to promote quality maintenance over time. A focus is placed on the enzyme alliinase, as the industry sponsor (Neem Biotech) required a rapid and accurate marker system for alliinase activity in stored garlic. Alliinase converts the non-volatile organosulphur compound alliin to the volatile compound allicin, and so changes in alliinase activity may be associated with changes in the organosulphur component of the VOC profile. How alliinase activity and more traditional quality parameters such as incidence of disease, sprouting, firmness and weight loss change over time in storage and whether any of these traits correlate with volatile organic compounds (VOCs) which may act as markers is assessed.

1. To study the postharvest biology of garlic and develop a marker system based on VOCs for garlic quality assessment for use in the biotech industry and other applications which require continuous high quality supply of garlic bulbs.
2. To assess the effects of storage conditions over time and optimise storage for the maintenance of biotechnologically relevant quality parameters of garlic.
3. To develop commercially viable methods for *in situ* VOC sampling in storage warehouses and explore the applicability of alternative VOC analysis tools for on-site quality monitoring.

2. Materials and Methods

2.1. Plant Material

Four cultivars of garlic, *Allium sativum* cv. ‘Altanero’, ‘Morado’, ‘Blanco’ and ’Violeta’ (Fig. 2.1) were sourced from a growing cooperative called Coopaman (Coopaman SCL, 45 Las Pedroñeras, Cuenca, Spain; 39° 27' North, 2° 40' West). Bulbs were of Category I quality, having been inspected for disease and damage, and 60-65 mm in diameter. They were sourced after harvest, drying and processing at their facility, prior to cold storage. Plants were lifted from the ground and left to dry in the field for 1-2 days before being transported to the processing facility where they were trimmed, cleaned of loose sheath, inspected and size graded. After this, they were collected into open-sided euro-pallet crates and left in in a well-ventilated area to complete the drying process. All garlic was grown in the same ~2,000 ha farmland of the cooperative and were grown with the same farming practises and fertiliser applications. Irrigation was supplied to all fields in accordance with recommendations from the regional irrigation advisory service (CREA, 2016). The soil type was a sandy loam and is broadly consistent over the farms.

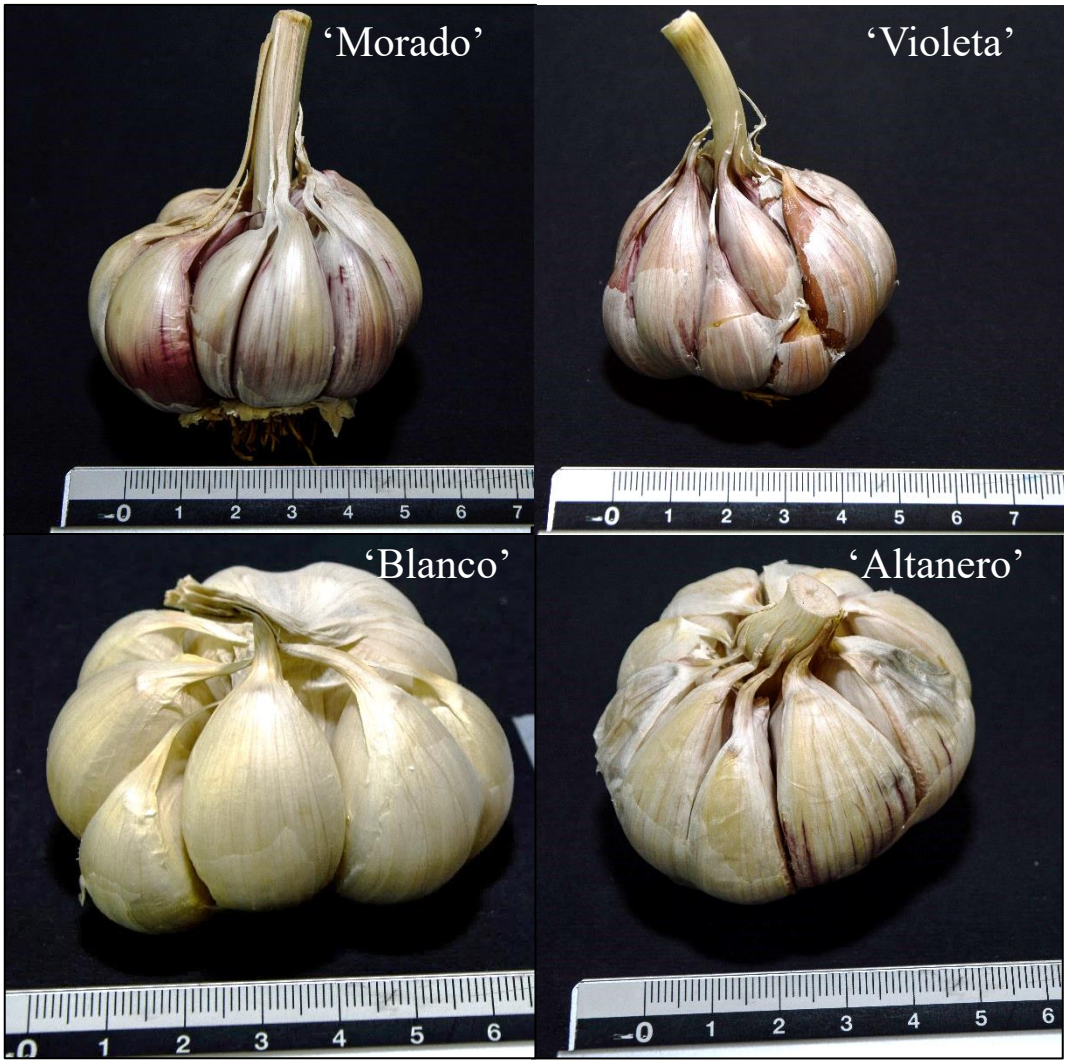


Figure 2.1: *Allium sativum* cv. ‘Altanero’, ‘Morado’, ‘Blanco’ and ’Violeta’. Note that there are three purple hardneck cultivars originating from Spain (Altanero’, ‘Morado’ and ’Violeta’) and one white softneck cultivar originating from China (‘Blanco’). Scale = cm.

2.2. Garlic Storage

Bulbs were stored in a large cardboard box with internal dividers between cultivars at -2 °C for the cold stored bulbs (-1.5 °C ± 0.6 °C) and 22 °C for the ambient stored (22 °C ± 3.5 °C) (ambient store) for a total of 8 or 14 months post-harvest, where the first two months were prior to the start of the experiment, consisting of the harvesting, drying and shipping time. Light was excluded from the bulbs throughout storage. Temperature and humidity data were monitored with a data logger (THC-4, InkBird Tech. Co, Shenzhen) to quantify the stability of storage conditions. Relative humidity levels remained at 65 % ± 10 % RH in both storage conditions over 6 months, measured for two weeks at the start and the end of the experiment.

2.3. Sampling Scheme

After storage, three bulbs of a single variety and treatment were selected at random from the storage box. These were then considered one biological replicate and all analyses were performed on material from these bulbs. Firstly, VOCs were collected from the whole, undamaged bulbs, after which, the three bulbs of garlic were weighed and broken into cloves by hand. The outer sheath, scape and basal plate were discarded and the remainder were re-weighed. Cloves were inspected for disease and any showing signs of decay were counted, weighed then discarded. The sprouting cloves were also counted and weighed at this stage, but these were not discarded. Subsequent experiments were performed from cloves taken at random from this pool of non-diseased cloves.

2.4. VOC Sample Collection

Three bulbs were selected at random and placed into a Nalophan bag (46 x 56 cm, Lakeland Ltd.) and sealed using a food bag storage clip. Nalophan was selected due to its low porosity and low VOC emissions. A 0.5 mL Eppendorf tube with its tip cut off, was inserted into the corner of the sampling bag by cutting off the corner and securing the cap with an elastic band. This served as a sampling port for TD tubes (Tenax TD/Sulficarb Tubes, Markes International). After an equilibration time of 2 h, 7.5 L of headspace was sampled through the TD tube at a rate of 60 mL/min using a "SpeediVac" High Vaccum Pump (Edwards High Vacuum Ltd, Crawley, UK), with tubes connected via a Q-Max Tube Holder (Supelco, USA) and flow rate calibrated with a GAP METER flow meter (GAP, Croydon, UK). Three replicates were collected for each cultivar and storage condition at every sampling point. Blanks of lab-air were sampled in an identical fashion.

2.5. Alliinase Enzyme Extraction

A sub-sample of healthy cloves weighing 50 g was taken at random and alliinase was extracted as per methods developed by Neem Biotech and adapted here to work more efficiently at low scale. Cloves were blended for 15 s in 150 mL chilled deionised H_2O and ice with a Philips HR1361 hand blender (Philips Electronics Co. Ltd, UK), keeping suspensions on ice during extraction. After 30 min on ice, the garlic suspension was passed through a handheld sieve lined with four layers of paper towel to remove large particulate matter and the pulp squeezed to release all free liquid. Celite Hyflo Supercel filter aid (2.5 g) (Fisher Scientific) was added and the mixture agitated with a magnetic stirrer for 5 min. The mixture was transferred to 50 mL Falcon tubes, centrifuged at 3,000 g for 10 min at 4 °C and the pellet discarded. The aqueous supernatant (500 μL) was aliquoted and frozen at -80 °C for Bradford protein assays. The pH of the solution was

then lowered to pH 4 using 3 M acetic acid, added dropwise whilst stirring with a magnetic stirrer and measuring the pH with a pH 510 pH/mV/°C meter (Eutech Instruments Pte. Ltd., Netherlands), causing the enzyme to precipitate. The mixture was then re-centrifuged at 3,000 g for 15 min at 4 °C and the supernatant discarded. The pellet was frozen, freeze dried for 72 h in a VirTis BenchTop Pro freeze drier (SP Scientific) and homogenised using a pestle and mortar into a uniform powder which was stored at -20 °C.

2.6. Alliinase Activity Assay

Alliinase activity was determined spectrophotometrically through the reaction between 4-MP (λ_{max} = 324 nm) and allicin, forming 4-allylmercaptothiopyridine, which has no absorbance in this spectral region (*Figure 2.2*) (Miron *et al.* 2002). UV-Vis measurements were performed in an Agilent Technologies Cary 60 UV-Vis spectrophotometer.

Reaction mixtures were made individually and immediately prior to use and consisted of 100 µL of 1 mM 4-mercaptopyridine (Fisher Scientific), 500 µL of 0.1 M pH 7 potassium phosphate buffer, 10 µL of 2 mM pyridoxal-5-phosphate (Fisher Scientific), 100 µL of 20 mM EDTA, 10 µL of 1 M alliin (Neem Biotech Ltd) and 280 µL dH_2O. Alliinase extract solution (500 µL), consisting of 1mg alliinase extract suspended in 500 µL of 0.1 M pH 7 phosphate buffer, was added to begin the reaction. The mixture was vortexed for 10 s and absorption was recorded 30 s after mixing and every 10 s for a total of 2 min. A standard curve was produced using 4-MP (0-0.66 mM in 0.11mM increments) and for all samples a blank was recorded using a reaction mixture lacking alliin, to account for absorbance by proteins within the extract. Absolute absorbance ranged from 0 to 0.98 and measurements were limited to the linear phase.

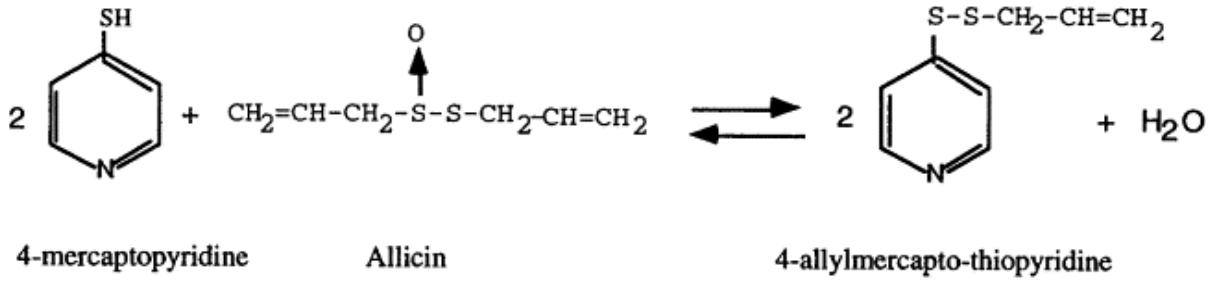


Figure 2.2: The reaction between 4-mercaptopyridine and allicin (Miron et al. 2002).

2.7. Bradford Protein Assay

The garlic extract was diluted in a 1:1 ratio with deionised H_2O to lower the protein concentration to a level within the linear phase of the Bradford assay. Garlic extract (10 µL) was mixed with 200 µL of pre-mixed Bradford reagent (Sigma, UK) in a Greiner 96-well flat-bottomed plate and read on a TECAN M200 PRO plate reader (Tecan Group Ltd, Switzerland). A standard curve was produced using BSA, at 0, 125, 250, 500, 750, 1000, 1500 and 2000 ng/mL concentration.

2.8. Bulb Water Content Evaluation

Five cloves of garlic were selected at random from each replicate of each sample. Cloves were weighed to 5 decimal places using a set of Mettler AM50 precision scales (Mettler Toledo Instruments, UK) before being cut longitudinally and dried in an electric oven at 80 °C for 7 days and reweighed.

2.9. Disease Assessment

The number and weight of diseased cloves was recorded for all bulbs. Diseased cloves were noted to have a much lower weight than non-diseased cloves due to associated carbon and moisture loss, hence disease rate was calculated by numbers of cloves, not weight. A clove was considered diseased if it displayed signs of pathogen attack, i.e. a region of dampness, discolouration, softening of tissues and/or evidence of sporulation (Mishra *et al.*, 2014 McDonald *et al.*, 2004).

2.10. TD-GC-ToF-MS Analysis

TD tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) using 5 min at 100 C, followed by 5 min at 280 °C, with a trap flow of 40 mL/min. Trap desorption and transfer occurred at 300 °C after heating at 20 °C/s starting from 25 °C, with a split flow of 20 mL/min into the GC (7890A; Agilent Technologies, Inc., Stockport, UK). Samples were run first with a 51:1 and then a 3.5:1 split ratio to increase sensitivity and dynamic range. VOCs were separated over a 60 m, 0.32 mm ID, 0.50 µm film thickness Rxi-5ms (Restek) at 2 mL/min constant flow of helium. The following temperature programme was used: initial temperature 40 °C for 2 min, 5 °C /min to 280 °C followed immediately by a post-run temperature of 300 °C for 5 min. The BenchTOF-dx mass spectrometer (Almsco International, Cincinnati, OH, USA) was operated with an ion source temperature of 275 °C, transfer line temperature of 200 °C and a mass range of 35–650 m/z. A retention time standard (C8–C20, Sigma Aldrich, Gillingham, UK) was prepared by injection of 1 µL of the standard directly into an open-ended TD tube and analysed identically to the samples.

GC–MS data were processed in ChemStation (E.02.01.1177; Agilent Technologies Inc., Stockport, UK) then deconvoluted and integrated with AMDIS (NIST, 2011), using a custom retention-indexed mass spectral library. MS spectra from deconvolution were searched against the NIST 2011 library (Stein, 2008) and only compounds scoring >80 % in forward and backward fit were included in the custom library. Compound identifications were based on match of mass spectra (>80%) and retention index (RI ± 15) (Beaulieu & Grimm, 2001).

Compounds which were not present in all three replicates of at least one experimental group were excluded, along with compounds known to be common contaminants.

Compounds present in all blank tubes at levels exceeding one tenth of the average abundance of samples were also excluded.

To normalise the data, the integrated signal of each compound was expressed as a percentage of the total signal for each sample. Where a compound was not detected in a sample, the missing values were replaced with an abundance $1/5^{th}$ of the minimum detected across all compounds and samples, as zero-inflated data can confound some of the statistical methods used in subsequent analysis. Finally, data were square root transformed to reduce bias towards highly abundant compounds in subsequent statistical tests.

2.11. Statistical Analysis

GC-MS data were analysed in R and MetaboAnalyst (R Version 3.4.2, R Core Team, 2019; MetaboAnalyst 3.0, Xia *et al.,* 2015). Permutational multivariate analysis of variance (PerMANOVA) was performed to identify differences in the VOC profile associated with experimental parameters, using the function 'adonis' in the "vegan" package in R (Oksanen *et al.,* 2019). Canonical analysis of principal coordinates discriminant analysis (CAPdiscrim) was performed in the "vegan" package in R to assess the significance of class discrimination according to experimental parameters (Legendre & Anderson, 1999; Anderson & Willis, 2003). Weighted correlation network analysis (WCNA) was performed in the "BiodiversityR" package in R to identify components of the VOC profile which varied most significantly between factors (Langfelder & Horvath, 2008). Breiman's random forest algorithm for classification and regression was used to analyse the relationship between VOCs, experimental conditions and physiological quality markers in garlic. A classification algorithm was performed on categorical data and a regression algorithm was performed on continuous variables, namely alliinase activity and disease rate. Random forest was performed in the R programme for statistical

analysis V 3.4.2 using the "randomForest" package (R Core Team, 2019; Liaw & Weiner, 2002). Classifications were plotted using Ordiplot in the "vegan" package and regressions were plotted using MDSplot in the "randomForest" package (Oksanen *et al.* 2019; Liaw & Weiner, 2002). The accuracy of random forest classifications was predicted by out-of-bag (OOB) error estimates, by which the mean prediction error is estimated by assessing how the accuracy of the algorithm changes in response to sub-sampling, thus avoiding the need for an independent validation dataset. The importance of components (individual volatiles) in random forest classifications was expressed as mean decrease accuracy (MDE) and regressions were reported as percent increase in mean square error (%IncMSE). MDE is a measure of the loss in predictive accuracy of the model if the component is permuted in a way which matches the overall distribution but assigns abundance values randomly across samples. %IncMSE is a measure of how close values are to a regression line; an increase in %IncMSE resulting from components being successively permuted gives an indication of the importance of each component to the accuracy of the regression line. The %IncMSE and MDE score of components was the basis for inclusion in the optimised random forest algorithm and subsequent plotting. Inclusion was based on achieving maximum resolution with the minimum number of compounds.

The relationship between alliinase activity, alliinase yield, bulb weight loss, firmness, % dry weight, % sprouting cloves and % healthy cloves and the variables storage time, storage temperature, harvest season and cultivar were assessed using a generalised linear model, using the "glm" statistical package within the R statistical interface V 3.4.2 R Core Team (2019).

3. Chapter 3: VOCs as Markers for Postharvest Quality Assessment of Garlic

3.1. Introduction

Garlic (*Allium sativum*) is an important commercial food crop, harvested for its subterranean storage bulbs, and a plant of considerable interest in the biotechnology industry, with 28 million tonnes produced worldwide in 2017 (FAO, 2017). The benefits of garlic on the cardiovascular system, *in vitro* antimicrobial properties and potential to reduce the risk of, or inhibit, cancer have been reported (Santhosha *et al.*, 2013, Luo *et al.*, 2016, Banerjee & Maulik, 2002; Ackermann *et al.* 2001; You *et al.*, 1989; Dorant *et al.*, 1993). These properties are predominantly attributed to organosulphur compounds derived from the enzymatic action of alliinase on the cysteine sulphoxide alliin, producing allicin (Cavallito & Bailey, 1944; Ankri & Mirelman, 1999). As such, there is a need for a molecular tool to assess alliinase activity and broader quality markers in garlic throughout storage.

Whilst current farming and storage practices aim to minimise disease, sprouting and damage, biochemical quality control is not routinely performed throughout the storage period. Bulbs are stored commercially in warehouses at -2 °C for up to 9 months to induce dormancy and prolong shelf life. However, it is not clear whether this storage regime is optimal for the maintenance of alliinase activity over time. The formation of different thiosulphinates and corresponding transformation products always relies first on the action of alliinase (Krest *et al.*, 2000). Hence, sufficient enzyme activity is necessary for a useful crop or medicinal plant and this particularly poses challenges for biotechnology firms extracting alliinase or allicin-derived organosulphur compounds from garlic. Post-harvest storage conditions have been shown to affect alliinase activity in onion (Uddin &

MacTavish 2003), although this study was performed over a relatively short storage period of 9 weeks and it is unclear if onion alliinase activity changes over time in a similar manner to garlic alliinase. Little is known about how alliinase activity in garlic is maintained over time in storage, however, diminishing alliinase activity from extracts of garlic had been observed, corresponding with the duration of time the garlic had been stored for (personal communication, Neem Biotech).

Alliinase activity loss over time during storage could be regulated at the transcriptional level, or post-translationally. There are a number of alliinase isozymes found in garlic, of which the isozymes found in the clove have high sequence homology (Rabinkov *et al.*, 1994; Cavagnaro *et al.*, 2003). A total of 9 isozymes have been identified to date, with statistical analysis suggesting the total number to be up to 14 (Ovesná *et al.*, 2015). The exon sequences of alliinase shows homology between garlic from different regions, however significant differences in the intron sequences were found between Spanish and Chinese garlic (Endo *et al*., 2014). In garlic bulbs, alliinase is localised in the vacuoles of bundle sheath cells, surrounding the phloem vessels, suggesting that alliinase, or compounds related to its metabolism, may be translocated to and from the clove during development (Ellmore and Feldberg 1994). Furthermore, alliinase genes are constitutively expressed during the growing cycle of garlic, with expression peaking during the germination phase, 7 days after sowing (Mitrová *et al.*, 2018). However, this study finished at day 55 of the growth period, at the initiation of bulbing and did not consider alliinase expression in the bulb or through storage. Little is known about how the activity of alliinase changes over time in storage; however the expression of alliinase genes is stable for at least the first 6 months of storage (Cho *et al.*, 2011). In this study, garlic was stored under three storage treatments: constant 20 °C for 6 months, storage at 20 °C for 3 months followed by 0 °C for 3 months and storage at 0 °C for 3 months

followed by 20 °C for 3 months, with neither storage time or temperature treatment affecting alliinase gene expression (Cho *et al.*, 2011).

A major component of garlic VOCs are organosulphur compounds (Martin-Lagos *et al.*, 1995). Cysteine synthase (AsCysK) is a key enzyme in the synthesis of organosulphur compounds in garlic and, like alliinase, is constitutively expressed throughout the growing cycle of garlic (Mitrová *et al.*, 2018). Cysteine synthase catalyses the reaction of serine with an allyl group of current unknown origin to produce S-allyl-L-cysteine, the precursor of alliin. Transcriptomic analysis identified 27 isoforms of cysteine synthase, with differential expression in isoforms between cloves and other vegetative or reproductive organs (Kamenetsky *et al.*, 2015). Furthermore, the diversity of S-alkenyl cysteine sulphoxides in garlic varied between hardneck and softneck garlic (Ovesná *et al.*, 2011). Alliin, S-allyl-l-cysteine sulfoxide, is synthesised from S-allyl-L-cysteine by a flavin-containing monooxygenase catalysing an S-oxygenation reaction. Alliin is synthesised both from stored S-allyl-l-cysteine during sprouting and through *de novo* synthesis in foliage during the growing phase (Yoshimoto *et al.*, 2015a).

Analysis of VOCs which are emitted by garlic throughout storage, could provide a tool to assess biochemical quality. GC-MS has been applied to perform post-harvest quality assessment of a range of crops (Li *et al.*, 2011; Ciesa *et al.*, 2013; Woolfenden, E. 2010; El-Mogy *et al.*, 2019) by monitoring changes in the profile of VOCs. Thermal desorption (TD) is a sampling method traditionally applied to air quality measurements and forensics as its portability is suited to use in the field and it has a higher sensitivity when compared to other VOC collection methods such as SPME (Rosier *et al.*, 2014; Gallego *et al.*, 2012). More recently, thermal desorption has been employed to perform postharvest quality assessment of produce (Tanaka *et al.*, 2018; Bell *et al.*, 2016). TD tubes allow for remote and passive sampling *in situ*, to measure average VOC profiles within a storage facility,

which is not possible with SPME or steam distillation based methods. The VOCs are then stable for several weeks once adsorbed onto the tube, affording time for transport to the lab for analysis. This can be performed in a matter of days from the completion of sampling, yielding results in a meaningful time frame for a crop which is stored for several months. As well as being entirely non-destructive and requiring no equipment on-site other than the tube and a spanner to remove the end cap, the dynamic range afforded by split injection on the TD-100 autosampler allows for the detection of compounds of <10 ppb level whilst also allowing relative concentrations of the highly abundant compounds to be measured without overloading the detector (Kim *et al.*, 2013; Woolfenden, E. 2010). Thermal desorption was found to produce superior reproducibility and recovery when working with garlic VOCs than activated-charcoal-TD or TENAX-solvent elution with < 6 % relative standard deviations in peak area (Pino, 1992).

This chapter studies the postharvest biology of garlic 'Altanero' over a 12 month time course at ambient and cold temperature, specifically in relation to quality parameters and VOC profile. The activity of alliinase extracts derived from these bulbs at different points through storage is measured and the expression of cysteine synthase genes are analysed. Physiological parameters such as disease incidence, sprouting and water content were measured. The VOC profile of the garlic was assessed over time and between storage temperatures. Compounds were putatively identified from the VOC profiles which discriminate between storage conditions.

3.2. Materials and Methods

3.2.1. Plant Material

Garlic cv. ‘Altanero’ was sourced from a growing cooperative in Spain as per Chapter 2.1.. The crop was planted from September to October of 2016 and harvested in July to August of 2017. Bulbs were of Category I quality, having been inspected for disease and damage, and 60-65 mm in diameter. Bulbs were shipped to the lab after harvest, drying and processing at their facility, which took approximately 2 months.

3.2.2. Garlic Storage

Bulbs were stored in a large cardboard box with internal dividers between cultivars at -2 °C and ambient temperature for 0, 3, 6 and 12 months commencing in September 2017. Light was excluded and temperature and humidity were monitored periodically through the storage period, as outlined in Chapter 2.2.

3.2.3. VOC Sample Collection and Analysis

Three bulbs were selected at random and placed into a Nalophan sampling bag as detailed in methods chapter 2.4. VOCs were collected on TD tubes (Tenax TD/Sulficarb Tubes, Markes International). After an equilibration time of 2 h, 7.5 L of headspace was sampled through the TD tube at a rate of 60 mL/min using a “SpeediVac” High Vaccum Pump (Edwards High Vacuum Ltd, Crawley, UK), with tubes connected via a Q-Max Tube Holder (Supelco, USA) and flow rate calibrated with a GAP METER flow meter (GAP, Croydon, UK).

TD tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) using TD, GC and MS settings outlined in methods chapter 2.10.

GC–MS data were processed in ChemStation (E.02.01.1177; Agilent Technologies Inc., Stockport, UK) then deconvoluted and integrated with AMDIS (NIST, 2011), using a custom retention-indexed mass spectral library. MS spectra from deconvolution were searched against the NIST 2011 library (Stein, 2008). Compound identifications were based on match of mass spectra (>80%) and retention index (RI ± 15) (Beaulieu & Grimm, 2001).

To normalise the data, the integrated signal of each compound was expressed as a percentage of the total signal for each sample. Where a compound was not detected in a sample, the missing values were replaced with an abundance 1/5th of the minimum detected across all compounds and samples., as zero-inflated data can confound some of the statistical methods used in subsequent analysis. Finally, data were square root transformed to reduce bias towards highly abundant compounds in subsequent statistical tests.

GC-MS data were analysed in R and MetaboAnalyst (R Version 3.4.2, R Core Team, 2019; MetaboAnalyst 3.0, Xia et al., 2015). Permutational multivariate analysis of variance (PerMANOVA) was performed to identify differences in the VOC profile associated with experimental parameters, using the function ‘adonis’ in the “vegan” package in R (Oksanen et al., 2019). Canonical analysis of principal coordinates discriminant analysis (CAPdiscrim) was performed in the “vegan” package in R to assess the significance of class discrimination according to experimental parameters (Legendre & Anderson, 1999; Anderson & Willis, 2003). Weighted correlation network analysis (WCNA) was performed in the “BiodiversityR” package in R to identify components of

the VOC profile which varied most significantly between factors (Langfelder & Horvath, 2008).

3.2.4. Alliinase Enzyme Extraction

Alliinase enzyme was extracted from 50 g garlic using an acid precipitation extraction method developed by Neem Biotech and adapted here to work more efficiently at low scale. Garlic was macerated in water, filtered and centrifuged to remove particulate matter before the pH of the supernatant was lowered to pH 4, precipitating the enzyme. The pollution was centrifuged into a pellet, the supernatant discarded and the pellet freeze dried, as per methods outlined in Chapter 2.5.

3.2.5. Protein Concentration and Alliinase Activity Assay

Protein concentrations were assessed with Bradford assays, as outlined in Chapter 2.7. Alliinase activity was determined spectrophotometrically through the reaction between 4-MP (λmax = 324 nm) and allicin, forming 4-allylmercaptothiopyridine, which has no absorbance in this spectral region, as per methods detailed in Chapter 2.6 (Miron et al. 2002).

3.2.6. Bulb Water Content

Five cloves of garlic were selected at random from each replicate of each sample. Cloves were weighed to 5 decimal places using a set of Mettler AM50 precision scales (Mettler Toledo Instruments, UK) before being cut longitudinally and dried in an electric oven at 80 °C for 7 days and reweighed.

3.2.7. Disease Assessment

The number and weight of diseased cloves was recorded for all bulbs. Diseased cloves were noted to have a much lower weight than non-diseased cloves due to associated carbon and moisture loss, hence disease rate was calculated by numbers of cloves, not weight. A clove was considered diseased if it displayed signs of pathogen attack, i.e. a region of dampness, discolouration, softening of tissues and/or evidence of sporulation (Mishra et al., 2014 McDonald et al., 2004).

3.2.8. RNA Extraction

RNA extraction was according to Jordon-Thaden et al. (2015), with minor modifications. Samples were ground in a pestle and mortar under liquid nitrogen to a fine, homogenous powder and stored in 1 g aliquots at -80 °C until used. CTAB extraction buffer contained 2 % (w/v) hexadecyltrimethylammonium bromide (CTAB), 1 % (w/v) polyvinyl pyrrolidone (PVP), 200 mM Tris-HCl buffer pH 8, 1.4 M NaCl and 0.2 % (v/v) β-mercaptoethanol (BME) was added immediately prior to the extraction.

Ground garlic clove (100 µg) was added to a chilled 1.5 mL Eppendorf tube containing 900 µL of CTAB buffer and BME. Samples were vortexed immediately for 2 min and incubated at 55 °C for 8 min, this was repeated once and then samples were centrifuged at 13,000 g for 5 min at 20 °C. Supernatant (750 µL) was transferred to a chilled 1.5 mL Eppendorf tube to which 750 µL 24:1 Chloroform: Isoamyl alcohol (Fisher, UK) was added and the phases were mixed by inverting the tube. Tubes were centrifuged at 13,000 g for 10 min at 4 °C and the aqueous phase pipetted into a 2 mL Eppendorf tube. To this, 40 µL of sarkosyl (Sigma, UK), 200 µL dH_2O and 750 µL Invitrogen Tri-reagent (Fisher, UK) was added and mixed by inverting. Samples were then centrifuged at 13,000 g for 10 min at 4 °C and the aqueous phase pipetted to a 1.5 mL Eppendorf tube. Chloroform

(200 µL) was added to the tube, before vortexing for 15 s, incubating at RT for 10 min and centrifuging at 13,000 g for 15 min at 4 °C. The aqueous phase was transferred to an Eppendorf tube and this step was repeated if the sample did not look clear and colourless.

The RNA was precipitated with the addition of 750 µL of isopropanol (Fisher, UK), which was inverted several times and then incubated for 10 min at RT. The RNA was pelleted by centrifuging at 13,000 g for 20 min at 4 °C. The supernatant was discarded, and the pellet washed in 75 % EtOH by vortexing, centrifuged at 13,000 g for 5 min at 4 °C. Then the supernatant was discarded and the pellet was left to dry for approx. 1h in a sterile laminar flow hood. The pellet was stored dry at -80 °C until use, upon which it was resuspended in 30 µL RNase free dH_2O.

Upon resuspension, the concentration and quality of RNA was checked by spectrophotometry using a NanoDrop 1000 (Thermo, UK) and agarose gel electrophoresis.

3.2.9. DNase and cDNA Synthesis

RNA extract (2000 ng) was diluted to 16 µL with nuclease free water and, 2 µL RQ1 10x DNase buffer (Promega, UK) and 2 µL RQ1 DNase were added. The sample was incubated for 30 min at 37 °C. Samples were tested via PCR with AsUBQF and AsUBQR primers (5'-AAGCCAAGATACAGGACAAG-3', 5'-GCATACCACCTCTCAATCTC-3', 153 nt amplicon, (designed by Prof Adriana Chiappetta, University of Calabria, Italy, using primer 3 software and based on database sequences; personal communication) followed by gel electrophoresis to ensure no DNA remained in the sample and if so, 2 µL RQ1 DNase STOP solution was added.

Once the samples were free of DNA, cDNA synthesis was performed using M-MLV RNase H- Reverse Transcriptase (Promega, UK). To DNase treated RNA (20 µL) 1 µL

Oligo (dt) was added and incubated in a thermal cycler at 70 °C for 10 min then chilled on ice for 10 min. 6 µL of 5 x first strand buffer, 1.5 µL 10mM dNTPs and 0.6 µL RNAsin (25 u) were added and incubated at 42 °C for 2 min. 1 µL M-MLV reverse transcriptase was added and the mixture was incubated at 42 °C for 60 min and inactivated by heating to 70 °C for 15 min.

3.2.10. PCR

Alliinase (*AsAll;* GenBank: Z12622.1 / AF409952) was amplified using *AsAllF* and *AsAllR* primers (5'-TGCTCATGCCCCTTTTTATC-3', 5'-TTGATTACTGCATGGCGAAG-3', 153 nt amplicon), cysteine synthase (*AsCysK; GenBank:* AY766093.1) was amplified using *AsCysKF* and *AsCysKR* primers (ATAGCAGCTGCGAAGGGTTA, 5'-GCATACCACCTCTCAATCTC-3', 152 nt amplico and both were normalised to a garlic ubiquitin housekeeping gene (*AsUBQ*; GenBank: KP116308), which was amplified using AsUBQF and AsUBQR primers (5'-AAGCCAAGATACAGGACAAG-3', 5'-GCATACCACCTCTCAATCTC-3', 153 nt amplicon) All primers were designed and validated by designed by Prof Adriana Chiappetta of the University of Calabria, Italy, using primer 3 software and based on database sequences and synthesised by Sigma-Aldrich, UK. PCR primers were checked by sequencing the PCR product and confirming the sequence was that of the target genes using BLAST (Basic Local Alignment Search Tool, NCBI, USA).

PCRs were carried out in 25 µL reactions (1 µL template, 0.5 µL 10 mM dNTPs, 5 µL GoTaq Green Buffer (Promega, UK), 1.5 µL 25 mM MgCl, 0.5 µL 10 µM forward and reverse primers, 0.125 µL Taq polymerase (Promega, UK) and 15.9 µl dH2O in an Applied Biosystems Veriti thermal cycler. The initial incubation was 5 min at 95 °C; followed by 35 cylces of 45 s at 95 °C; 45 s at 58.5 °C; 30 s at 72 °C and a final 5 min at

72 °C. PCR products were visualised via gel electrophoresis on a 2 % agarose gel with Bioline 1 Kb Plus ladder and SafeView nucleic acid stain on a UV transilluminator.

3.2.11. qPCR

qPCR was performed using qPCRBIO SyGreen Blue Mix Lo-ROX ready mix (PCRBiosystems, UK) in a Roche Light Cycler 96 real time PCR system (Roche, Germany). The program consisted of an incubation at 95 °C for 2 min; 40 cycles of 3 step amplification 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a melting curve. PCR was performed in 20 µL reactions consisting of 10 µL qPCRBIO SyGreen Blue Mix Lo-ROX (2X) (PCR Biosystems), 0.8 µL 10 µM primers, cDNA (40 ng) made to 20 µL with nuclease free water. Reactions were run in technical triplicates from three biological replicates and included negative controls. Analysis was based on the delta –delta Ct (ΔΔCt) method by comparison to AsUBQ (Livak & Schmittgen, 2001). PCR products were sequenced (Eurofins Genomics) and the sequences searched using BLAST (BLAST, NCBI) to confirm the identity of the amplified sequence.

3.2.12. Statistical Analysis

Physiological and enzymatic data were assessed using a generalised linear model, using the "glm" statistical package within the R statistical interface V 3.4.2 R Core Team (2019).

3.3.Results

3.3.1. Percent Disease

The incidence of disease within samples increased sharply after 6 months of ambient storage, with significant differences to all other time points and temperature treatments (GLM, $p < 0.001$). There were no statistically significant differences in disease incidence between garlic stored at either temperature for the first 6 months of storage, nor did they differ from the baseline disease incidence found at pre-storage (time 0). There was no significant increase in disease incidence from pre-storage to 12 months of storage in cold stored garlic (Fig. 3.1).

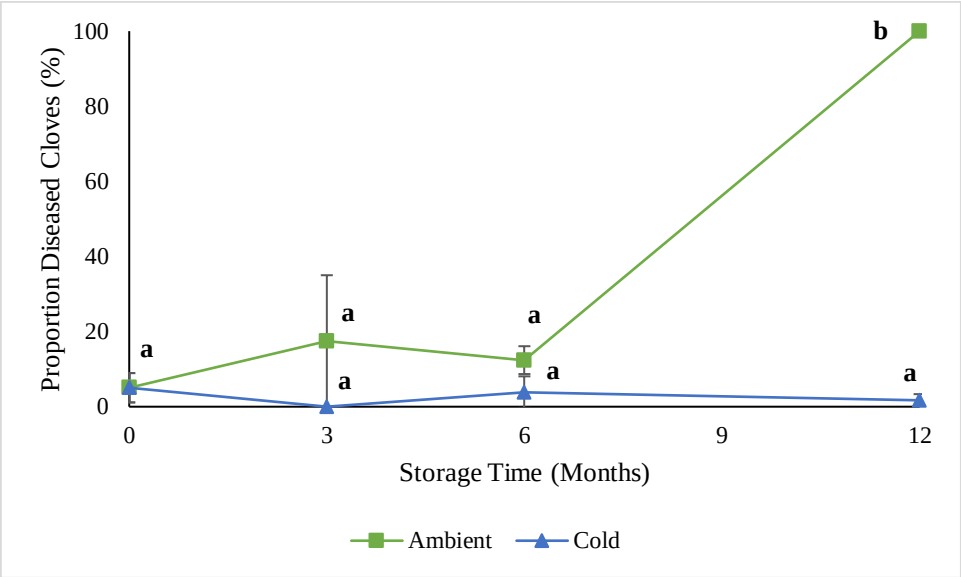


Figure 3.1: Proportion of diseased garlic cloves stored at ambient (■) and cold (▲) temperatures for 0, 3, 6 and 12 months. Letters indicate statistically different values between the different temperatures and time points, based on GLM and LSD, alpha =0.05, N = 3 replicates of 3 bulbs, error bars = SD.

3.3.2. Water Content

Throughout storage, the water content of garlic remained constant, with no significant changes in either ambient or cold stored garlic (ANOVA, P_{Time} = 0.074, P_{Temp} = 0.673, $P_{TimeXTemp}$ = 0.737). Ambient stored garlic had an average dry weight of 36.9 % and cold stored garlic of 34.8 % (Fig. 3.2). At specific time points, there were some significant differences in water content, with ambient stored bulbs having a significantly higher water content than cold stored bulbs at 3 months of storage. No bulbs varied significantly from pre-storage levels.

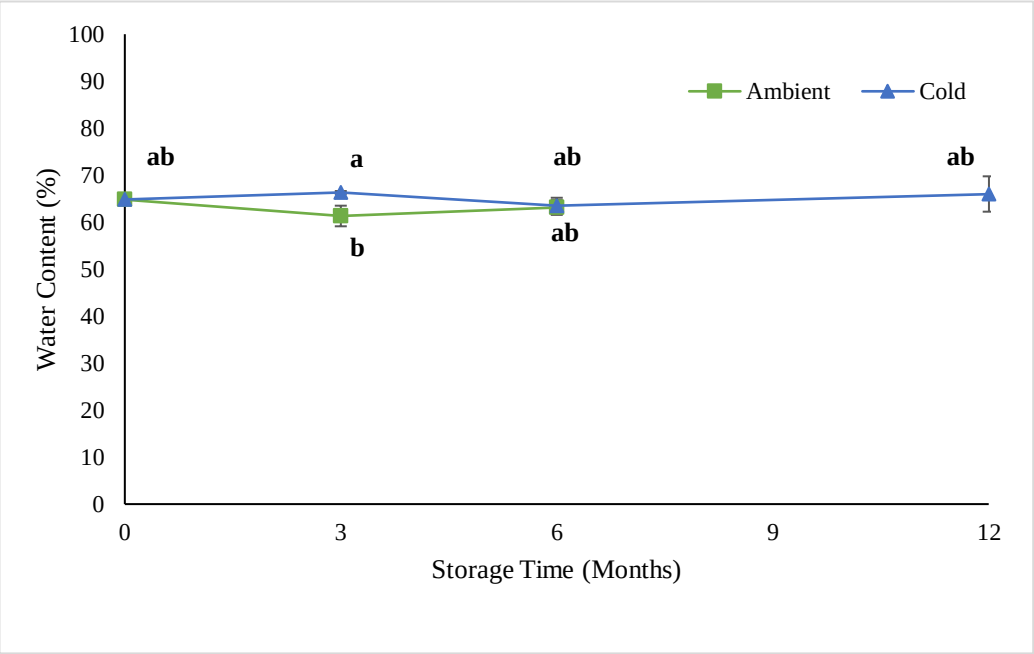


Figure 3.2: Percent water content of garlic cloves, stored at ambient (■) and cold (▲) temperatures for 0, 3, 6 and 12 months. Water content calculated by FW-DW/FW. Letters indicate statistically different values between the different temperatures and time points, based on GLM and LSD, alpha = 0.05, N = 3 replicates of 5 cloves, error bars = SD.

3.3.3. Extraction Yield

Extraction yields increased significantly after 3 months cold storage, before falling back to pre-storage yields at 6 months and significantly decreasing below pre-storage yields at 12 months (LSD, Fig. 3.3). Ambient stored garlic retained an alliinase extract yield which did not differ from pre-storage levels for the first 6 months of storage; by 12 months no material remained from which to perform extractions. Neither storage time (P = 0.804), temperature (P = 0.173) or the interaction of time x temperature (P = 0.581) were significant factors (GLM).

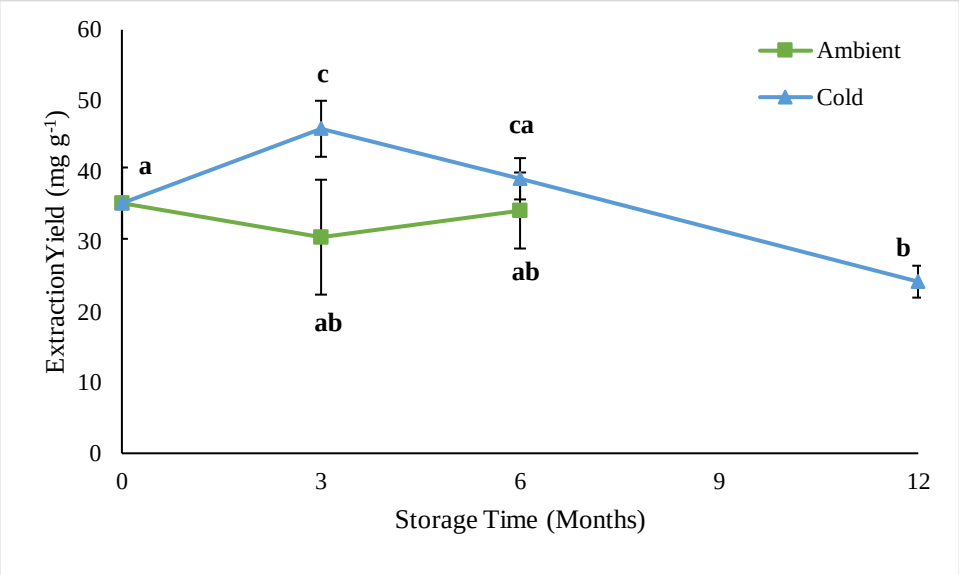


Figure 3.3: Extraction yield (mg) per g fresh weight peeled garlic cloves stored at ambient (■) and cold (▲) temperatures, stored for 0, 3, 6 and 12 months. Letters indicate statistically different values between the different temperatures and time points, based on LSD, alpha = 0.05, N =3 replicates of 50 g FW, error bars = SD.

3.3.4. Alliinase Activity

Alliinase activity decreased significantly from pre-storage levels over time at both storage temperatures (GLM, $p < 0.001$, Fig. 3.4). Activity decreased significantly more in cold stored garlic throughout the first 6 months of storage (GLM, $p < 0.001$) after 3 months of storage, alliinase activity was significantly lower in garlic stored cold, while the loss in activity at ambient was not statically significant. After 6 months there was significant loss of activity at both storage temperatures but the magnitude of activity loss at ambient was significantly less compared to cold storage (53.0 % loss vs. 84.8 % loss compared to pre-storage activity). However, after 12 months' storage at ambient conditions the garlic had decayed completely, and no enzyme activity was detectable while cold stored garlic retained 21.4 % of pre-storage enzyme activity.

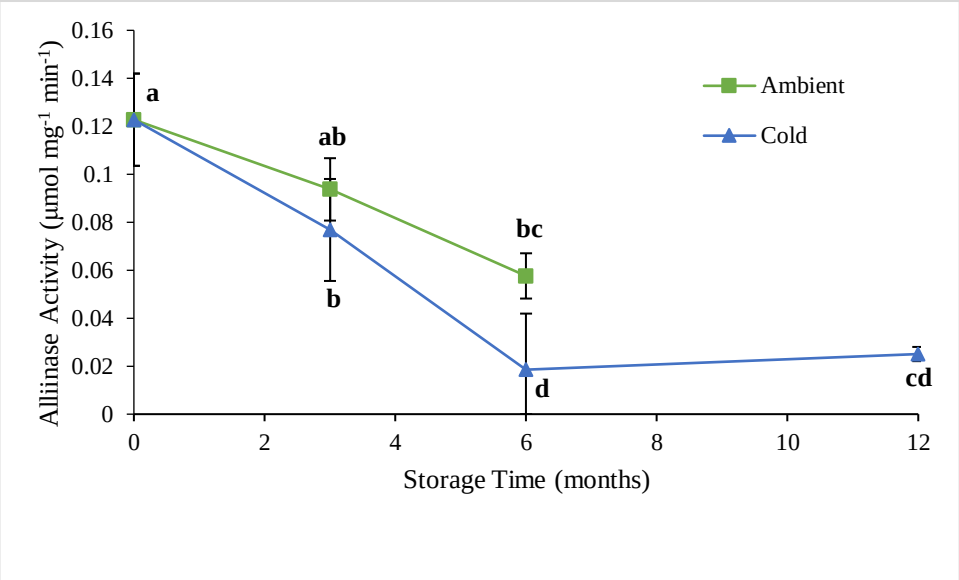


Figure 3.4: Alliinase activity of enzyme extracts from garlic stored at ambient (■) and cold (▲) temperatures, for 0, 3, 6 and 12 months. Enzyme activity was normalised to total soluble protein. Letters indicate statistically different values amongst different temperatures and time points, based on GLM and LSD, alpha = 0.05, N = 3 replicates of 50 g FW, error bars = SD.

3.3.5. Cysteine Synthase Gene Expression

In order to examine gene expression levels, transcript levels were taken as a proxy. Cysteine synthase expression was maintained at a stable rate throughout storage and did not rise between 6 and 12 months of storage in the cold (Fig. 3.5). When PCR products were sequenced, clear sequences were obtained from products amplified using UBQ and CysK primers (Supplementary Fig. S3.1), indicating single PCR products. When searched with BLAST, *Allium sativum* CysK and Ubq genes were identified (Supplementary Table S3.2). Although the primers used to study alliinase expression were previously found to amplify all alliinase genes, the sequences from products amplified here included multiple peaks at each base position and were not identified by BLAST. Subsequently, no further conclusions were drawn from this data, however the expression data can be seen in the supplementary data (Fig. S3.4). Melting peaks for all qPCR reactions resulted in well defined, individual peaks for each PCR primer pair (Fig. S3.2)

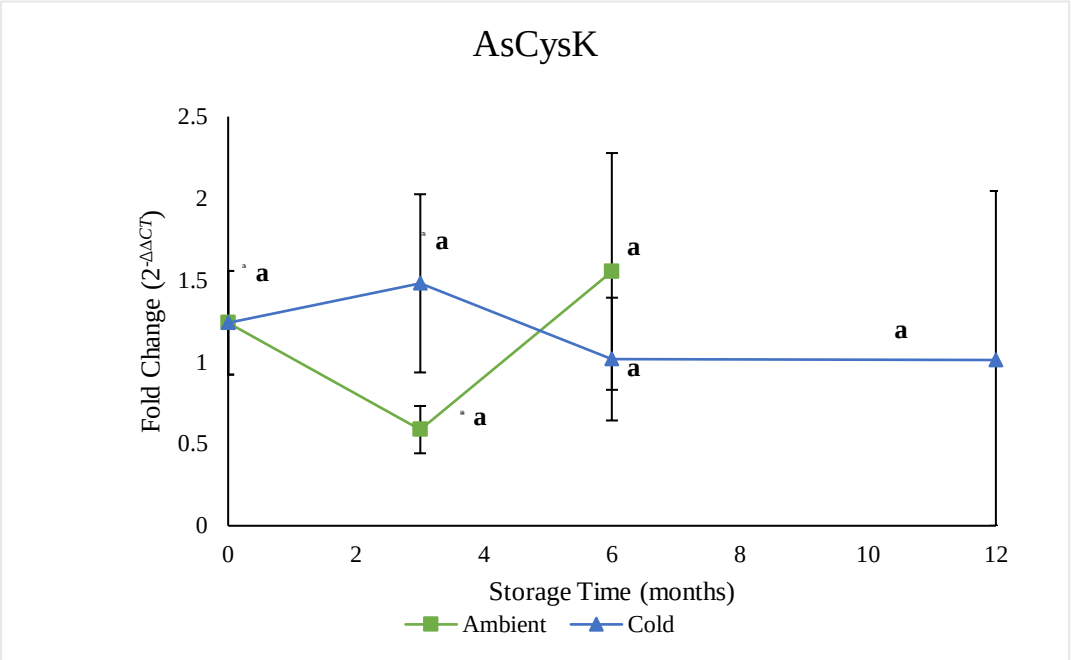


Figure 3.5: Expression of Cysteine Synthase (CysK) normalised to Ubiquitin (UBQ). Letters indicate statistically different values between the different temperatures and time points, based on GLM and LSD, p = 0.05, N = 3 cloves, error bars = SD.

3.3.6. Composition of the VOC Profile

A total of 117 compounds were quantified from all garlic 'Altanero' samples across all four time points and two temperatures of storage by comparison of mass spectra to NIST library and retention index data, of which all were putatively identified (Supplementary Table S3.1). Major groups of compounds were hydrocarbons (55), terpenes (15), organosulphur (13), alcohols (11) and aldehydes (10). Other VOCs were ketones (5), esters (4), furans (3) and carboxylic acids (1). The most abundant individual compounds were organosulphur compounds and included diallyl disulphide (37 %), diallyl sulphide (24 %) and ally methyl sulphide (18 %). On average, sample profiles comprised 92 VOCs with a range of 57 to 117 compounds per sample.

3.3.7. Effect of storage time and temperature on VOC Profiles of garlic

Storage time was not a significant factor affecting VOC profiles, however temperature was significant (PerMANOVA, P = 0.166, R^2 = 0.073 and P = 0.022, R^2 = 0.124 respectively). There was no significant interaction between time and temperature (PerMANOVA, P = 0.138, R^2 = 0.077). CAP showed partial separation of the samples based on storage time and temperature (Fig. 3.6). None of the stored samples were discriminated from pre-storage, time 0 samples, however, storage for 12 months in the cold resulted in a distinct VOC profile compared to VOC profiles of all other stored samples. When only storage time was considered in the CAP model, again there was partial discrimination of samples based on VOC profile (percentage correct classification of 77.8% at P = 0.05) (Fig. 3.7) with samples stored for 12 months separated from pre-stored (0 month) samples, and samples stored for 6 months. In addition, the VOC profile from garlic bulbs stored for 6 months was separated from all other stored samples. CAP could not separate VOC profiles based on storage temperature.

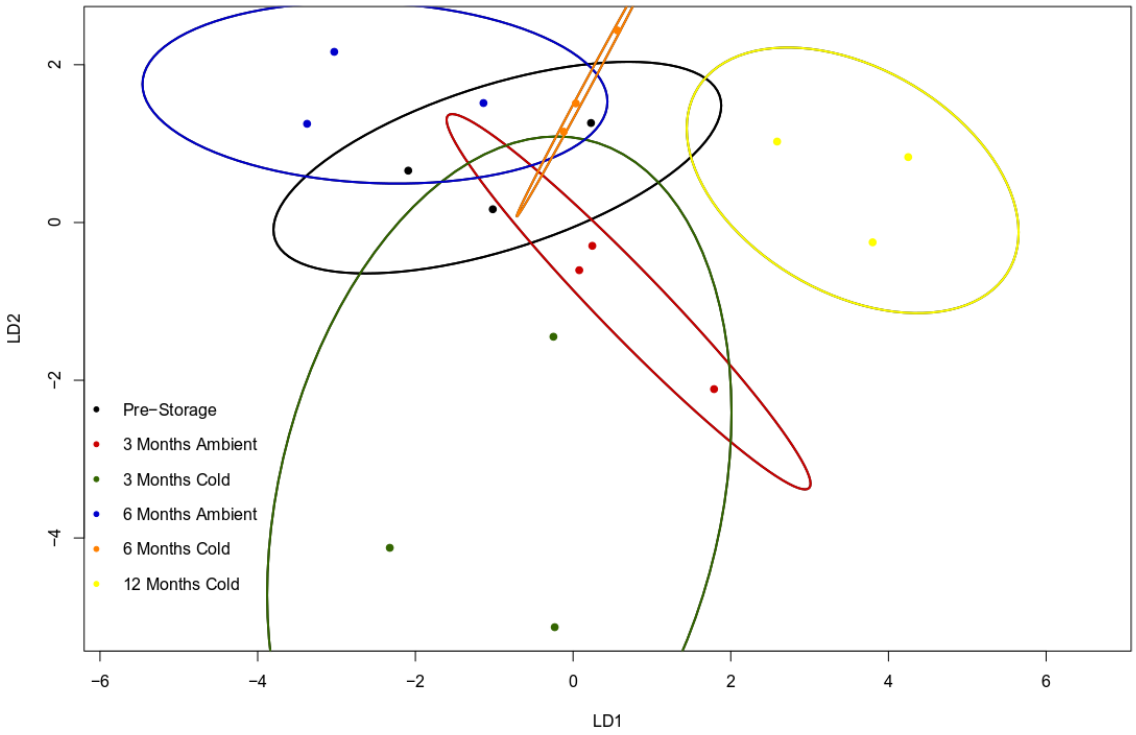


Figure 3.6: Canonical Analysis of Principal Coordinates based on all VOCs from garlic cv. Altanero using TD-GC-ToFMS. A CAP model was produced for samples stored at 0, 3, 6 and 12 months, and ambient and cold temperatures, combined into a single category of time x temperature. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval. The percentage of correct classification was overall 55.6% at a confidence of P = 0.05 (Percent successful classification by group: pre-storage = 33.3 %, 3 months ambient = 33.3 %, 3 months cold = 66.6 %, 6 months ambient = 66.6 %, 6 months cold = 33.3 %, 12 months cold = 100%) N = 3 replicates of 3 bulbs.

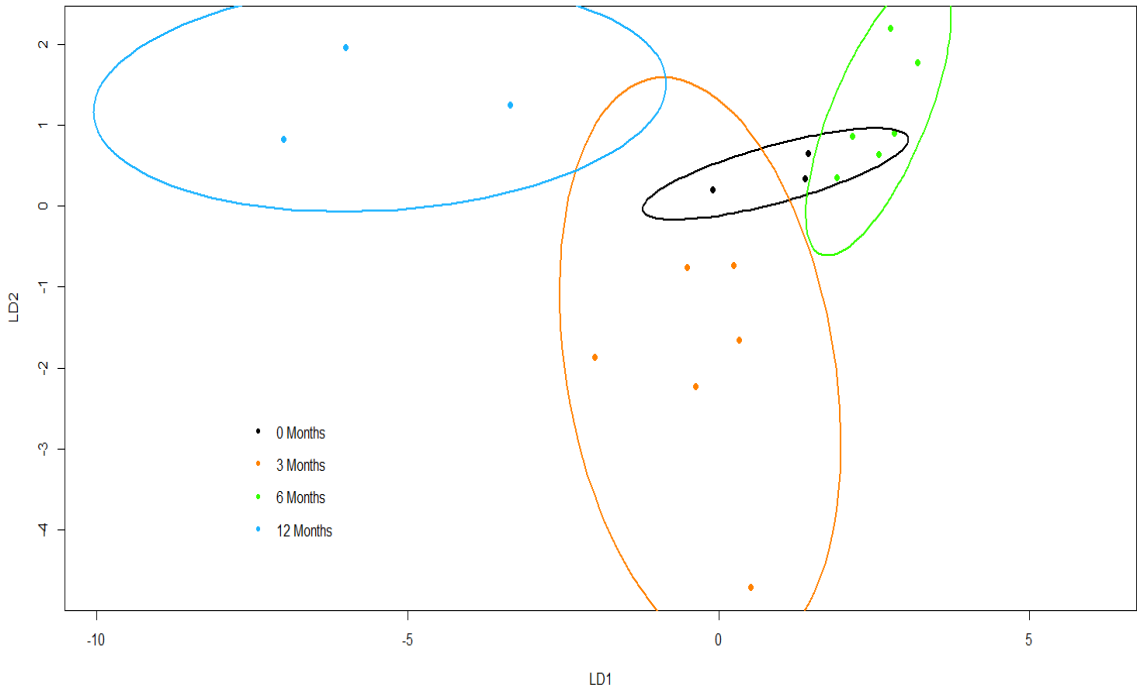


Figure 3.7: Linear discriminant plot from CAP of VOC profiles from garlic cv. Altanero cold stored for 0, 3, 6 and 12 months. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 77.8% at a confidence of P = 0.05 (Percent successful classification by group: 0 months = 66.6 %, 3 months = 66.6 %, 6 months = 83.3 %, 12 months = 100%). N = 3 for 0 and 12 months, N = 6 for 3 and 6 months.

3.3.8. Identification of VOC markers for storage time and temperature

WCNA of VOC profiles of garlic *cv.* clustered the VOCs into seven modules of which two changed significantly with storage temperature (brown and grey) and one with storage time (turquoise, Fig. 3.8a). The brown module (temperature) included eight organosulphur and one cyclic hydrocarbon, and the grey module (temperature) contained two terpenes, two cyclic hydrocarbons and one phenol. The turquoise module (time) was dominated by terpenes (9) and contained three ketones, three alkanes, three esters and two alkenes. The VOCs associated with storage temperature were predominantly

organosulphur compounds (8 out of 14), whereas the VOCs associated with time did not contain any organosulphur compounds.

Across storage time, a number of compounds were only present after 12 months of storage (epizonarene, cis-calamenene, α-muurolene, 2,3-dimethylhexane, α-copaene, δ-amorphene, 3,7(11)-selinadiene, α-cubebene and β-elemene) and these have potential to be used as markers for the end of garlic shelf-life. Conversely, 6,6-dimethyl-undecane and 3-methyl-decane were present at all time points besides pre-storage and so the absence of either of these compounds may be a good indicator of the freshness of garlic. Furthermore, if 6,6-dimethyl-undecane and 3-methyl-decane were present, but none of the compounds indicative of 12 months storage were present, then this would indicate that the garlic is either 3 or 6 months old. To determine if the garlic is either 3 or 6 months old, the presence or absence of 2,3-dimethylhexane and γ-cadinene may be examined; γ-cadinene is present at 3 months but absent at 6 months and the inverse is true of 2,3-dimethylhexane (Fig. 3.8b).

In relation to temperature of storage, the abundance of diallyl sulphide and 3-ethenyl-3,4-dihydrodithiine were not distinct between pre-storage and ambient storage, suggesting that these four compounds may be of value as marker compounds of cold storage. However, abundance of supraene and phenol appeared to decline dramatically in stored garlic, irrespective of the storage temperature and several other VOCs including allyl-mercaptan and 3-ethenyl-3,6-dihydrodithiine showed differential abundance amongst pre-stored, ambient and cold stored garlic (Fig. 8c). The % abundance of the VOC profile accounted for by the eight organosulphur compounds was 12.0 % in pre-storage garlic, 9.0 % in ambient stored garlic and 3.3 % in cold stored garlic. All eight of the organosulphur compounds fell in % abundance from pre-storage to cold stored samples (Fig. 3.8c).

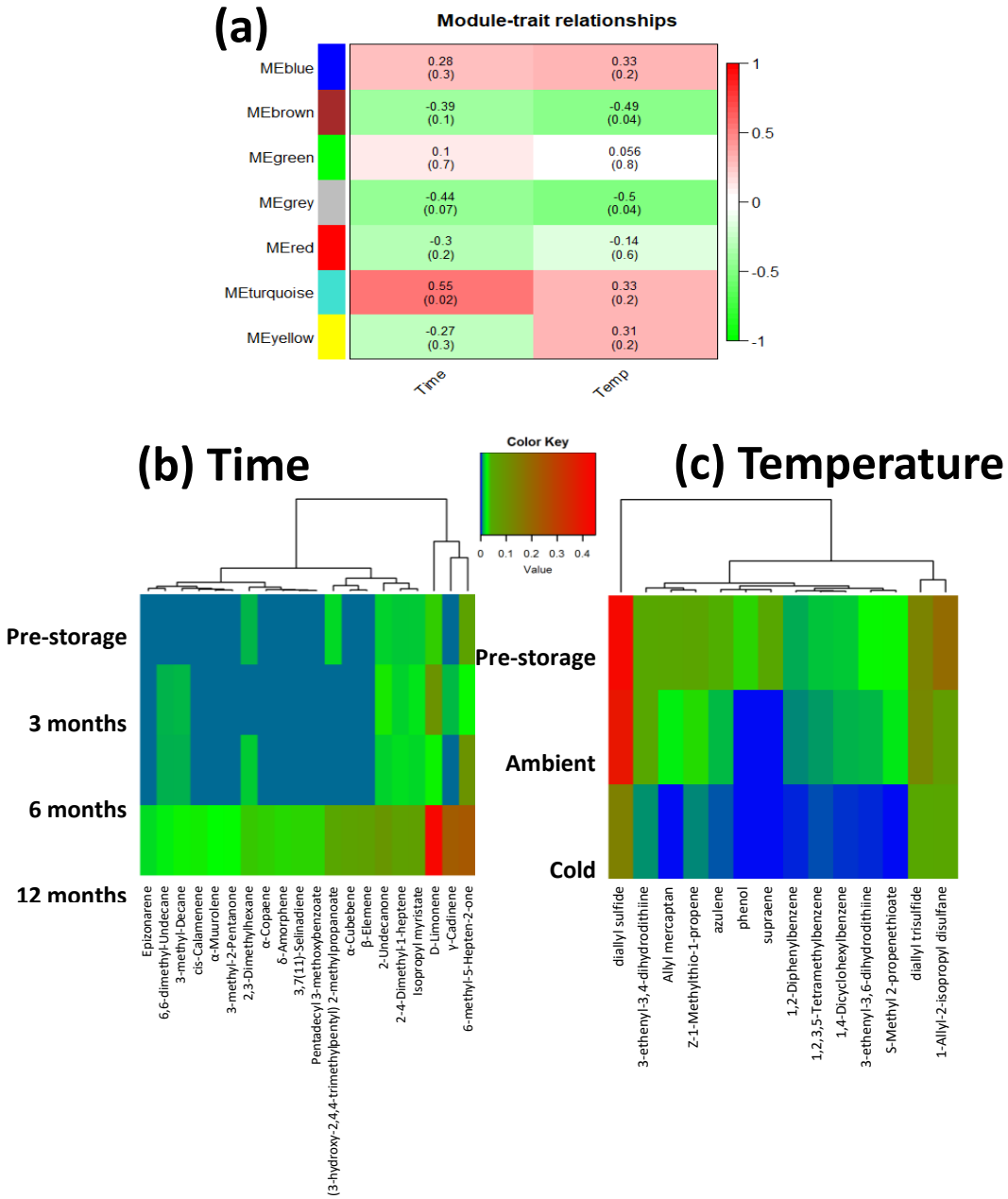


Figure 3.8: Correlation analysis of VOCs from garlic cv. Altanero stored for 0, 3, 6 and 12 months at ambient and cold. **(a)** WCNA modules: score and significance according to Pearson analysis (P values in brackets; red, positive and green, negative correlations with variable). **(b)** and **(c)** heat maps of significant VOCs in significantly correlating WCNA modules according to **(b)** storage temperature and **(c)** time (blue low, green intermediate and red, high VOC relative abundance).

As the majority (8 out of 13) of organosulphur compounds detected here were found to be significantly associated with storage temperature, the analysis was repeated on a reduced VOC dataset consisting of all 13 organosulphur compounds. This found that the organosulphur component of the VOC profile varied significantly between storage temperature (PerMANOVA, $p = 0.037$, $R^2 = 0.140$). Partial separation was achieved between samples according to temperature, with 6 months ambient being discriminated from 6 months cold (Fig. 3.9). However, 3 months ambient and 3 months cold samples were not separated.

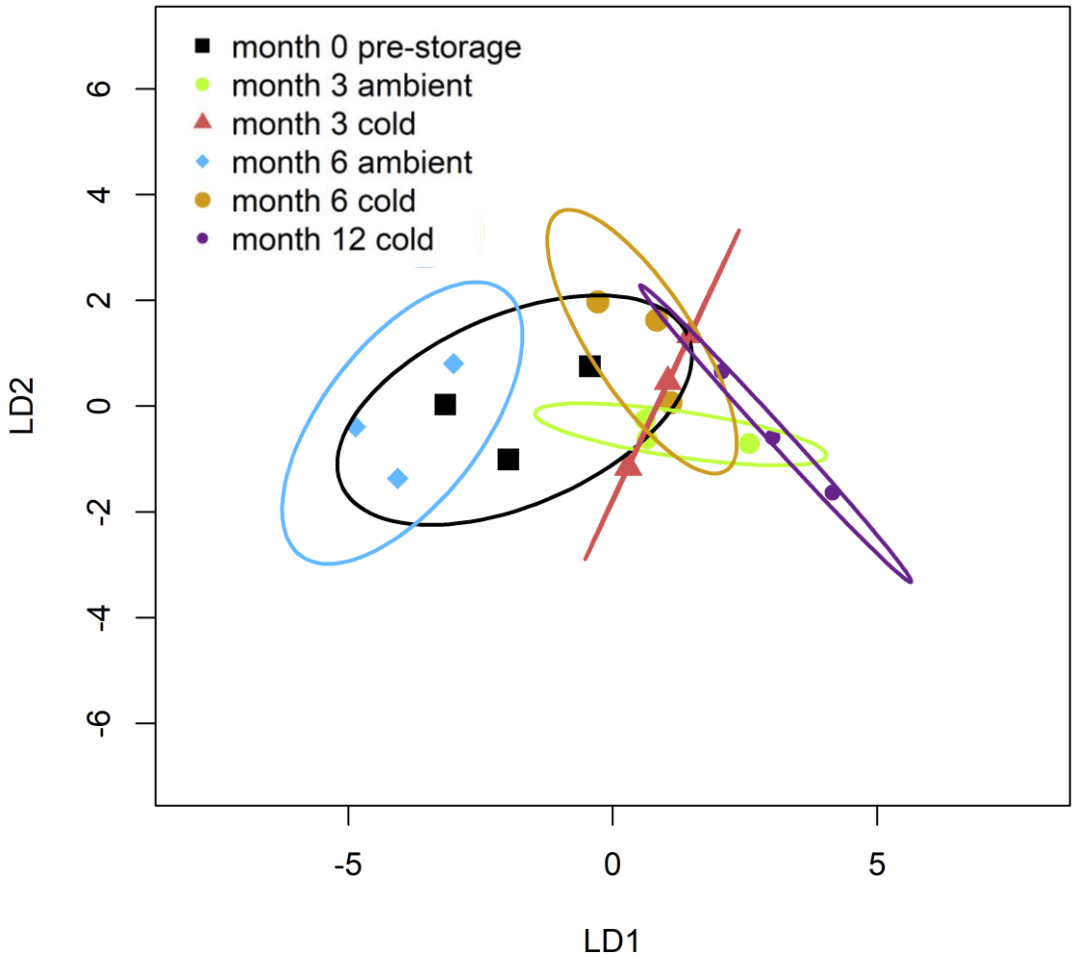


Figure 3.9: Linear discriminant plot from CAP analysis of organosulphur VOCs. Time and temperature were combined into a single category of time x temperature for the analysis. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 44.4 % at p = 0.05 (percent successful classification by group: 0 months = 33.3 %, 3 months ambient = 66.6 %, 3 months cold = 33.3 % 6 months ambient = 66.6 %, 6 months cold = 0 % 12 months cold = 66.6 %). N = 3 replicates of 3 bulbs.

3.3.9. Validation of VOCs identified by WCNA as storage markers

The 14 VOCs varying significantly with storage temperature, as identified by WCNA, were significantly different according to storage temperature (PerMANOVA, $P = 0.002$, $R^2 = 0.340$). Furthermore, VOCs discriminated pre-storage samples from ambient and cold stored samples (Fig. 3.10). However, ambient and cold stored samples were not discriminated. The 20 VOCs varying significantly with storage time (Fig. 8b), as identified with WCNA, successfully discriminated between storage time (PerMANOVA, $P = 0.002$, $R^2 = 0.351$). All storage times were discriminated from one another in a CAP model, with discrimination occurring exclusively in the first dimension of the linear discriminants (Fig. 3.11).

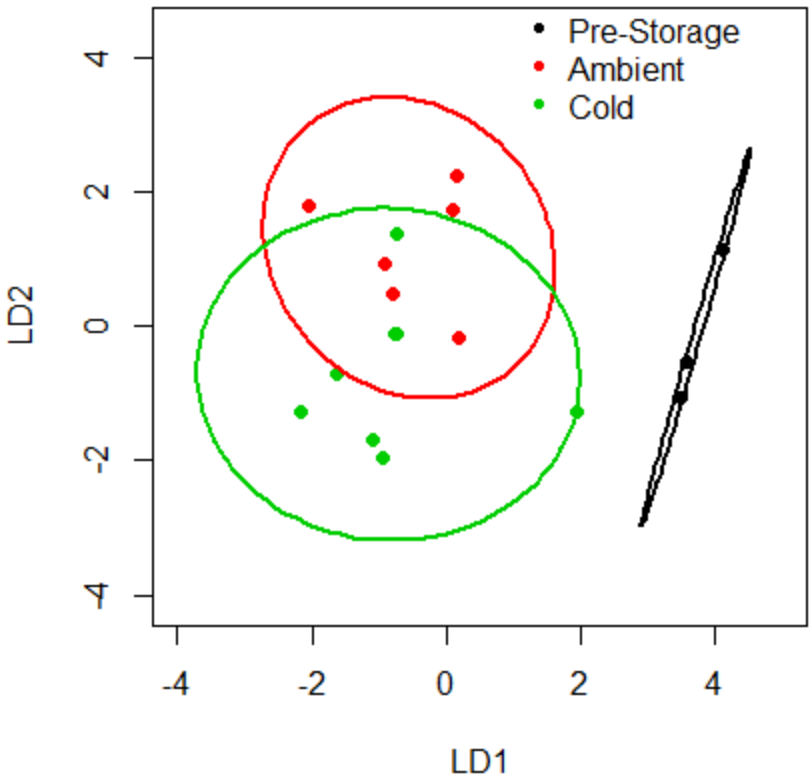


Figure 3.10: Linear discriminant plot from CAP analysis of 14 VOCs found to be significantly associated with storage temperature by WCNA (Fig. 8c). The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 72.2 % at p = 0.05 (percent successful classification by group: pre-storage = 66.6 %, ambient = 66.6 %, cold = 77.8 %). N = 3 replicates of 3 bulbs.

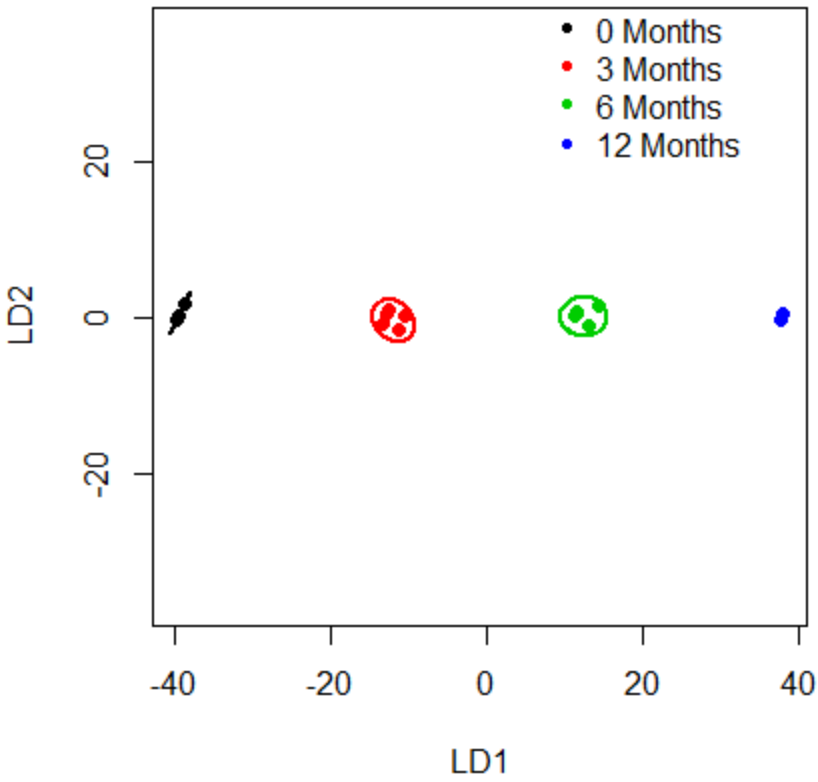


Figure 3.11: Linear discriminant plot from CAP analysis of 20 VOCs found to be significantly associated with storage time by WCNA (Fig. 8b). The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95 % confidence interval and the percentage of correct classification was 100 % at p = 0.05 (percent successful classification by group: 0 months, 3 months, 6 months and 12 months = 100 %).

3.3.10. Multitrait analysis of VOCs to identify markers of alliinase activity

Alliinase activity and proportion of diseased cloves were introduced into the WCNA model to identify VOCs associated with these traits. Traits again fell into seven modules (Fig. 3.12a). Alliinase activity shows a similar pattern in relation to storage temperature based on the cluster dendrogram to 1-allyl-2-isopropyl disulphide and di-2-propenyl trisulphide (Fig. 3.12c). These two VOCs show a clear difference in abundance between cold and ambient stored garlic suggesting that they may be useful markers for storage condition. However, there are no VOCs which share the gradual and consistent reduction seen in alliinase activity over time of storage (Fig. 3. 12b).

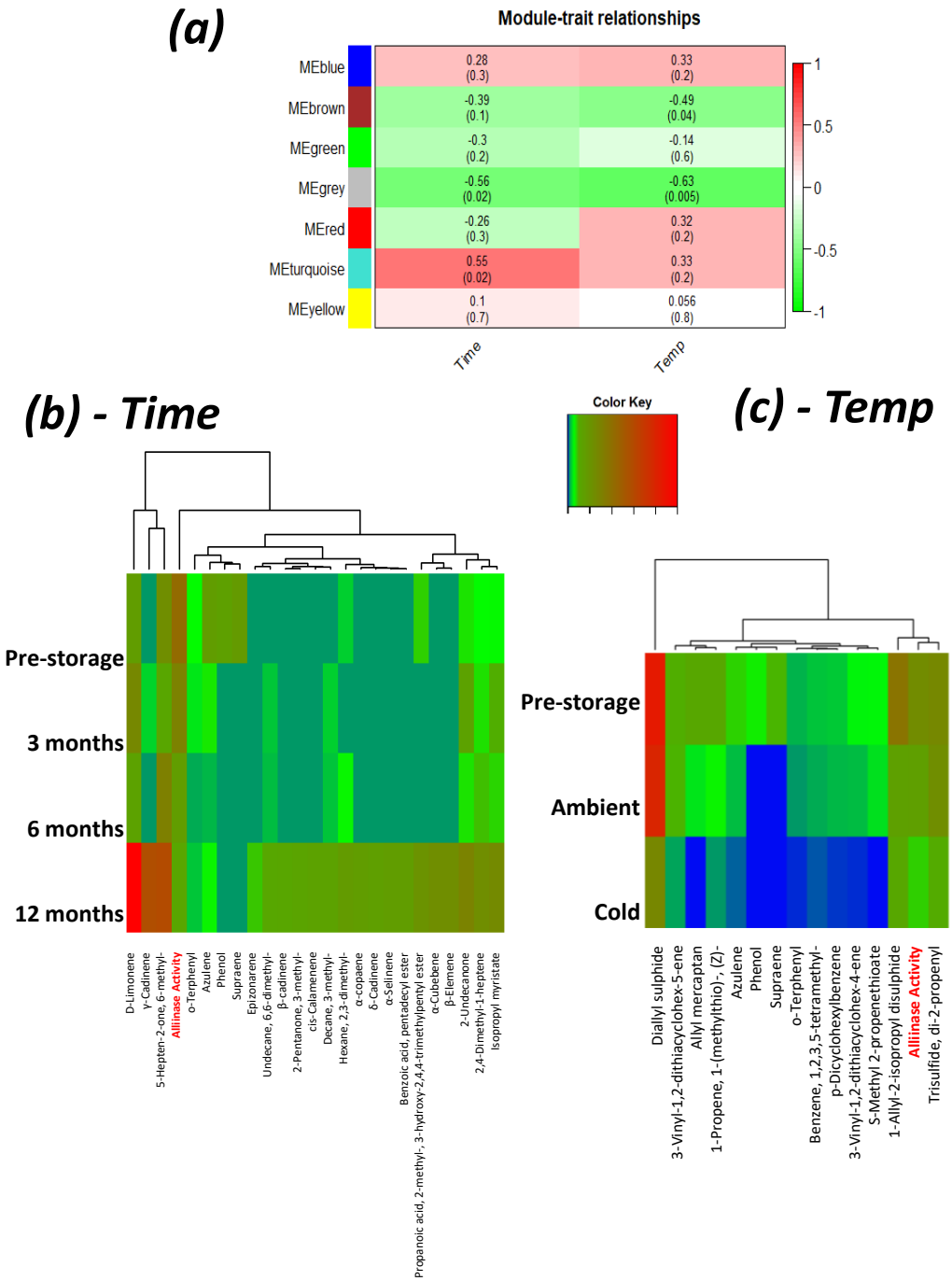


Figure 3.12: Multitrait analysis of VOCs and alliinase activity from garlic cv. Altanero stored for 0, 3, 6 and 12 months at ambient (22 °C) and cold (-2 °C). **(a)** WCNA modules: score and significance according to Pearson analysis (P values in brackets; red, positive and green, negative correlations with variable). **(b)** and **(c)** heat maps of significant VOCs in significantly correlating WCNA modules according to **(b)** storage time. **(c)** temperature. (blue low, green intermediate and red, high VOC abundance).

3.4. Discussion

3.4.1. Cold storage reduces disease and water loss but accelerates loss of alliinase activity

Cold stored garlic did not exhibit a significant increase in disease from pre-storage levels, even after 12 months of storage but ambient stored garlic showed a dramatic increase in disease incidence between 6 and 12 months. This supports findings in the literature, where common spoilage organisms such as *Penicillium hirsutum*, are known to have increased infectivity and growth rates at ambient temperatures (Bertolini & Tian, 1996). *Penicillium hirsutum* was shown to have faster rates of spore germination, *in vitro* mycelial growth and infectivity to both wounded and unwounded garlic. Colonisation of unwounded cloves was significantly reduced from an average of 23% to 0% when the temperature was reduced from 20 °C to -2 °C over a 4 month storage period. In the data presented here, whilst ambient stored garlic did have a greater disease incidence when compared to cold stored garlic, it took 12 months instead of 4 months for this effect to be seen. This can be attributed to several factors. Firstly, unlike the bulbs studied by Bertolini & Tian (1996), the bulbs studied in this thesis were not deliberately infected with any spoilage organisms at the start of the study, the reduced pathogen load may explain the delayed onset of disease in ambient stored garlic. Secondly, here the garlic bulbs were kept whole and a degree of protection may be conferred by having an intact clove and bulb sheath. Thirdly, humidity levels here were considerably lower, at 65 % ± 10 % RH as opposed to 85-90 % RH in the Bertolini & Tian (1996) study. This is important as a relative humidity over 70 % RH favours fungal spoilage organisms (Shiina *et al.*, 2005).

Although ambient storage reduced the yield of crude alliinase extract, alliinase activity remained higher after 6 months of storage compared to cold stored bulbs. Thus over a storage period of below 6 months, ambient storage may be preferable to cold storage for garlic specifically targeted to biotechnological applications, with reduced energy costs.

The increased retention of alliinase may also be of relevance to the food industry, since alliinase is essential for the conversion of alliin to allicin and subsequent breakdown to flavour components (Jones *et al.*, 2007). Whilst cold storing garlic is currently required to provide a year-round supply of garlic, ambient temperature storage may provide better flavour for high value niche markets. Whilst the alliinase activity of extracts from garlic bulbs has not been monitored through storage before, storage of onions for ~7 months at ambient temperature did not significantly affect alliinase activity (Hanum *et al.*, 1995). However, it is unclear how similar garlic alliinase activity maintenance over time in storage is to onion alliinase, nor were the effects of cold storage considered by Hanum *et al.* (1995).

3.4.2. Water content

Throughout storage, the water content of the garlic across both temperatures remained remarkably constant. However, weight loss from garlic bulbs throughout storage is reported (Croci & Curzio, 1983). Weight loss may occur due to respiration and desiccation (Ward, 1976). Further research confirmed that weight loss cannot be explained by environmental factors such as temperature and humidity alone, and that sprouting and metabolism played a key role (Vazquez-Barrios, 2006). Additionally, the disruption of the epidermis by shoot and root growth out of the clove could increase the rate of water loss (Fraga, 1998). However, here only one replicate of 3 months ambient stored bulbs showed 13.7 % sprouting, with all other samples at both temperatures having no sprouted cloves (Supplementary Fig. S3.3). This may mean that the increase in the proportional rate of water vs dry matter loss from sprouting as reported by Fraga (1998) did not occur here, as most samples were free from sprouting. Therefore, evidence is presented here that in healthy, non-sprouting garlic, water loss occurs at a rate

proportional to the loss of carbon through respiration in a ~ 1:2 dry matter to water ratio, consistent with the ratio of dry matter to water content in the bulbs.

3.4.3. Storage conditions affect alliinase activity and VOC profiles but not cysteine synthase gene expression

Storage time elicited significant changes in some components of the VOC profile, with all time points being discriminated from one. However, the reasons for this change remain unclear as disease incidence did not increase significantly in cold stored samples, nor are VOC changes mirrored by a change in alliinase activity which did not change significantly over this period. Alliinase activity fell during storage from 0 to 6 month at both storage temperatures, suggesting that storage time is a stronger determinant of alliinase activity than storage temperature.

The alliinase primers used here were non-specific, indeed they are homologous to all nine alliinase genes from SV2-SV10 as identified by Ovesná et al. (2015). However, we did not see a single amplicon, which may suggest heterogeneity in the genes amplified, or potential off-site amplification. It is possible that more specific primers would therefore reveal changes in alliinase expression that were not detectable here.

3.4.4. VOCs

A broader range of VOCs from garlic is reported here than in previous studies, with a total of 117 compounds detected, which may be attributed to the high sensitivity and dynamic range of the sampling method and detector (Lee *et al.*, 2003). The ability to concentrate volatiles from a large amount of headspace onto the sorbent material of the TD tubes allowed for minor VOC constituents to be detected. The high dynamic range afforded by analysing samples at two split ratios, coupled with the high sensitivity of the ToF-MS also aided in the detection of minor VOCs and relative quantitation of major VOCs. Furthermore, where some studies focussed on the organosulphur component of the VOC profile, this thesis examined all VOCs of garlic and identified compounds from a broader range of chemical groups than the literature (Supplementary Table S3.3). In comparison to the 117 compounds detected and putatively identified here, fewer were found in the literature: Calvo-Gómez*et al.* 2004 reported 47 compounds, of which 8 were concurrent with this chapter; Yu *et al*. (1989) reported 35 compounds, of which 9 were concurrent; Lee *et al.* (2003) reported 21 compounds, of which 8 were concurrent; Vernin *et al.* (1985) reported 7 compounds, of which 5 were concurrent; and Molina-Calle *et al.* (2016) reported 45 compounds, of which 13 were concurrent (Supplementary Table S3.4).

Some studies reported VOCs of garlic distillates and extracts after maceration and heat treating and subsequently saw several organosulphur VOCs which we did not, probably due to the extensive synthesis of allicin and a more complete thermal degradation process. These included allyl methyl trisulphide (Vernin *et al.*, 1985); thiazole, 1-propenyl methyl disulphide and methylthiocyclopentane (Calvo-Gómez *et al.*, 2004); and 1-(1-propenylthio)propane, 5-methyl-1,2,3-thiadiazole and diallyl tetrasulfide (Lee *et al.*, 2003).

Allicin was not detected in any of the samples here. This may be due to the labile nature of allicin (Freeman & Kodera, 1995; Chong *et al.*, 2015) but also the non-destructive sampling method employed, which minimised allicin production by the bulb. However, allicin is highly abundant in the VOC profile of crushed garlic (Block, 2010). Indeed, experiments performed by HPLC (Bocchini *et al.*, 2001) and supercritical fluid extraction liquid chromatography atmospheric-pressure chemical ionisation mass spectrometry (SFE-LC-APCI-MS) detected allicin as the major component of the VOC profile (Calvey *et al.*, 1997). Furthermore, some studies reported detecting allicin using GC-MS, however the yield was low, with most of the allicin reacting to diallyl disulfide and vinyldithiins, which constituted the most abundant components of the VOC profile (Mondy *et al.*, 2001). However, Mondy *et al.*, 2001 crushed the sample before sampling, thus releasing allicin in greater quantities.

The major VOC constituents vary between studies according to extraction method (Lee *et al.*, 2003). Here, diallyl disulphide was the most abundant VOC, accounting on average for 37 % of the total abundance across all samples, which is lower than HS-SPME, in which diallyl disulphide accounted for 58 % of the (Lee *et al.* 2003). Diallyl disulphide accounted for 88 and 98 % of the total in steam distillation and solid-phase trapping solvent extraction, respectively (Lee *et al.*, 2003). Furthermore, changing the coating of the SPME fibre caused significant differences in compound abundance, with the abundance of diallyl trisulphide and allyl sulphide varying by ten and twenty times their relative abundance between fibres (Lee *et al.*, 2003). Also detected were non-natural breakdown products of allicin, namely 3-Vinyl-1,2-dithiacyclohex-4-ene and 3-Vinyl-1,2-dithiacyclohex-5-ene, which were previously reported in solvent extraction mass spectrometry, and arise due to decomposition under the conditions of the GC column (Brodnitz, 1971).

The fact that organosulphur compounds associated with storage temperature were less abundant in cold stored garlic (12.0 % to 3.3 %), may suggest a lower rate of allicin synthesis as these are principally breakdown compounds of allicin (Brodnitz *et al.*, 1971). Alliinase activity decreased more rapidly in cold stored garlic, which may account in part for this reduction. The other possible explanations are that alliin concentrations within the bulb decreased or that the cellular compartmentalisation of alliinase and alliin were better maintained at cold temperatures. Alliin concentration appears to largely stay constant or increase in concentration through storage. In fact, alliin concentration was shown to increase in the clove throughout ambient storage, more than doubling over 83 days (Bloem *et al.*, 2011), whilst Hughes *et al.* (2006) found alliin remaining largely unchanged after 6 months of cold storage and Ichikawa *et al.* (2006) reported no significant changes from pre-storage levels in garlic stored at -3 °C or 23 °C across 9 time points over 150 days storage. Ichikawa *et al.* (2006) did, however, report a significant increase in alliin concentration at 90, 120 and 150 days compared to pre-storage levels in the same garlic cultivar when stored at 4 °C. Pre-storage factors including fertilisation with sulphur have been shown to increase alliin content of garlic (Huchette *et al.*, 2004) and there is also a genetic and environmental influence on alliin concentration (Montaño *et al.*, 2011). Data is presented here that show no clear change in cysteine synthase expression, however not all of the biosynthetic steps of alliin were considered, so it is unclear if this would correspond to alliin concentration. It may be the case that lower rates of internal sprouting in cold stored garlic as reported by Pellegrini & Orioli, (2000) reduced tissue damage and allicin synthesis.

In conclusion, alliinase activity fell significantly over time at both storage temperatures, however cold storing garlic lead to a more rapid decrease in alliinase activity, suggesting cold storing may not be optimal for alliinase activity maintenance. VOCs did not correlate with alliinase activity, which may suggest that activity loss is not a result of a failure to

compartmentalise alliin and alliinase, otherwise an increase in organosulphur VOCs would be expected. Disease rates increased significantly in ambient storage only after 12 months of storage. The water content of bulbs did not change substantially over time in storage at either temperature and no time points were significantly different from pre-harvest levels. VOCs could be used to discriminate between storage time using a CAP model of compounds identified from WCNA, however they could only partially discriminate between storage temperature. It is not clear whether the findings presented here are applicable to different cultivars of garlic, or stable across multiple growing seasons; this is explored in Chapter 4.

4. Chapter 4: Varietal and Seasonal Variance in Stored Garlic

4.1. Introduction

Garlic exhibits a high degree of variation in key pre-harvest agronomic traits such as bulb size, yield, flavour and colour, which are influenced by cultivar and cropping year (Volk & Stern, 2009; Waterer & Schmitz, 1994). However, less is known about how alliinase activity varies across cultivar or cropping year, or how they affect post-harvest quality. This presents difficulties in accurately predicting quality traits across different cropping years or amongst cultivars.

Planting method and cultivar has been shown to have significant impacts on average bulb weight, yield (t/ha), the number of cloves per bulb and revealed interactions between planting method and cultivar, with some cultivars performing better under different planting regimes (Haque *et al.,* 2002). As well as changes in physiological parameters of garlic, the biochemistry of garlic changes too with cultivar, year of harvest and cultural practises. Changes in soil sulphur levels from 6.45 mg kg^{-1} to 8.41 mg kg^{-1} (30.4 % increase) increased allicin levels by 33.7% and likewise increased soluble sugar and soluble protein concentrations in garlic (Yang *et al.*, 2004). Similarly, changes in soil zinc availability altered soluble protein and free amino acid content (Yang *et al.,* 2005). This suggests that even subtle changes in availability of plant micronutrients in soil have the ability to cause significant changes to the bulb biochemistry and nutritional quality and may provide a mechanism for observed differences in biochemical quality of the same cultivar grown at different locations.

The diversity of S-alk(en)yl cysteine sulphoxide content varies amongst different garlic cultivars, specifically between bolting and non-bolting cultivars (Ovesná, *et al.*, 2011).

Furthermore, the biochemistry of phenolic compounds of garlic has been shown to change with cultivation location and genotype in garlic grown in Spain (Beato *et al.,* 2011). Whilst a difference was not detected between white and purple or white and Chinese garlic cultivars, a significant difference was found in total phenolics and ferulic acid between Chinese garlic cultivars and purple garlic cultivars (Beato *et al.,* 2011). This suggests that genotype has significant effects on a broad range of medicinally relevant organosulphur and phenolic compounds in garlic. Furthermore, there was a significant effect of planting location on caffeic, vanillic, p-hydroxybenzoic, and p-coumaric acids (Beato *et al.,* 2011). In that study, garlic was grown in the south of Spain, in four locations (Córdoba, Santaella, Granada and Mengíbar), all of which were 37°N, more southerly than the planting location used for the garlic used in this study of 39 °N. Despite the modest distance between these farms and the identical agronomic treatment, temperature and rainfall varied between sites, which may account for some of the differences observed. However, organosulphur compounds and alliinase activity were not studied by Beato *et al.* (2011) and so it is unclear if they too would have varied according to planting location.

In this chapter, the effect of inter-cultivar variance on postharvest quality was studied in three cultivars of garlic: two were Spanish hard-necked purple cultivars (‘Morado’ and ‘Violeta’) and one was a Chinese soft-necked white cultivar (‘Blanco’). Furthermore, these cultivars were grown on the same farm in a second, consecutive year to explore the interseasonal variance from one cropping year to another. Emphasis was placed on alliinase activity maintenance over time at two storage temperatures and traditional quality measures such as disease, sprouting and firmness were also measured as well as the effects of cultivar, season and storage temperature on VOC profiles.

4.2. Materials and Methods

4.2.1. Plant Materials and Growth Conditions

Garlic *"Morado, Blanco"* and *"Violeta"* was grown by Coopaman SCL, a garlic farming cooperative in the Las Pedroñeras, Spain (39° 27' North, 2° 40' West), a region with protected geographical indication (PGI) on garlic products. All garlic cultivars over both years were grown in the same ~2,000 ha farmland of the cooperative and were grown with the same practises and fertiliser applications. Irrigation was supplied to the crops in accordance with recommendations from the regional irrigation advisory service, SIAR (CREA, 2016). The soil type was a sandy loam and is broadly consistent over the farms. Bulbs were of Category I quality, having been inspected for disease and damage, and 60-65 mm in diameter. They were sourced after harvest, drying and processing at their facility, prior to cold storage. Plants were lifted from the ground and left to dry in the field for 1-2 days before being transported to the processing facility where they were trimmed, cleaned of loose sheath, inspected and size graded. After this, they were collected into open-sided euro-pallet crates and left in a well-ventilated area to complete the drying process.

Planting began in early September and was completed in October for all varieties, with the early maturing *Blanco* and *Violeta* sown first, but no delay in planting occurred amongst cultivars. The garlic used in season one was planted in 2016 and harvested in July-August 2017 and season two was planted in 2017 and harvested in July-August of 2018. The overall time it takes to sow the crops varies between farms slightly due to the methods and machinery used to plant the bulbs and their respective areas being planted. Year to year variation in planting duration also exists as the cloves cannot be planted in heavy rain.

Irrigation was supplied to all fields in accordance with recommendations from the regional irrigation advisory service, Servicio Integral de Asesoramiento al Regante de

Castilla La-Mancha (SIAR) (CREA, 2016). Irrigation began immediately after sowing, where cloves are watered to help them establish in the soil and was maintained throughout the growing season until bulbs began to dry before harvest. Early maturing crops have a high water demand in February and March and later maturing crops having highest demand in March-April, corresponding with rapid vegetative growth and during this time, water was readily supplied.

4.2.2. Clove Firmness

Clove firmness was measured with a Bishop model FT 327 Fruit pressure Tester (Facchini SRL, Italy), using a 0.5 cm^2 penetrometer tip. The outer sheath of the clove and the upper epidermis of the clove were removed with the peeler provided with the penetrometer. Cold stored garlic was returned to room temperature over night before testing. The pressure probe was slowly pushed into the peeled area at a uniform rate, midway from the basal plate to the tip of the bulb, angled directly towards the centre of the clove. The maximum force exerted to push the probe 5 mm into the clove was reported in kilogram force (kgf), as per industry standards, where 1 kgf = 9.807 N.

4.2.3. Garlic Storage

Bulbs were stored in a large cardboard box with internal dividers between cultivars at -2 °C and ambient temperature for 0, 3, 6 and 12 months commencing in September 2017 for season one and September 2018 for season two. Light was excluded and temperature and humidity were monitored periodically through the storage period, as outlined in Chapter 2.2.

4.2.4. Bulb weight loss over time

Ten whole, undamaged bulbs of garlic were selected at random from each cultivar of garlic. Five were stored at ambient conditions (20 °C ± 1 °C) and five -2 °C cold stored (-1.5 °C ± 0.6 °C). Each bulb was numbered with a permanent marker and weighed at the start of the experiment and again after 3 and 6 months of storage at their respective temperature.

4.2.5. VOC collection and analysis

Three bulbs were selected at random and placed into a Nalophan sampling bag as detailed in mathods chapter 2.4. VOCs were collected on TD tubes (Tenax TD/Sulficarb Tubes, Markes International). After an equilibration time of 2 h, 7.5 L of headspace was sampled through the TD tube at a rate of 60 mL/min using a "SpeediVac" High Vaccum Pump (Edwards High Vacuum Ltd, Crawley, UK), with tubes connected via a Q-Max Tube Holder (Supelco, USA) and flow rate calibrated with a GAP METER flow meter (GAP, Croydon, UK).

TD tubes were desorbed using a TD100 thermal desorption system (Markes International Ltd., Llantrisant, Wales, UK) using TD, GC and MS settings outlined in methods chapter 2.10.

GC–MS data were processed in ChemStation (E.02.01.1177; Agilent Technologies Inc., Stockport, UK) then deconvoluted and integrated with AMDIS (NIST, 2011), using a custom retention-indexed mass spectral library. MS spectra from deconvolution were searched against the NIST 2011 library (Stein, 2008). Compound identifications were based on match of mass spectra (>80%) and retention index (RI ± 15) (Beaulieu & Grimm, 2001).

To normalise the data, the integrated signal of each compound was expressed as a percentage of the total signal for each sample. Where a compound was not detected in a sample, the missing values were replaced with an abundance 1/5th of the minimum detected across all compounds and samples., as zero-inflated data can confound some of the statistical methods used in subsequent analysis. Finally, data were square root transformed to reduce bias towards highly abundant compounds in subsequent statistical tests.

GC-MS data were analysed in R and MetaboAnalyst (R Version 3.4.2, R Core Team, 2019; MetaboAnalyst 3.0, Xia et al., 2015). Permutational multivariate analysis of variance (PerMANOVA) was performed to identify differences in the VOC profile associated with experimental parameters, using the function 'adonis' in the "vegan" package in R (Oksanen et al., 2019). Canonical analysis of principal coordinates discriminant analysis (CAPdiscrim) was performed in the "vegan" package in R to assess the significance of class discrimination according to experimental parameters (Legendre & Anderson, 1999; Anderson & Willis, 2003). Weighted correlation network analysis (WCNA) was performed in the "BiodiversityR" package in R to identify components of the VOC profile which varied most significantly between factors (Langfelder & Horvath, 2008).

4.2.6. Alliinase Extraction

Alliinase enzyme was extracted from 50 g garlic using an acid precipitation extraction method developed by Neem Biotech and adapted here to work more efficiently at low scale. Garlic was macerated in water, filtered and centrifuged to remove particulate matter before the pH of the supernatant was lowered to pH 4, precipitating the enzyme. The pollution was centrifuged into a pellet, the supernatant discarded and the pellet freeze dried, as per methods outlined in Chapter 2.5.

4.2.7. Protein Concentration and Alliinase Activity Assay

Protein concentrations were assessed with Bradford assays, as outlined in Chapter 2.7. Alliinase activity was determined spectrophotometrically through the reaction between 4-MP (λmax = 324 nm) and allicin, forming 4-allylmercaptothiopyridine, which has no absorbance in this spectral region, as per methods detailed in Chapter 2.6 (Miron et al. 2002).

4.2.8. Physiological Assessment

Disease incidence was assessed with methods outlined in chapter 2.9. and bulb moisture content was measured in accordance with chapter 2.8. The number of sprouting cloves was assessed by visual inspection and a clove was considered to be sprouting if it had a shoot protruding more than 1 mm from the apical tip of the clove.

4.2.9. Statistical analysis

Physiological and enzymatic data were assessed using a generalised linear model, using the "glm" statistical package within the R statistical interface V 3.4.2 R Core Team (2019).

4.3. Results

4.3.1. Clove firmness

Clove firmness was measured in the second season and varied significantly amongst storage times and cultivars ($p = 0.006$ and $p = 0.012$ respectively). There were significant interactions between variables, with time influencing the effect of temperature ($p < 0.001$), time influencing the effect of cultivar ($p = 0.032$) and temperature influencing the effect of cultivar ($p < 0.001$). Firmness in 'Blanco' decreased over time at both temperatures, with a significant loss occurring after 3 months in ambient storage and 6 months in cold storage (Fig. 3). Under cold storage, 'Blanco' had a significantly lower firmness across all storage time points than either 'Morado' or 'Violeta' ($p = 0.034$). 'Morado' and 'Violeta' both display the opposite trend at ambient compared to cold storage (Fig. 4.1). At ambient storage, firmness falls significantly after 3 months before returning to pre-storage levels in 'Violeta' and becoming significantly firmer than pre-storage in 'Morado' (Fig. 4.1). Conversely, in cold storage, firmness increases significantly in both cultivars at 3 months before falling to pre-storage firmness at 6 months (Fig. 4.1).

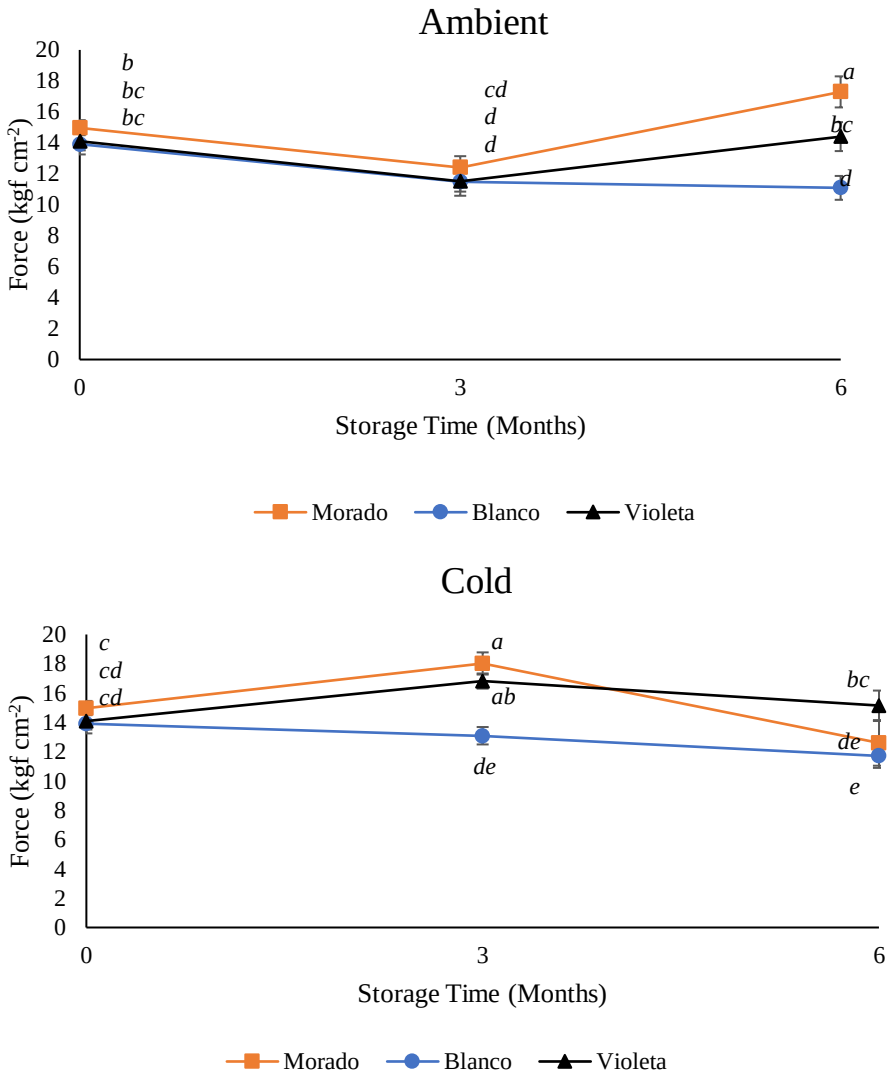


Figure 4.1: Force required to penetrate cloves of garlic with a pressure-testing probe, stored at ambient and cold conditions for 0, 3 and 6 months. Letters indicate statistically different values between the different cultivars and time points, based on ANOVA and LSD, alpha = 0.05, N = 3 cloves in triplicate, error bars = SD.

4.3.2. Bulb Weight Loss

Bulb weight loss was measured in the second season, and bulbs lost significant amounts of weight over time in storage ($p < 0.001$, Fig. 4.2). There was a significant cultivar effect, with ‘Blanco’ losing a higher proportion of its weight than ‘Morado’ or ‘Altanero’ in ambient conditions ($p < 0.001$; Table 4.1). Weight loss occurred to a greater extent in ambient stored garlic than cold stored over the 6 month period ($p < 0.001$; Table 4.1).

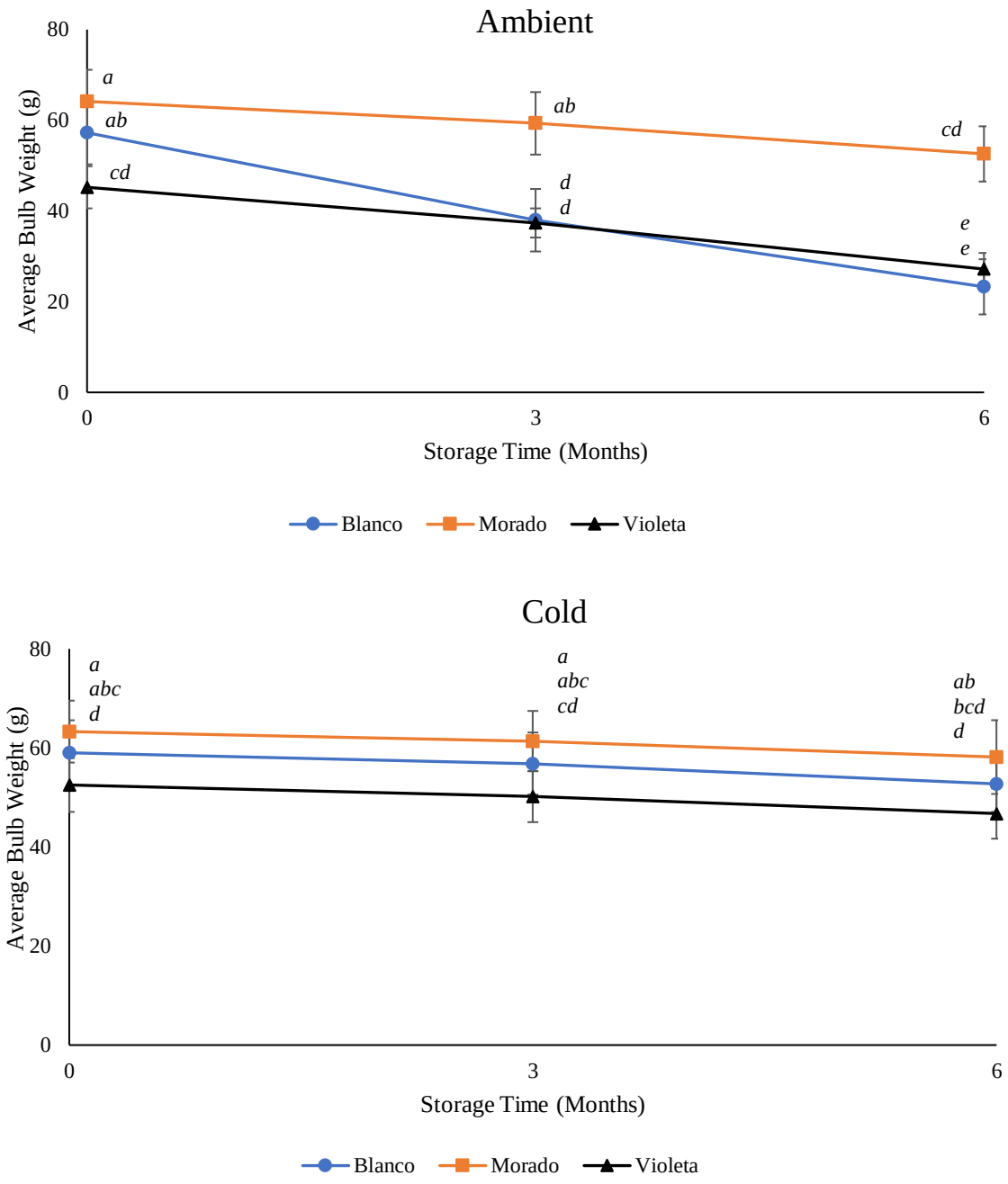


Figure 4.2: Fresh weight of garlic bulbs, stored at ambient and cold conditions for 0, 3 and 6 months over one storage season. Letters indicate statistically different values between the different cultivars and time points, based on ANOVA and LSD, alpha = 0.05, N =3 bulbs, error bars = SD.

Table 4.1: Percent weight loss of garlic bulbs in ambient and cold storage over a 6 month period.

		Ambient		Cold	
		First 3 Months	Second 3 Months	First 3 Months	Second 3 Months
Weight Loss	Blanco	33.7 %	38.7 %	3.7 %	7.2 %
	Morado	7.5 %	11.4 %	3.0 %	5.3 %
	Violeta	17.4 %	27.2 %	4.5 %	6.8 %

4.3.3. Bulb Water Content

Bulb water content (WC=(FW-DW)/FW) remained remarkably constant throughout the storage period in both harvest seasons, across cultivars and storage temperatures, despite the loss of weight. Water content for the first experimental season was 62.7 % across all samples, whilst for the second season it was 63.1 %. Cultivars had significantly different water contents, with 'Blanco' averaging 64 % 'Morado' 63.0 %, and 'Violeta' 61.7 % ($p = 0.003$). Ambient stored garlic had slightly lower water content, of 61.8 % vs 63.7 % in cold storage, but the effect of storage temperature on water content was not significant ($p =0.28$). Water content fell from 63.7% at 0 months to 63.4 % by 3 months and 62.1 % by 6 months over storage time, irrespective of temperature ($p < 0.001$).

4.3.4. Disease

Disease rates varied significantly between cultivars and were significantly higher in ‘Blanco’ when stored at ambient conditions ($p = 0.016$; $p < 0.001$) (Fig. *4.3*). Across both years, disease rates were low throughout the storage period in cold stored garlic, with only ‘Blanco’ experiencing a very slight although statistically significant increase in rate of disease after 3 months of cold storage in season two ($p < 0.05$).

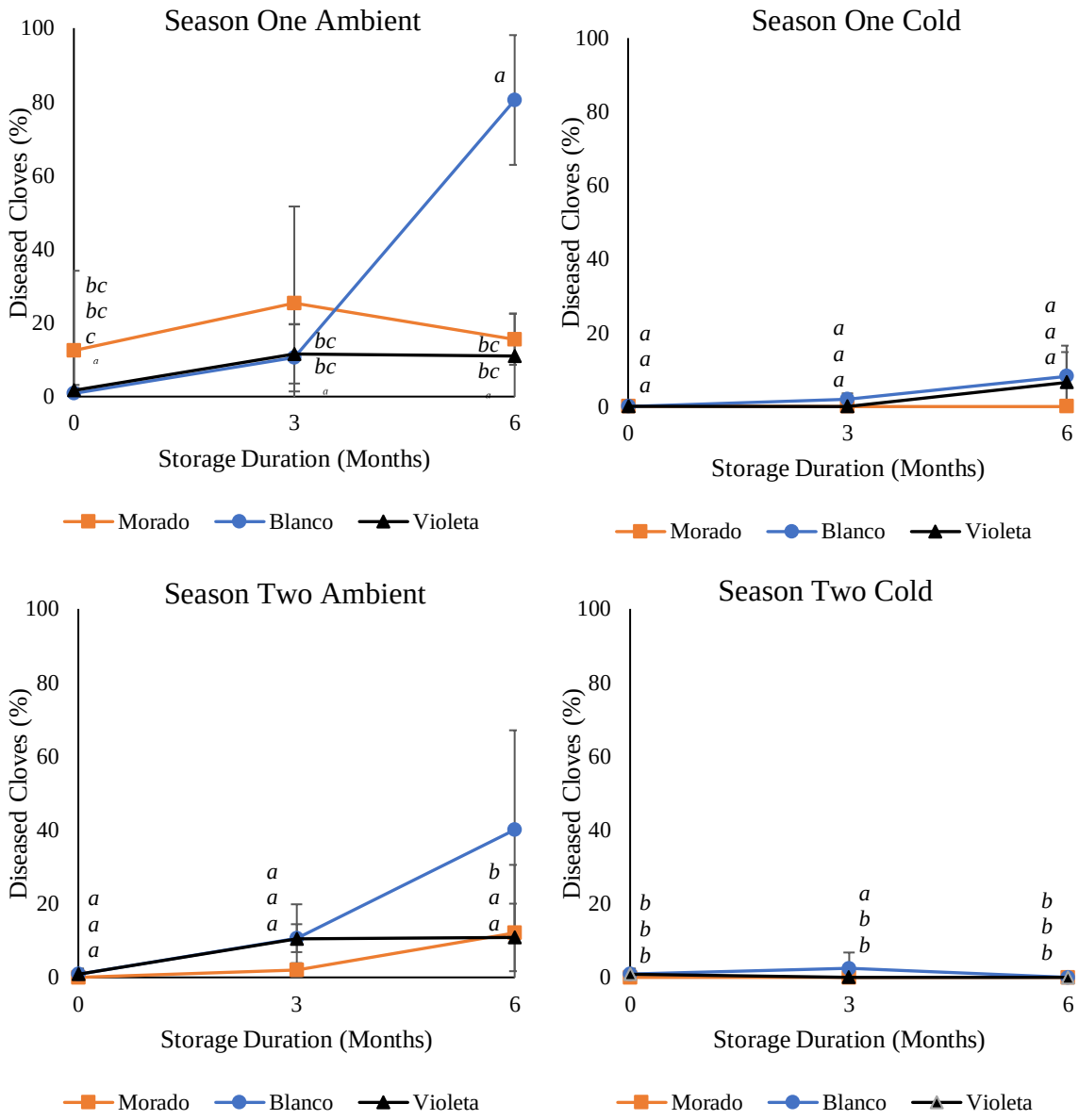


Figure 4.3: Prevalence of disease in garlic bulbs, stored at ambient and cold conditions for 6 months over two storage seasons. Letters indicate statistically different values between the different cultivars and time points, based on ANOVA and LSD, alpha = 0.05, N =3 bulbs, error bars = SD.

4.3.5. Sprouting

All three cultivars across both storage years showed no sprouting prior to storage treatment (*Fig. 4.4*). Temperature and cultivar had significant effects on the occurrence of sprouting ($p < 0.001$; $p = 0.009$), whereas storage time and year of harvest did not have a significant effect on sprouting ($p = 0.091$; $p = 0.075$). Garlic cloves stored at ambient conditions did not exhibit a significant increase in sprouting after 6 months. However, *Blanco* in season one and *Morado* in season two had a subtly but significant higher occurrence of sprouting at 3 months. In cold stored garlic, *Blanco* sprouted significantly more in season two after 3 months of storage, and after 6 months storage in season two. *Violeta* exhibited significantly higher proportions of sprouting in season one after 6 months of cold storage. The incidence of sprouting did not exceed 20% of the total sample at any treatment. Sprouting was assessed on the same bulbs used for VOC analysis and is not intended as a proxy for the entire batch of stored garlic.

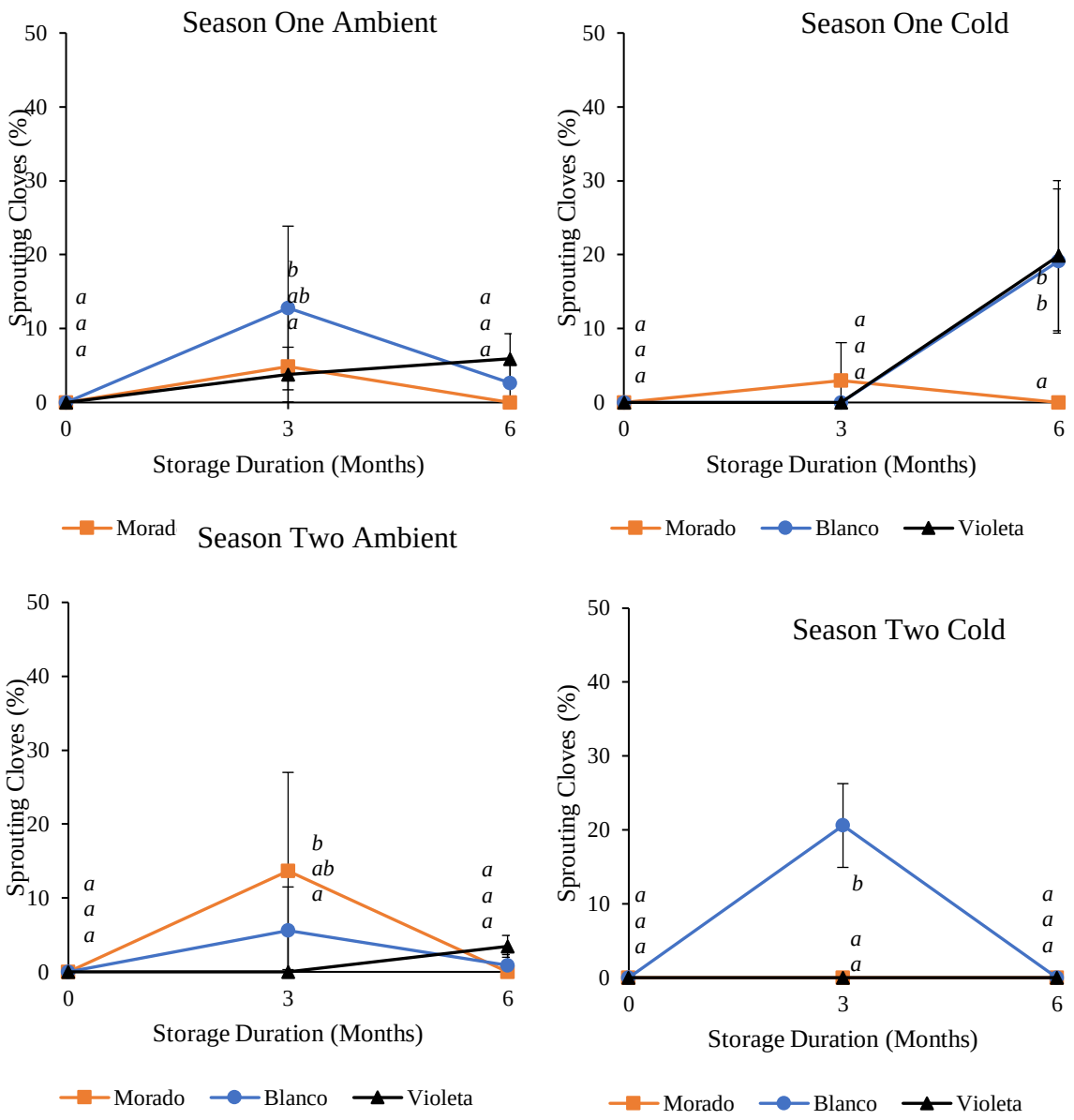


Figure 4.4: Percent sprouting cloves from three garlic bulbs, stored at ambient and cold conditions for 6 months over two storage seasons. Total number of cloves ranged from 24 to 61 per replicate. Letters indicate statistically different values between the different cultivars and time points, based on ANOVA and LSD, alpha = 0.05, error bars = SD.

4.3.6. Alliinase Activity

Change in alliinase activity over time varied significantly between the two seasons. In the first season alliinase activity immediately after harvest was significantly higher than in the second experimental year, where samples had on average a 4-fold lower activity level at harvest ($p < 0.001$). Alliinase activity fell significantly after the first 3 months of storage in both cold and ambient storage in the season one ($p < 0.05$). The activity continued a downward trend at both storage temperatures between 3 and 6 months, with the loss of activity remaining linear in *Violeta* and *Blanco* ($R^2 > 0.989$ & $R^2 > 0.990$ respectively). In the season two, the activity started at a much lower value and did not follow the same downward trend as in season one. There was little change in the activity of extracts across time and alliinase activity was marginally higher than after 6 months in season one (*Fig.* 4.5).

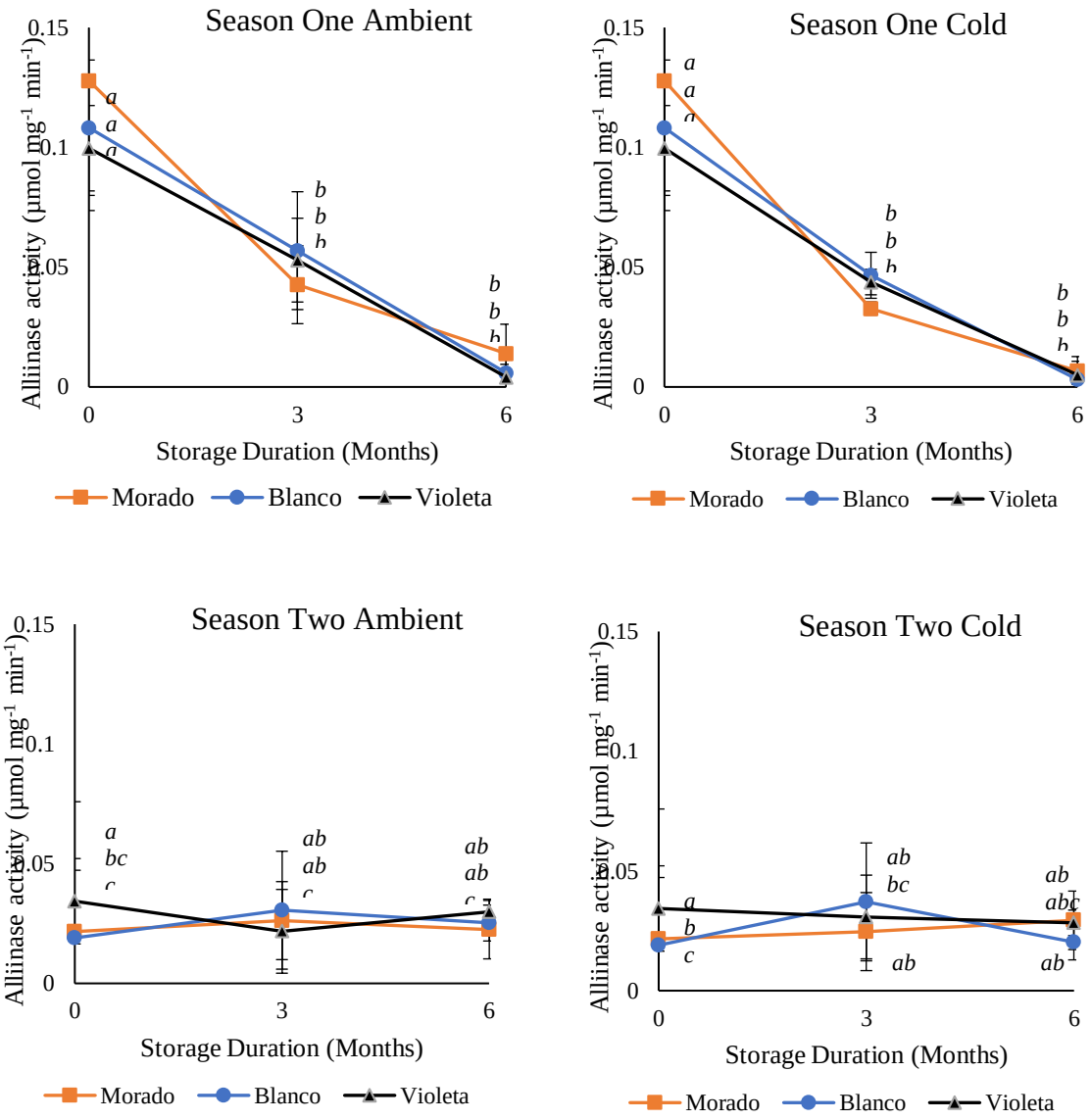


Figure 4.5: Alliinase activity of extracts from garlic bulbs stored at ambient and cold conditions for 6 months over two storage seasons. Letters indicate statistically different values between the different cultivars and time points, based on ANOVA and LSD, alpha = 0.05, N =3, error bars = SD.

4.3.7. Volatile Profile

A total of 223 VOCs were quantified across all samples, of which 221 were putatively identified by comparison of mass spectra to NIST library and retention index data. Major compound groups included organosulphur, alcohols, alkanes, alkenes, terpenes, alcohols, esters, cyclic hydrocarbons and organic acids. The VOC profile differed significantly with storage time and between storage temperature, cultivars and cropping year (Table 4.2). Storage temperature accounted for the highest proportion of the variance within the dataset (R^2 = 0.08), closely followed by storage time (R^2 = 0.07), cropping year (R^2 = 0.07) and the interaction between storage time and temperature (R^2 = 0.06). Of all the factors, cultivar had the least effect on VOC profile; however, significant differences were still detected.

Table 4.2: Output of PerMANOVA examining the effect of the factors: storage year, storage time, storage temperature and cultivar on the VOC profile of garlic 'Morado', 'Blanco' and 'Violeta'. All interactions were included in the model and all significant values reported. R^2 = proportion of variance within the dataset explained by variable; Pr (>F) = p significance value, where < 0.05 is considered significant; Residuals = proportion of variance within the dataset not explained by any factor or interaction in the model.

Factor	R^2	Pr (>F)
Year	0. 06997	< 0. 0001
Time	0. 07135	< 0. 0001
Temperature	0. 08187	< 0. 0001
Cultivar	0. 02725	0. 0287
Year : Time	0. 01762	0. 0218
Year : Temperature	0. 03222	0. 0005
Time : Temperature	0. 06254	< 0. 0001
Year : Time : Temperature	0. 02080	0. 0084
Residuals	0. 48644	-

4.3.8. Identification of VOCs that correlate with changes in alliinase activity

Alliinase activity was found to correlate most strongly with allyl methyl sulphide (AMS, C225) in a random forest regression which explained 5 .79 % of the variance in the dataset (*Fig. 4.6*a). A cut-off point was selected at C214 and all compounds above this line (allyl methyl sulphide (C225), 1,4-dicyclohexylbenzene (C206) and methyl propyl sulphide (C214)) were included in an MDS plot of a second random forest analysis, with 60.11 % of the variance of the dataset explained (Fig. 4.6b). Samples discriminated in terms of alliinase activity in the first and second dimension, with samples with high alliinase activity clustering at the negative end of both dimensions (Fig. 4.6b).

Furthermore, C225, allyl methyl sulphide (AMS), stood out as having a particularly high predictive score (Fig. 4.6a). When the abundance of AMS was plotted against alliinase, samples with an alliinase activity below 0.074 μmol mg^{-1} min^{-1} did not have detectable levels of AMS. Samples with an alliinase activity above 0.074 μmol mg^{-1} min^{-1} always had a detectable level of AMS (Fig. 4.7). However, once detectable, there was no simple relationship to predict alliinase activity from AMS relative abundance. Nine samples had high alliinase activity and detectable levels of AMS whilst 81 had lower alliinase activity without detectable levels of AMS.

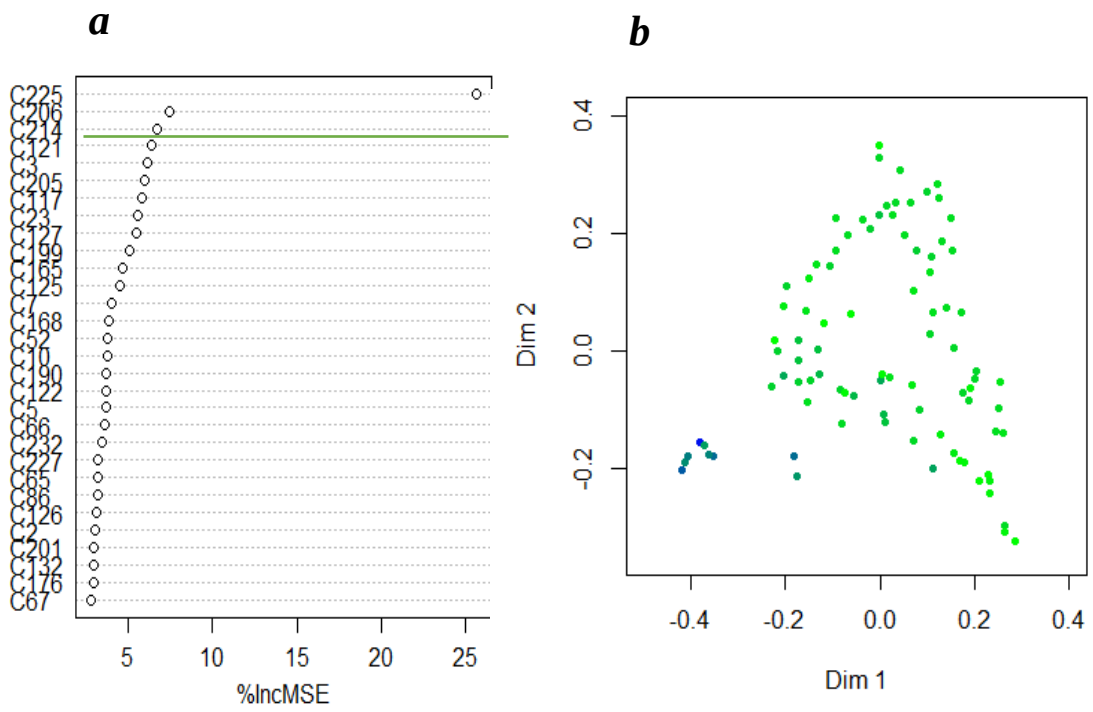


Figure 4.6: **a)** significant features identified by random forest regression to predict alliinase activity. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when out of bag (OOB) during permutation. Green line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b)** MDS plot of samples plotted against two dimensions of the random forest, with blue colouration indicative of high alliinase activity and green colouration indicative of low alliinase activity. Shading is displayed on a gradient in which samples with high alliinase activity (maximum of 0.19 µmol mg-1 min-1) are blue and those with lower activity (minimum of 0 µmol mg-1 min-1) are green.

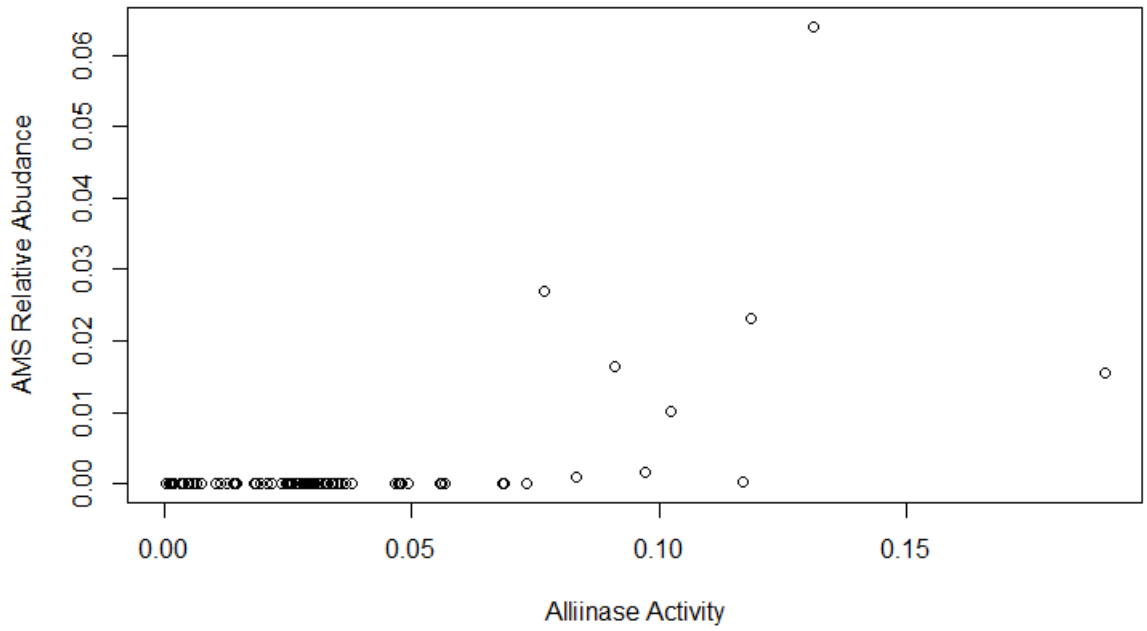


Figure 4.7: The relative abundance of allyl methyl sulphide (normalised to total VOC abundance, where 1=100% of the VOC profile and 0 = 0 %), and plotted against alliinase activity (umol mg-1 min-1).

4.3.9. VOC profiles correlate weakly with proportion of diseased cloves

Disease incidence in stored garlic bulbs was correlated with volatile profile in a random forest regression which explained 15.68 % of the variance in the dataset (Fig. 4.8a). A cut-off point was selected at 5 %IncMSE and all compounds above this line (5-hexen-2-one (C69), 5-hexen-1-ol (C68), 2-ethyl-1-hexanol (C17), 5-hexenal (C70), diallyl sulphide (C135) and methyl-pyrazine (C222)) were included in an MDS plot of a subsequent random forest analysis, with 19.81 % of the variance of the dataset explained (Fig. 4.8b). Samples with the highest rates of disease (blue colour) are broadly found in the negative area of dimension 2, but do not cluster distinctly from samples with low disease (Fig. 4.8b).

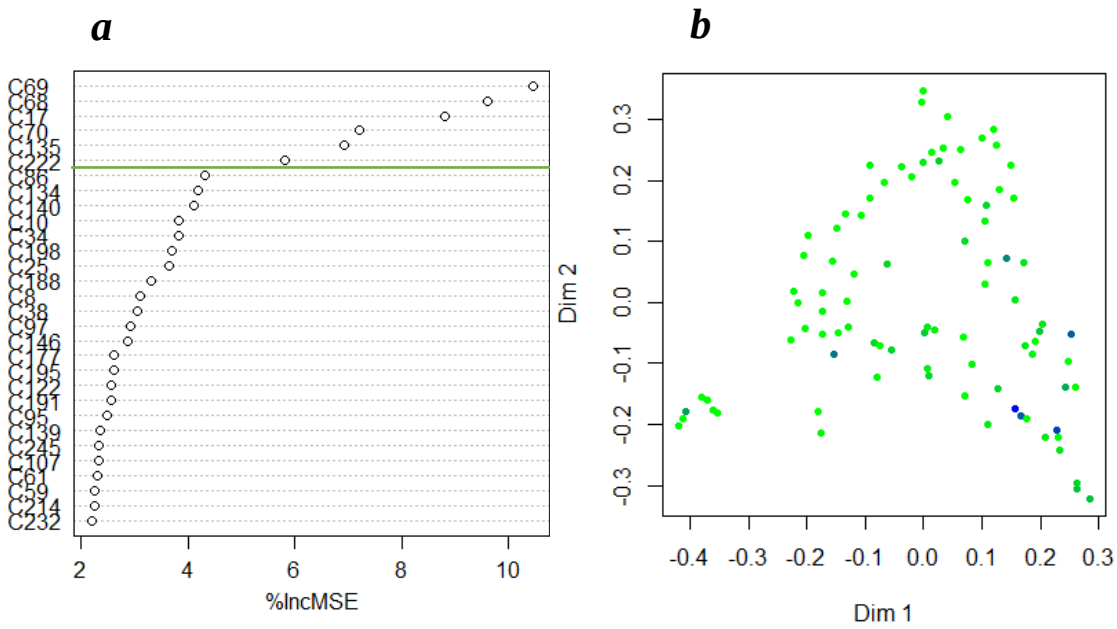


Figure 4.8: **a)** significant features identified by random forest regression to predict disease rate in stored garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Green line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b)** MDS plot of samples plotted against two dimensions of the random forest, with blue colouration indicative of high disease incidence and green colouration indicative of low disease incidence. Shading is displayed on a gradient in which samples with 100 % disease incidence are blue and those with 0 % are green.

4.3.10. VOCs discriminate between years of harvest

Discrimination between growing years was achieved with an OOB estimation of error of 0 % when the entire volatile profile of the stored garlic was considered. The top ten important compounds for the random forest algorithm based on mean decrease accuracy were included in a second classification analysis (Fig. 4.9a; Table 4.3). The second algorithm performed with a 12.22% OOB estimate of error rate and when re-run on the original dataset, the algorithm correctly classified storage year for all samples and when plotted, showed a significant discrimination between years (Fig. 4.9b). This shows that a number of compounds in the VOC profile of garlic have contrasting abundance between sampling years.

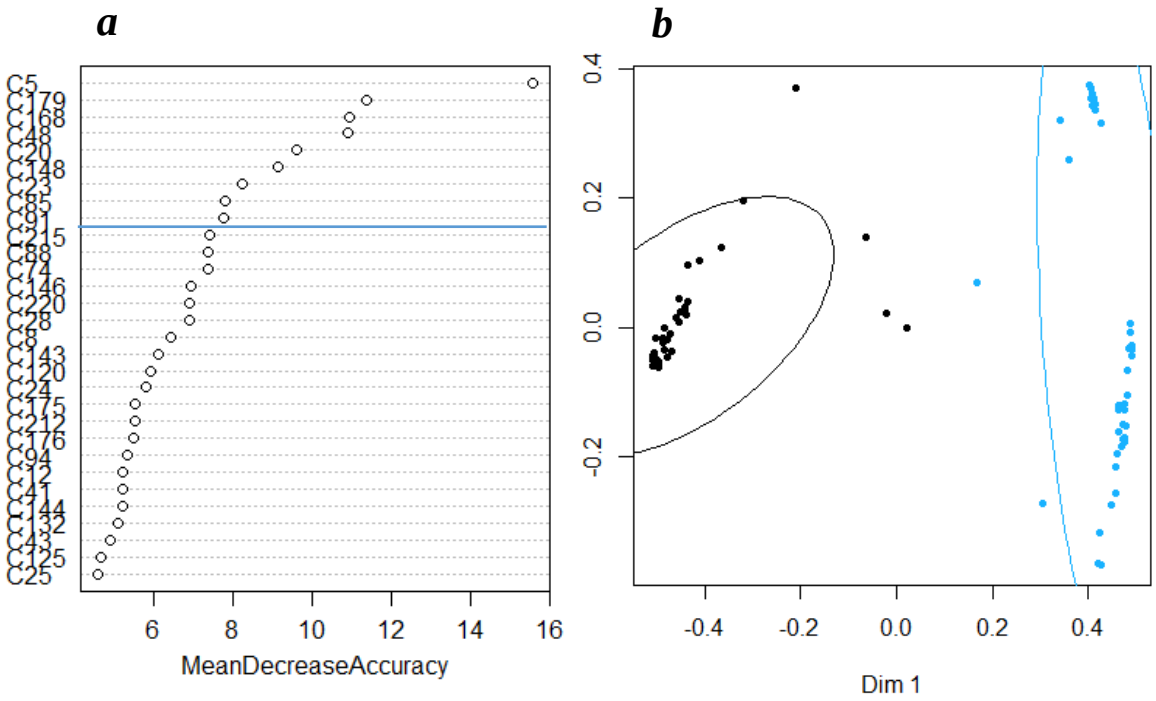


Figure 4.9: **a)** significant features identified by random forest classification to predict sampling season in stored garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Blue line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b)** Ordiplot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses (SD), where black = season one and blue = season two. N = 45.

Table 4.3: compounds with mean decrease accuracy > 7.5 in the random forest classification algorithm predicting year of harvest between stored garlic.

Compound Name	**Compound Number**
1,3-cyclopentadiene	C5
γ-muurolene	C179
hexanoic acid	C168
1-methoxy-2-propanol	C48
2-butyl-1-octanol	C20
4-methyl-dodecane	C148
2-methyl-1-propanol	C23
benzaldehyde	C85
1,3-di-tert-butylbenzene	C91
Propanoic acid	C215

4.3.11. VOCs do not discriminate between storage times

Discrimination between storage times was performed with an OOB estimation of error of 12.22 % when the entire volatile profile of the stored garlic was considered. The top six important compounds for the random forest algorithm based on mean decrease accuracy were included in a second classification analysis (Fig. 4.10a; Table 4.4). The second algorithm performed with a 21.11 % OOB estimate of error rate and when plotted, showed a degree of clustering across the dimensions according to storage time, but too much overlap to separate them (Fig. 4.10b)

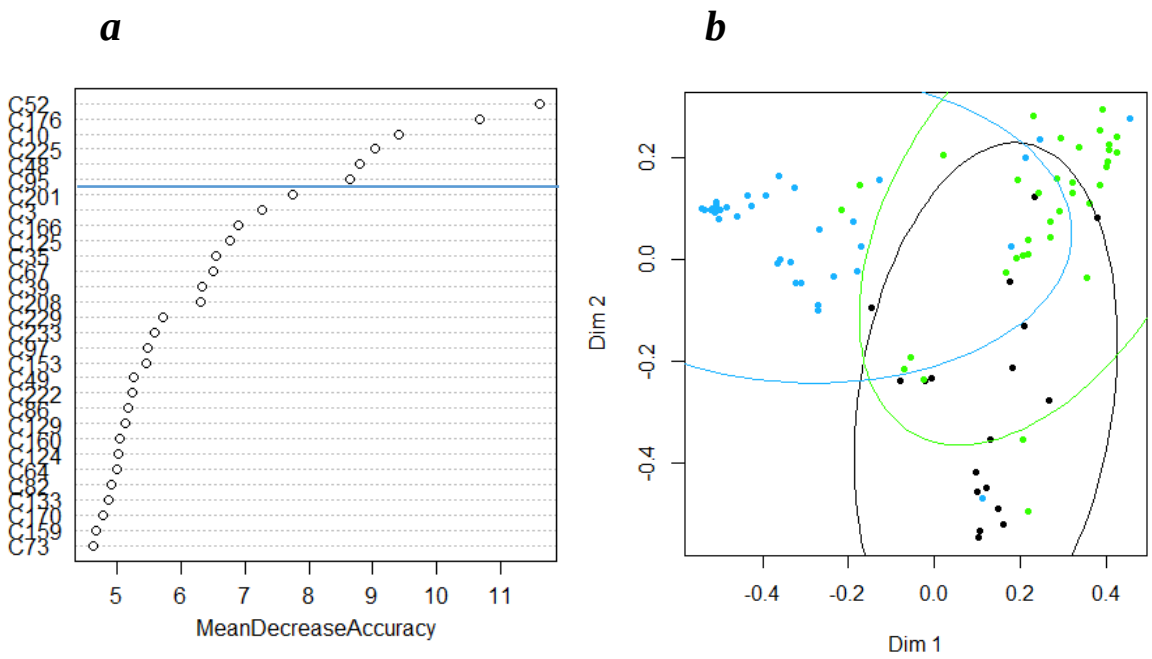


Figure 4.10: **a)** significant features identified by random forest classification to predict storage time in stored garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Blue line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b)** Ordiplot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses (SD), where black = 0 months, green = 3 months and blue = 6 months. N = 18 for 0 months and N = 36 for 3 and 6 months.

Table 4.4: compounds identified as being having a high mean decrease accuracy in the random forest classification algorithm predicting time of storage in garlic.

Compound Name	Compound Number
3-Carene	C52
Mesitylene	C176
1-Butanol	C10
Allyl-methyl-sulphide	C225
1-Methoxy-2-propanol	C48
1-Ethyl-4-methyl-benzene	C95

4.3.12. VOCs do not discriminate between storage temperatures

Analysis by random forest explained 16.67 % of the variance in the dataset when the entire volatile profile of the stored garlic was considered. The top ten important compounds for the random forest algorithm based on mean decrease accuracy were included in a second classification analysis (Figure 4.11a; Table 4.5). The second algorithm explained 20.00 % of the variance in the dataset, however it showed a low degree of clustering across the dimensions according to storage temperature and all temperatures overlapped (Figure 4.11b). Analysis of the dataset by cultivar did not generally improve discrimination of time or temperature (Supplementary Fig. S4.1 - S4.6). The only exception was in ‘Morado’ garlic, where VOCs discriminated between temperature, with cold stored discriminating from and both ambient and prestorage (Supplementary Fig. S4.4). Likewise, analysis of data from season one and season two separately did not result in discrimination between time, temperature or cultivar (Supplementary Fig. S4.7 - S4.12).

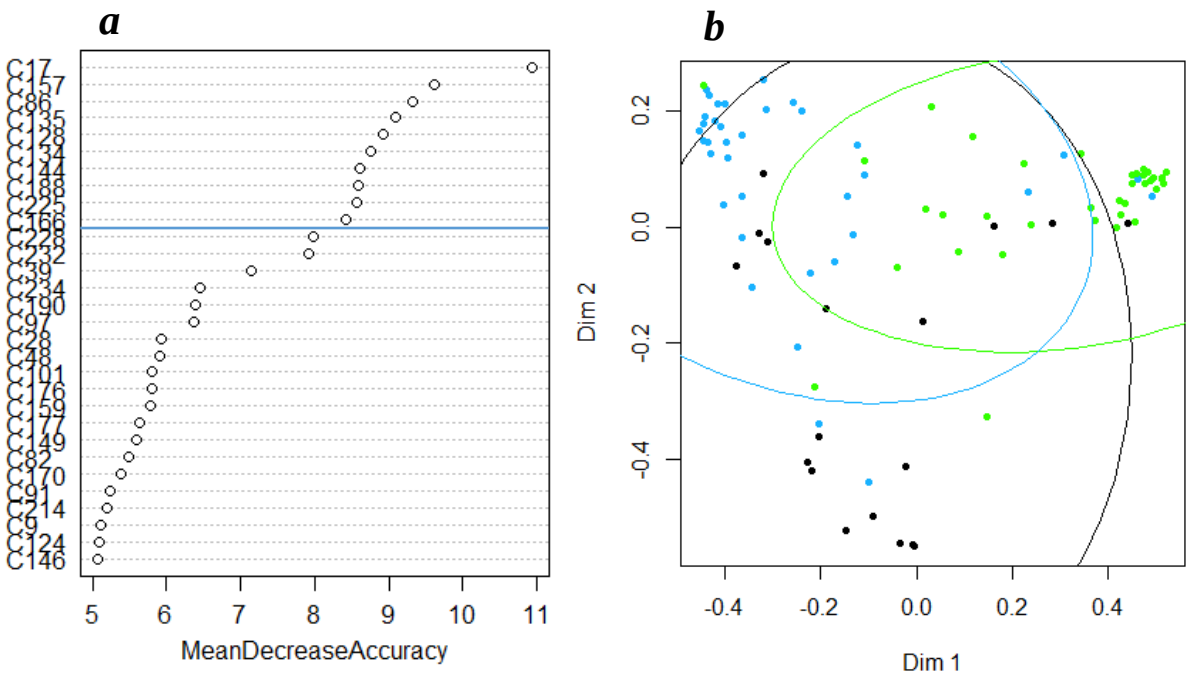


Figure 4.11: **a)** significant features identified by random forest classification to predict storage temperature in stored garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Blue line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b)** Ordiplot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses (SD), where black = 0 months, green = 3 months and blue = 6 months. Garlic bulbs of Cv. Morado, Blanco and Violeta were ambient and cold stored and measured at 0, 3 and 6 months over two consecutive sampling years. N = 18 samples of 3 bulbs for pre-storage and N = 36 for ambient and cold stored garlic.

Table 4.5: compounds identified as being having a high mean decrease accuracy in the random forest classification algorithm predicting storage temperature in garlic.

Compound Name	Compound Number
2-Ethyl-1-Hexanol	C17
2-Pentyl-furan	C157
Benzene	C86
Diallyl sulphide	C135
Decanal	C128
Diallyl disulphide	C134
Dodecane	C144
Nonanal	C188
Allyl methyl sulphide	C225
Hexanal	C166

4.3.13. VOCs did not discriminate amongst cultivars

The whole VOC profile of garlic did not discriminate or cluster based on cultivar in a random forest analysis (Fig. 4.12 a & b).

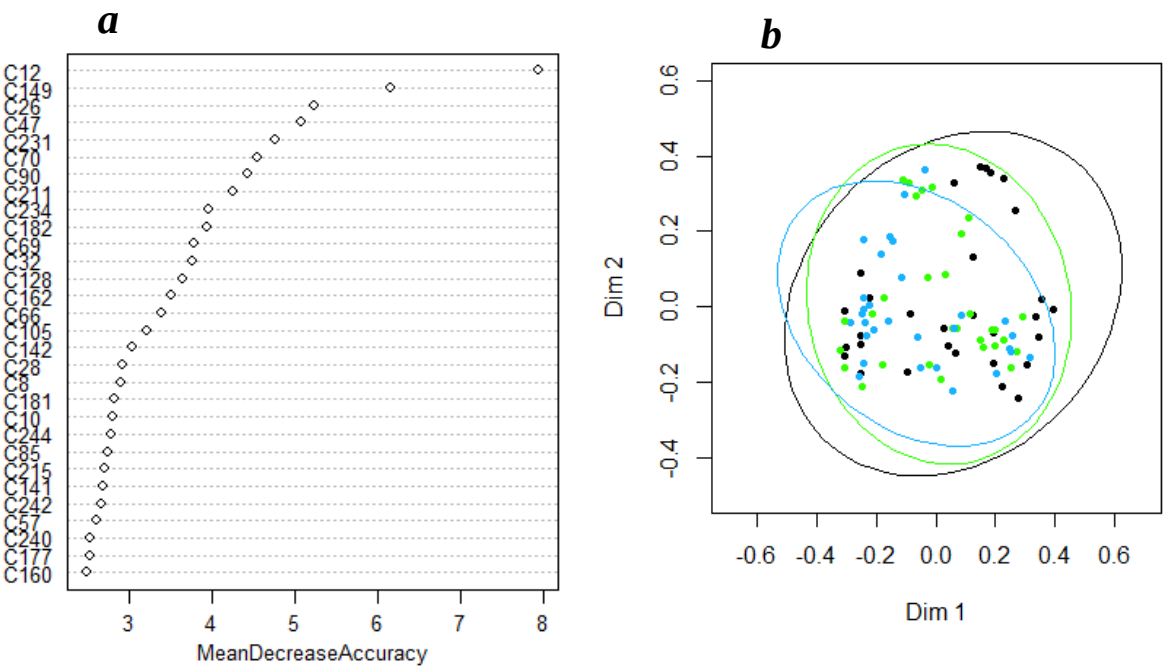


Figure 4.12: **a)** significant features identified by random forest classification to predict the cultivar of garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. **b)** Ordiplot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses (SD), where black = 'Morado', green = 'Blanco' and blue = 'Violeta'. N = 30 samples of 3 bulbs.

4.4. Discussion

The seasonal nature of garlic cropping requires bulbs to be stored for prolonged periods of time to facilitate year round supply, during which time quality falls (Block, 2010). The temperature at which storage occurs influences key quality traits over time in storage, including sprouting, weight loss and disease incidence (Rahim & Fordham, 1988; Mann & Lewis, 1956). Storage conditions also affect biochemical quality traits, however there is less research into alliinase activity itself (Martins *et al.,* 2016; Ichikawa *et al.,* 2006; Kodera *et al.,* 2002). Confounding the matter is the high degree of variance seen between cultivars, growing locations and year of harvest (Waterer & Schmitz, 1994; Panthee *et al.,* 2006; Maaβ &Klaas, 1995; Sharma *et al.,* 2004). Here, the crop management practises employed in both years did not change markedly as all farms within the cooperative grow garlic in the same way, however we do not know which farmer planted and grew specific varieties on specific years. It is therefore possible that slight differences existed in how the crops were grown, including individual farmers having slightly differing preferences in work method.

There is a strong and broadly linear relationship between irrigation and garlic yield (Sadaria *et al.,* 1997). Rainfall across the seasons differed quite substantially (Supplementary Fig. S4.13a and S4.13b). In February 51.6mm rain fell in 2016 and only 18.8mm fell in 2017. Both years had a similar amount of rainfall in March (16.8 mm and 20.8 mm respectively), but again 2016 was wetter in April and May (34.7 mm vs 22.4 mm per month average for April and May). 2017 saw marginally more rain fall in June and July, near harvest, with an average rainfall for these months of 18.6 mm versus 5.2 mm in 2016. The crops were, however, irrigated, so it is not clear how the total supply of water changed across the two cropping years, but it is assumed not to be substantially different and it is known that neither crop experienced a water shortage. The temperature

across the two cropping years changed only subtly, with year two having a colder winter and experiencing some mild frosts in January (Supplementary Fig. S4.14a and S4.14b). The first cropping year did not experience any frosts, which may have implications for pest and disease pressures (Zheng *et al.*, 2010; Maude, 2018). In the second year, winter temperatures also fell more sharply and earlier in the season, with temperatures falling quickly in October. Spring warming began earlier in the second season but progressed at a slower rate.

Firmness is a desired trait in high quality garlic and is known to decrease over time in storage, particularly when stored in facilities without temperature control (Volk *et al.*, 2004; Curzio & Urioste, 1994). In results presented here, Chinese garlic had a significantly lower resistance to penetration than either of the purple Spanish cultivars. A high resistance to penetration may be associated with a resistance to mechanical damage from harvest and processing. Wounded garlic has greater opportunity for pathogens to enter the bulb and leads to poor storage characteristics (Bertolini & Tian, 1996; Tian & Bertolini, 1995).

The average firmness of garlic across all cultivars fell significantly after 3 months of storage, before returning to pre-storage levels at 6 months. This contrasts with previous work (Cantwell, 2003), in which the firmness of garlic stored at 0 °C decreased steadily over time in storage from 18 kgf cm^{-1} to 13.5 kgf cm^{-1} over a 6 months period. However, a decrease was found here in *cv. Blanco*, so there appears to be a strong cultivar effect on firmness loss over time in storage, or the lower temperature used in this study had significant effects on firmness maintenance through storage. Park *et al.* (2000) reported that ambient stored cloves initially lost less firmness than cold stored cloves, but by 7.5 months, the cold stored garlic (0 °C) was significantly firmer than the ambient stored. The firmness of *Morado* and *Violeta* increased over the first 3 months of cold storage, in agreement with findings that firmness of Korean garlic *cv. Danjang* increased (9.5 - 10.3

N) over 45 days in 0 °C stored garlic (Park & Kim, 2015). Iranian white garlic bulbs lost firmness over time in storage (12 ± 8 °C, 6 months) regardless of harvest time or curing temperature after harvest (Bayat *et al.*, 2009; Bayat, 2004). This was in agreement with a study on white Mexican garlic *cv. Perla*, in which penetration resistance fell in all temperature and humidity treatments, but where bulbs stored at 0 °C remained firmer than those at room temperature over 190 days (6.3 months) (Vazquez-Barrios *et al.*, 2006). The mechanisms behind this are not fully understood in garlic, however an analysis in onion found that cultivars with high firmness at harvest delayed softening throughout storage. High firmness was associated with thick cell wall/middle lamellae, high dry matter and uronic acid contents. Cellulose was found to be roughly equivalent between the cultivars studied and decreased in all lines throughout storage while the loss in firmness was due to degradation of the middle lamellae which facilitated easier cell separation under force (Coolong *et al*., 2008). So if there were variations in cell wall composition or structure across the garlic cultivars studied here, that may have affected changes in firmness during storage

Results presented here confirm that garlic has a water content of approximately 64 % after harvest and that this is reasonably stable throughout storage at both cold and ambient temperatures (Kwon *et al.*, 1985; Lawson, 1996). The rate of water loss in all cultivars studied here was found to increase over time in storage. This had been recognised previously and was ascribed to the physiology of the developing shoot and roots that change the shape of the clove and cause breaks in the epidermis, facilitating more rapid water loss (Fraga & Enríquez, 1998). As water loss here increased at both storage temperatures in the second 3 months of storage, it suggests both storage temperatures experienced a degree of shoot and root development.

Weight loss over storage occurred in all cultivars, at both storage temperatures and was larger at the higher temperature, in agreement with previous research (Vázquez-Barrios

et al., 2006). The Chinese cultivar *Blanco* lost significantly more fresh weight compared to the two Spanish cultivars when stored at ambient temperature. Weight loss occurs through transpiration, as water is lost from the bulb at a rate influenced by temperature and relative humidity, and through carbohydrate metabolism, which corresponds with sprouting and loss of dormancy (Croci & Curzio, 1983; Tekalign *et al.*, 2012; Salama *et al.*, 1995; Hariprakash & Nambisan, 1996; Vázquez-Barrios *et al.*, 2006). As storage conditions were identical amongst the cultivars and this analysis was only performed on non-diseased bulbs, it may be the case that the Chinese cultivar is more metabolically active throughout storage or has weaker control of transpiration.

Fungal and bacterial spoilage organisms have previously been shown to be more pathogenic at higher temperature and humidity (Bertolini &Tian, 1996; Tournas, 2005). *Blanco* suffered from a significantly higher incidence of disease than the Spanish cultivars, which may be attributable to genotypic variance (Rengwalska & Simon, 1986). Soil-borne spores are the principal infective agent of garlic (Tyson & Fullerton, 2004). It is possible that Spanish garlic cultivars have a higher resistance compared to Chinese garlic cultivars, which have a longer history of being grown in these soils and exposed to these specific pathogens.

Sprouting is a commonly used index of garlic quality in storage and when sprouting occurs, the shelf life is considered to have ended (Vázquez-Barrios *et al.*, 2006; Fraga, 1998). Storage at 20 °C or room temperature induced sprouting in garlic *cv. Perla* over a 190 day storage period, however here there was a negligible increase in sprouting over 180 days which could not be reproduced over the second storage year. Dormancy in garlic is a period of approximately 90 days after harvest where the cloves are unresponsive to environmental stimuli that would otherwise promote sprouting (Pellegrini *et al.*, 1988). As this time had certainly elapsed by the third time point, it is concluded that storage at

room temperature (22 °C ± 3.5 °C) inhibits sprouting and is a viable method for storing Spanish garlic for up to 6 months.

Sprouting was assessed by visual inspection of the clove and looking for a green sprout at the apical tip of the clove. This has limitations as it cannot assess how large the internal sprout has grown, and metrics such as sprouting index cannot be calculated. To dissect the cloves to assess this would have been incompatible with the other experiments performed in this study. The crudeness of this method may be a contributing factor as to why an increase in sprouting was not detected. However, a statistically significant, if modest, increase in sprouting was detected in ambiently stored garlic despite considerable year to year variance in the data.

The differences in results between the two storage seasons cannot be attributed to high variance between cloves as to when they sprout because the variance within each year was small. It may instead be that there are priming effects of biotic or abiotic stresses experienced during growth, or lack thereof, that influence the rates at which cloves lose dormancy. It is also known that handling methods of garlic prior to cold storage and delays in getting bulbs into cold storage significantly reduce the dormant period in storage and there is likely a degree of variance between years in this study in this parameter (Mann & Lewis, 1956; Takagi, 1990).

Whilst flavour precursors and biochemical quality of garlic has been relatively well studied in garlic under different storage conditions, little is known about how alliinase activity changes over storage at different temperatures. It was previously shown that the highest expression of the alliinase gene was during GSII of garlic, which is the first shoot, approximately 7 days after planting, and that alliinase gene expression in leaves then fell throughout the growing season (Mitrova *et al.*, 2018). However, this study did not consider the enzyme activity and we present evidence that alliinase activity does not

correlate with alliinase gene expression. Furthermore, the instability of alliinase has been widely reported (Lawson & Wang, 2001; Rabinkov *et al.*, 1993). In this work there was a very marked difference in the alliinase activity after harvest between the two storage seasons and greatly varying loss of activity over time in storage. Cultivars tended not to vary significantly from one another throughout storage at either temperature, suggesting that seasonal variance had a higher bearing on alliinase activity than cultivar or storage conditions. Alliinase activity was maintained just as well in garlic stored at ambient temperature as in that stored at -2 °C for 6 months and in some samples the activity was marginally higher. This may suggest that cold storage is not necessarily optimal for alliinase activity maintenance, however it does keep disease, sprouting and weight loss to a minimum.

A total of 223 VOCs were detected across all the garlic samples which were present in all replicates of at least one sample condition. A larger number of volatiles were putatively identified here from garlic, including a broader array of non-sulphur containing VOCs than those reported in the literature (Molina-Calle *et al.*, 2016; Calvo-Gomez *et al.*, 2004; Lee *et al.* 2003; Mondy *et al.*, 2001; Pino, 1992). This is attributed to the non-destructive sampling method, as crushing releases an overwhelming abundance of sulphurous volatiles which would overload the instrument if enough headspace was collected to detect the less abundant VOCs. Some studies rely on derivatisation steps to detect compounds which would otherwise not be detected, such as in the assessment of thiol compounds from crushed garlic (Warren *et al.*, 2013). Warren *et al.* (2013) detected 1-propanethiol, 1-butanethiol and thiophene amongst others, whilst here thiophene and methanethiol were detected without the need for derivatisation.

VOCs correlated with alliinase activity in a regression random forest algorithm, with samples separating well in the second dimension of the plot. This provides the potential for a molecular marker system based on just three compounds, namely AMS, 1,4-

dicyclohexylbenzene and methyl propyl sulphide to gauge alliinase activity of garlic in storage. AMS in particular performed well as a predictor of high alliinase activity and warrants further study to elucidate the mechanisms behind AMS emission in high alliinase garlic. VOCs also successfully discriminated between storage years, with an error rate of 12.2%, however, samples did not discriminate well based on disease rate. Volatiles discriminated less well by storage time and temperature and did not discriminate by cultivar except when analysed by PerMANOVA. PerMANOVA is a multifactorial analysis and so can consider the effect of time, temperature, year and cultivar simultaneously. This gives PerMANOVA a higher degree of statistical power and suggests that the effects of time, temperature and cultivar on the VOC profile are significant, but are too complex for methods such as random forest to inspect. However, PerMANOVA cannot identify the compounds responsible for this difference. The accuracy of these marker systems have not been validated against a novel dataset, so it is not certain as to whether they would be applicable to a third storage year, a novel garlic cultivar or growing location. With more seasonal replicates, it may become evident that certain volatiles are descriptive of poor harvest years, where alliinase activity is lower than expected.

In onion, volatiles can discriminate between healthy, blue-rot (*Penicillium sp.*) and soft-rot (*Burkholderia cepacia* and *Fusarium sp.*) infected bulbs (Wang *et al.* 2015). Styrene was detected in the garlic sample here and was previously reported as a VOC indicative of infection in *Alliums*, with emission from onions infected with *F. oxysporum* and *Aspergillus niger, Erwinia carotovora subsp. carotovora* and *Penicilium sp*. (Vikram *et al.*, 2005; Fischer *et al.*, 1999). However, styrene was not found to associate with disease rate in statistical analyses here.

Soft-rot onions had higher levels of ethanol, 1-propanol, 1-propanethiol, acetic acid and methyl propyl disulphide compared to healthy or *Penicillium* infected bulbs (Prithiviraj

et al., 2004). Methyl propyl disulphide was detected in the garlic samples here but not identified by the analyses as being associated with disease rate. 3-methyl-1-butanol was unique to soft-rot bulbs and styrene was specific to both diseased sample sets and absent in healthy bulbs (Prithiviraj *et al.*, 2004). 3-methyl-1-butanol was also detected in the garlic samples here but again not present in the results of our analyses as being associated with disease rate. However, studies on onion may be of limited relevance to garlic. Even if garlic is infected by the same pathogen, is not known if it would emit the same volatiles as those in onion, as the substrate of a microorganism has been shown to significantly alter the VOC profile (Wheatley *et al.,* 1997). Fungal volatiles also vary significantly during interspecific interactions, with variance also between substrate and interaction status (Evans, *et al.*, 2008).

In conclusion, alliinase activity fell significantly in season one but started and remained low throughout storage in season two over both temperatures. This suggests a high degree of variation between seasons regarding alliinase activity in the three cultivars studied here, despite the farming, processing and storage being uniform over the two years. Putative VOC markers for alliinase activity were identified, as were those for storage year. The inclusion of multiple cultivars and cropping seasons added variability into the VOC dataset that made identifying VOCs that changed significantly over storage time and temperature more challenging.

5. Chapter 5: Applied Aspects of VOC Marker Systems

5.1. Introduction

5.1.1. Cold Stress in Stored Garlic

Although commercial storage and transport of garlic aims to maintain an even temperature of -2 ^{o}C, the storage temperature of garlic may be non-isothermic at times due to the limitations of refrigerating large areas such as warehouses. If garlic freezes, the crystallisation of water within the cloves cells causes cell lysis and upon thawing, the damage is readily apparent by visual inspection (personal communication, Neem Biotech; James *et al.*, 2009). Cold damage from freezing results from either mechanical injury to cells during ice crystal formation or from cell dehydration, as water is drawn out of solution as it crystallises, concentrating the solutes in the cell (Pearce, 2001; Muldrew & McGann, 1990; Enüstün *et al.*, 1978). However, temperatures below the desired -2 °C of the cold-store that do not cause freezing may elicit a cold stress response, which may have implications for alliinase activity or quality traits in garlic.

Whilst pure liquids have a precise point of freezing, garlic has a freezing range. This is because garlic bulbs are non-homogenous and vary in factors such as solute content. Garlic has a freezing range of -2.6 to -3.2 °C, with a mean of -2.7 °C (James *et al.*, 2009). However, garlic can in fact be stored below these temperatures with no ill effect. For example, garlic was stored at -3 °C by Volk & Rotindo (2004) in relation to spring planting viability of cold-stored garlic and -4 °C to reduce the incidence of *Penicilium hirsutum*, and no freezing injury was reported (Tian & Bertolini, 1996). Garlic can be cooled below its freezing point without ice crystals forming due to the phenomenon of supercooling. The nucleation point of garlic was found to be much lower than previous literature, with -7.7 °C reported as the highest temperature at which ice nucleation occurred James *et al.*, (2009). Some guidelines quote values as high as -0.8 °C as the

nucleation point of garlic, suggesting that there may be variation in the cold tolerance of garlic (Gast, 2001)

Garlic, like most temperate plants, acquires frost tolerance in a process called cold acclimation (Thomashow, 1999). This is a process induced by low, non-freezing temperatures which reprograms gene expression and metabolism, notably leading to an accumulation of proteins and metabolites known to have protective effects against freezing injury (Zhu *et al.*, 2007; Thomashow, 1999). Metabolites such as sucrose, proline, galactinol, trehalose, fructan, maltose and glycine have been shown to increase in plants in response to cold stress and their protective properties have been documented (Stitt & Hurry, 2002; Kaplan & Guy, 2005; Chen & Murata, 2002). These responses are typically induced at temperatures from 0-15 °C, however acclimation temperatures vary across species (Thomashow, 1999; Ruelland *et al.*, 2009). Broad changes in metabolism in response to cold shock in *Arabidopsis* have been shown to cause VOCs to change more markedly than is seen in heat shock, with 311 metabolites altered in response to cold shock, providing a potential basis for cold stress detection (Kaplan *et al.*, 2004). In garlic, cold temperature conditioning of cloves at 5 °C for 5 weeks significantly increased phenolic and anthocyanin content during bulb development (Dufoo-Hurtado *et al.*, 2013).

In this Chapter, experiments were conducted to test the effects of exposing garlic bulbs to temperatures below the usual storage temperature, but not so cold as to cause the cloves to freeze. This temperature range was selected since when cells are lysed due to ice crystal formation, the damage is visually apparent upon thawing (James *et al.*, 2009). The cell lysis results in a failure of the compartmentalisation of alliinase from alliin and the production of a strong odour which is easily detectable (Neem Biotech, personal communication). This is sufficient in the industry for quality control and more complex tools are not required. The concern instead is that lower than planned storage temperatures that do not cause nucleation of ice crystals may affect alliinase activity or other bulb

quality characteristics and leave no visible trace. Analyses were performed here to explore more subtle changes in the VOC profile that may be elicited by cold stress.

5.1.2. Application of VOC analysis on a warehouse scale

It is envisaged that the methods developed here in this thesis are to be applied to industry, where it is hoped that the VOC profile can be used to predict alliinase activity and quality traits in garlic and monitor them through storage. However, translating lab based experiments to work well on a much larger scale, such as in a warehouse presents some challenges. Firstly, sampling is to be performed on entire crates of garlic at a time, as sub-sampling may lead to the measurement of a VOC profile which is less representative of the whole. Secondly, the temperature in the warehouse is much lower than in the lab, causing VOCs to become less volatile and therefore potentially harder to detect. Thirdly, a method of sampling must be developed which complements existing quality control methods, without creating a significant amount of added work.

Warehouse monitoring of VOCs has been applied to stored crops other than garlic. In particular, research has focussed on climacteric fruit due to the long-standing literature on ethylene signalling in ripening fruit (Ivanov *et al.*, 2005; Lelièvre *et al*., 1997). Similarly to garlic, apples are stored for many months in cool temperatures. Throughout storage, apples begin to ripen and release ethylene; this VOC can be monitored over time in storage and inferences regarding ripeness and shelf-life can be made (Lang & Hübert, 2012; Fonollosa *et al.*, 2012).

5.1.3. VOC Analysis using Pulsed Fame Photometric Detection (PFPD)

Thermal desorption time of flight mass spectrometry (TD-GC-ToFMS) is a very sensitive method for VOC analysis, with high dynamic range and excellent putative compound identification abilities due to predictable and unique mass spectral patterns and retention indices. However, the major problem of this system to industry is the cost of purchase and maintenance of the machine and the need to hire a specialist to operate it. Whilst this can be mitigated in part by performing the analysis off-site, it would delay the result. Once specific VOCs have been identified and a quality control method has been developed, compounds no longer need to be identified by mass spectra and can instead be detected by a more cost effective detector and verified using known compound standards.

Several detectors have been developed and are increasingly being used in food science applications for quality control. One such development is that of electronic noses, or eNoses, which comprise active materials which detect specific odours and convert the signal into electrical signals. There are a range of technologies used in eNoses, which interact with different volatiles, including metal oxide semiconductors, metal oxide semiconductor field effect transistors, surface acoustic waves, quartz crystal microbalances, conducting organic polymer sensors, intrinsically conducting polymers and optical fibre bundles (Nordberg *et al.*, 2000; Hartmann *et al.*, 1994; Ricco *et al.*, 1998, Sisk *et al.*, 2003).

Another method showing much promise in this field is pulsed flame photometric detection (PFPD). Unlike eNoses, PFPD detectors are not specific to single compounds but instead detect specific elements: 28 elements can be detected by PFPD, of which 13 elements give a delayed light emission (chemiluminescence) following combustion and subsequently have far greater selectivity and sensitivity (Amirav & Jing, 1995). Sulphur

is one such compound, where the light emission from the sulphur atom in any volatile compound containing sulphur after the flame of the hydrogen fuel gas, carbon from the organosulphur compound or any other co-eluting hydrocarbons. This gives a low amount of background noise and coupled with combustion pulse rates of 1-10 Hz, where column effluent is collected and burnt in a pulse, gives the PFPD high sensitivity, in the 0.5-39.5 ppb range, or 4.6 to 13.6 pg mL^{-1} (Kim, 2005; Catalan *et al.,* 2006).

PFPD has been used extensively for detecting pesticide residues in crops, including *Alliums* (Ueno *et al.,* 2003; Salvador *et al*, 2006; Amirav & Jing, 1998). It has also been employed to perform postharvest quality assessment of rice, grapefruit and tomato among others. Sulphur compounds detected by PFPD were found to discriminate between Jasmine, Basmati and Jasmati rice (Mahattanatawee *et al.,* 2014). In another study, PFPD was used to analyse the sulphur VOC constituents of grapefruit juice, where 13 compounds were identified and significant differences in the VOC profile of reconstituted and fresh grapefruit juice were identified (Jabalpurwala *et al,* 2010). Also, PFPD analysis found two newly reported sulphur VOCs in tomato and detected significant differences in the VOC profile in response to maturity stages (Du *et al.,* 2015). The unique and complex sulphur chemistry of *Alliums* makes PFPD an excellent candidate for a more cost effective VOC monitoring tool than MS.

This chapter considers whether the approach of using VOCs for quality assessment in garlic could be applied to detect non-isothermic or temperature abuse in storage, the need to expand existing methods to warehouse scale and the need for future VOC analysis platforms to meet the cost requirements of industry.

5.2. Materials and Methods

5.2.1. Cold Stress

Bulbs of garlic *cv. Morado* (season two: August 2017-2018) were cold stored at -2 °C in accordance with methods in Chapter 2.2 for 5.5 months, after which they were transferred to a separate incubator and cold stressed for 96 h at -5 °C ± 0.05 °C. After the cold stress, the bulbs were returned to the -2 °C incubator and stored for the remainder (14 days) of the 6 month storage period with the unstressed samples. In this experiment, data for unstressed samples were previously reported in chapter 4.

VOC collection was performed as per Chapter 2.2 and analysed as in Chapter 2.10 and 2.11. Alliinase was extracted by methods in Chapter 2.5, assayed as per Chapter 2.6 and normalised as in Chapter 2.6. Firmness was assessed as per Chapter 4.2.2 and water content by Chapter 2.8.

5.2.2. Warehouse VOC Collection

Volatiles were sampled from garlic stored in -2 °C refrigerated warehouses in Spain at 3 month intervals, beginning at approximately 2 months postharvest (Season 3: 2018-2019). Volatiles were collected onto Tenax/Sulficarb packed open ended thermal desorption tubes with a 1cm diffusion pathway (Markes International, UK). Tubes were placed in open sided euro-pallet crates of garlic with one end cap removed and left to passively collect volatiles for 7 days before being recapped and shipped back to the laboratory for analysis. Tubes were desorbed and volatiles analysed in line with TD-GC-ToF-MS protocols outlined in Methods Chapter section 2.10. Compounds were identified based on a comparison with the pre-existing custom compound library as identified from previous lab work and no new compounds were added to this library to exclude any contaminants from the warehouse air.

5.2.3. GC-PFPD Volatile Analysis

GC-PFPD experiments were carried out at the Open University in the laboratory of Dr. Geraint Morgan. Headspace (500 mL) was sampled onto custom-made stainless-steel sorbent tubes of 81 mm length, 5 mm outer diameter and 1 mm wall thickness, packed with 200 mg Carbograph 1TD 40/60 and 200 mg Carboxen 1003 40/60 sorbent material (loaned from Insect Research Systems Ltd). The sorbent material was packed to a depth of 27 mm, corresponding with the heated zone of the Optic-4 Gas Chromatograph Inlet System used for desorption (GL Scientific, Europe). Samples were desorbed at 250 °C with a 1:5 split ratio and volatiles were separated on a 20 m, 18 μm ID DB-5-ms column in an Agilent Technologies 7890A GC System with 1 mL min^{-1} flow rate, using the following temperature program: 40 °C 1 min, 20 °C min^{-1} ramp to 300 °C, 3 min hold. Compounds were detected with a Model 5380 pulsed flame photometric detector (OI Analytica, UK).

Samples were collected and analysed in tandem on a GC-MS to perform putative compound identification based on NIST database, as in Chapter 2.10. The sampling and injection method were identical. Volatiles were separated on a 15 m, 25 μm ID DB-5-ms column in an Agilent Technologies 7890A GC System with 1.1 mL min-1 flow rate, using the temperature program outlined for the PFPD. Compounds were detected with an Agilent 5975C Inert XL El/Cl MSD quadrupole mass spectrometer. Statistical analysis of both PFPD and GCMS data were performed as detailed in Chapter 2.11.

5.2.4. Material for PFPD analysis

Garlic was sourced from Spain and Chile (Season 3: May 2019), which due to the difference in seasons, allowed analysis of garlic of different ages after harvest at the same time. The Spanish varieties were *Morado* and *Violeta* and had been cold stored at -2 °C for approximately 9 months. The Chilean variety was unknown, of purple colouration, had not been cold stored and it was sampled at approximately 3 months after harvest for the experiment.

5.3. Results

5.3.1. Cold Stress

5.3.1.1. Effects of Cold Stress on Garlic

Cold stress did not have a significant impact on alliinase activity (Fig. 5.1; $p = 0.413$). However, water content was significantly higher in bulbs that were cold stressed, with stressed bulbs having a water content 3 % greater on average than unstressed bulbs (Fig. 5.2; $p = 0.001$). Furthermore, cold stressed bulbs had a significantly lower firmness than control bulbs (Fig. 5.3; $p = 0.025$). The average force required to penetrate the cloves fell from 18.0 kg cm^{-1} to 15.2 kg cm^{-1}, with the firmest clove in the control group requiring 22.2 kg cm^{-1} for penetration and the weakest clove in the stressed group requiring just 12.4 kg cm^{-1}.

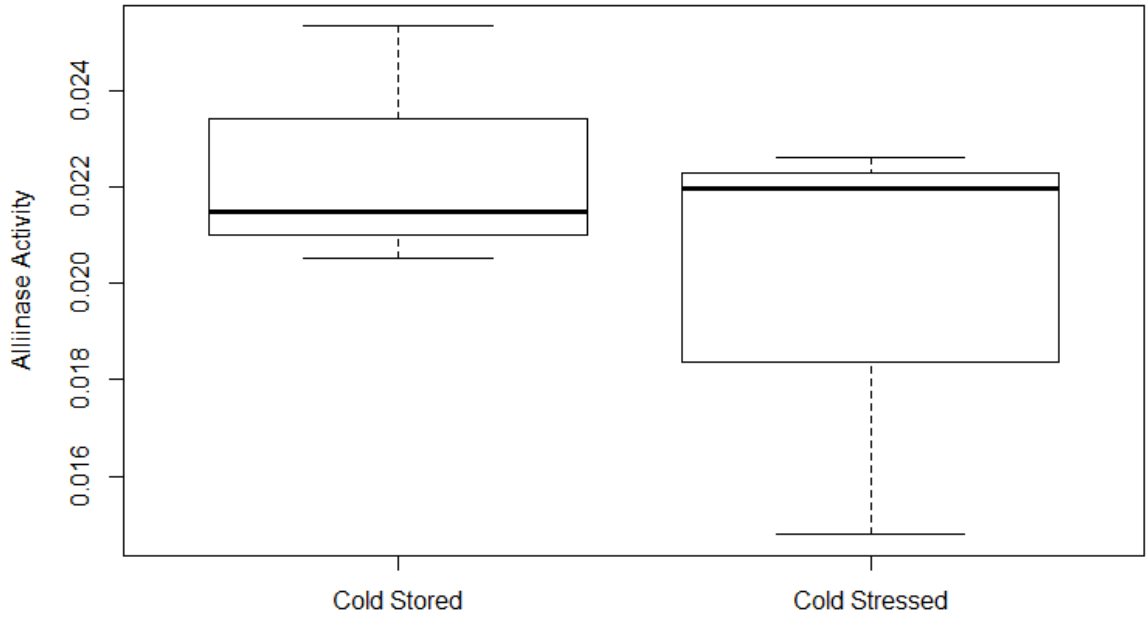


Figure 5.1: Alliinase activity (µmol mg^{-1} min^{-1}) of extracts from garlic cold stored for 5.5 months at -2 °C, cold stressed for 96 h at -5 °C and returned to -2 °C for a 10 day recovery period. N = 9.

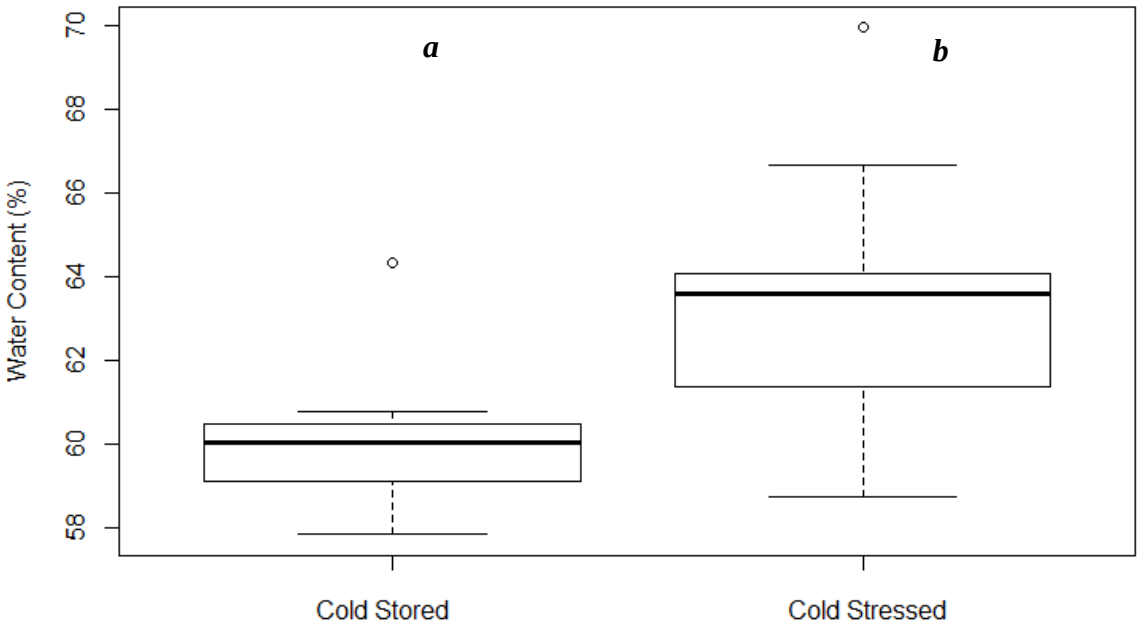


Figure 5.2: Water content (FW-DW/FW) of garlic cloves cold stored for 5.5 months at -2 °C, cold stressed for 96 h at -5 °C and returned to -2 °C for a 10 day recovery period. Letters denote significance $p < 0.05$. N = 5 cloves per sample in triplicate.

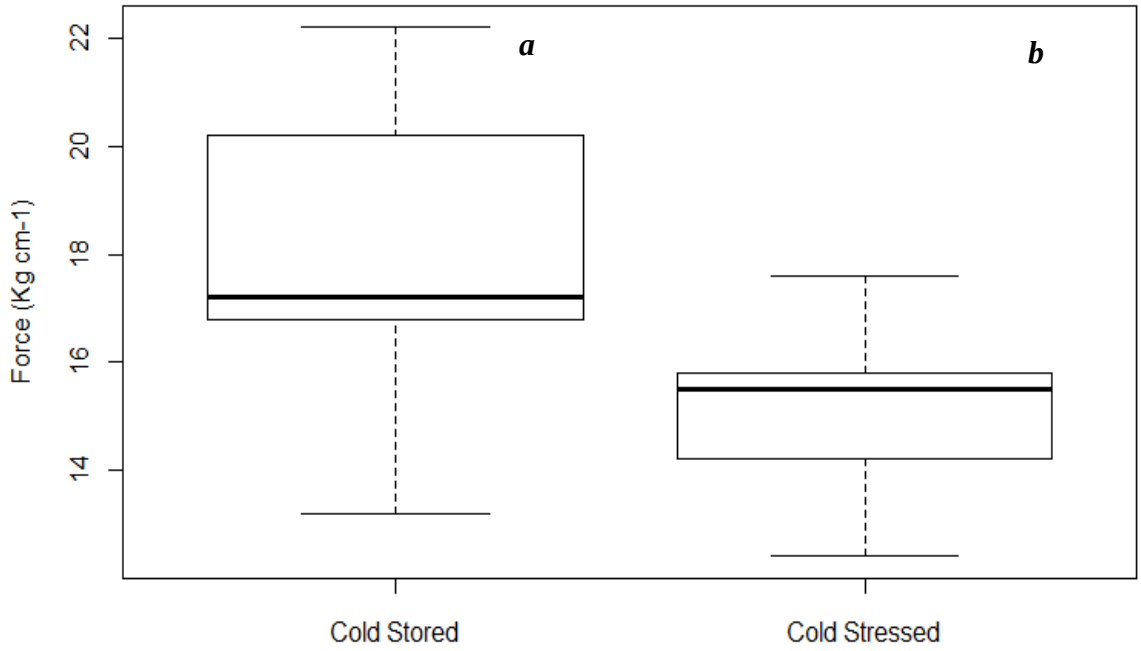


Figure 5.3: Clove firmness, as measured by resistance to penetration of garlic cloves cold stored for 6 months at -2 °C and cold stressed for 96 h at -5 °C. N = 5 cloves per sample in triplicate. Letters denote significance $p < 0.05$.

5.3.1.2. Volatile profile of cold-stressed garlic

The overall VOC profiles of garlic did not change significantly between unstressed controls and garlic subjected to the cold stress treatment (PerMANOVA; $p = 0.142$). However, WCNA identified a module of compounds which changed significantly between stressed and unstressed cloves (METurquoise, Figure 5.4a) with a negative correlation to the stress. This module consisted of 19 volatiles which varied in abundance significantly between treatments (Figure 5.4b). When reanalysed with PerMANOVA, the model ran 719 permutations, with complete enumeration, due to the small size of the data and the compounds did not discriminate between stressed and unstressed ($p = 0.101$). The module did, however, contain some compounds which did not vary as markedly as others, so the selection was further refined using visual inspection of the heatmap (Fig. 5.4b). Seven compounds were removed, namely: 1-methyl-4-propyl-benzene, 2,6-dimethyl-heptadecane, 1-heptene, 3,7-dimethyl-decane, 2,8-dimethyl-undecane, 4,8-dimethyl-tridecane and 3-methyl-pentadecane. This reduced dataset of 12 compounds was analysed with CAP and stressed samples discriminated strongly from unstressed (Fig 5.5).

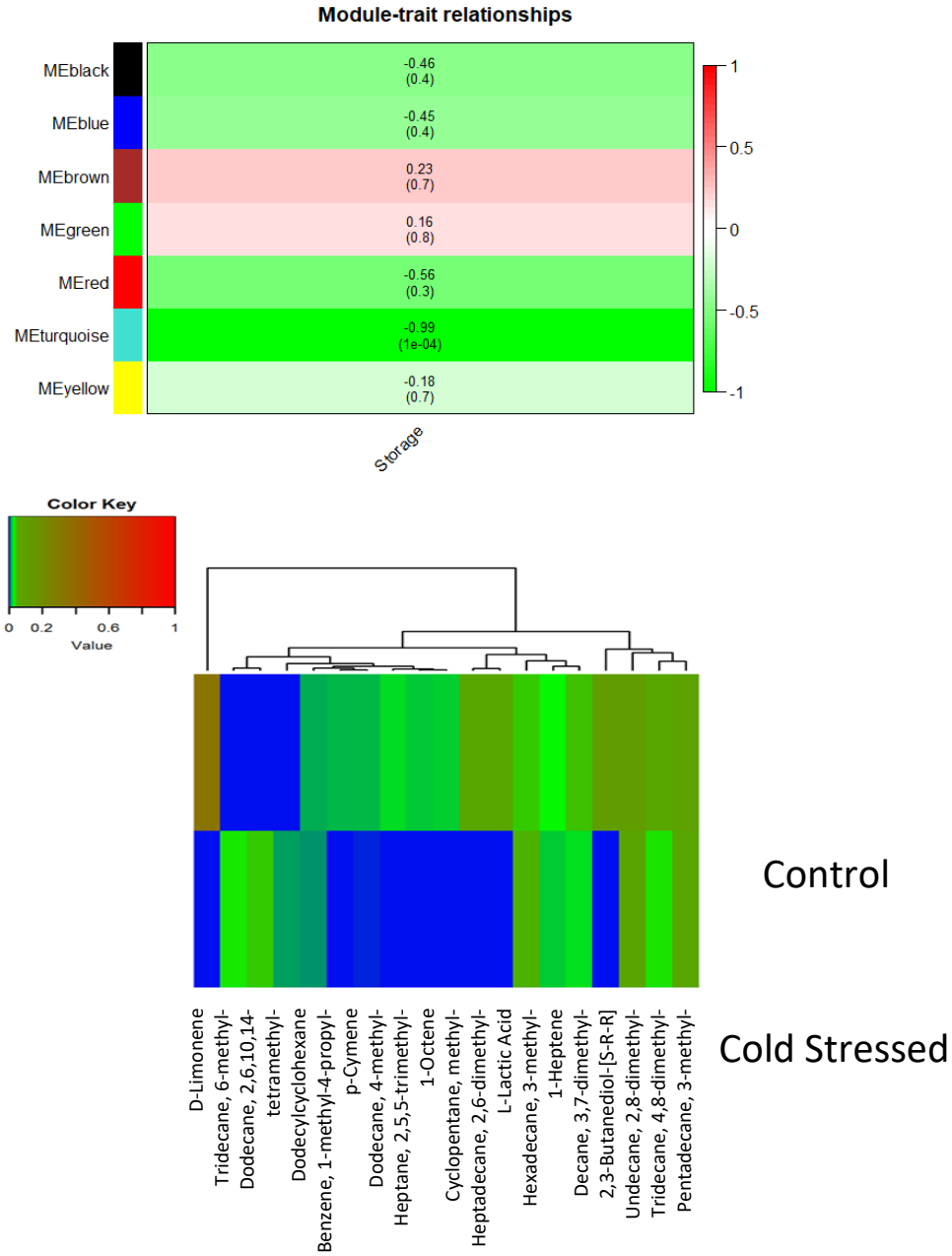


Figure 5.4: WCNA of VOCs from garlic cv. Morado stored for 6 months at -2 °C and -5 °C stressed. (a) WCNA modules: score and significance according to Pearson analysis. P values in brackets; red, positive and green, negative correlations with storage type (stressed vs control). (b) heat maps of significant VOCs in significantly correlating WCNA modules according to storage.

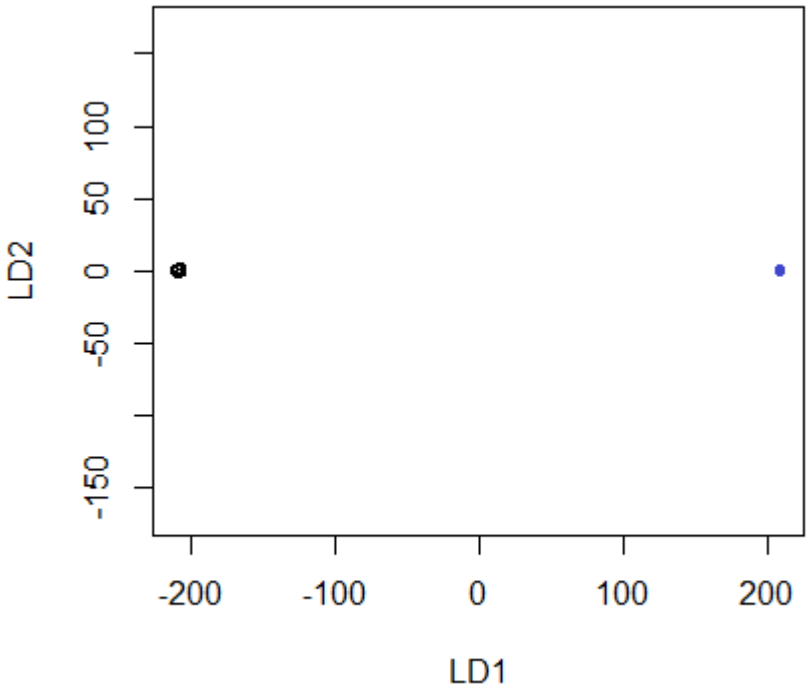


Figure 5.5: Linear discriminant plot from CAP analysis of 12 VOCs found to be significantly associated with storage temperature by WCNA (Fig. 8c). The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 100 % at p = 0.05, where black = control and blue = cold stressed. N = 3 bulbs.

5.3.2. Warehouse Trials of VOC Analysis

Collecting VOCs from warehouse air detected 143 compounds, versus the 244 compounds detected in the lab. Whole VOC profile varied significantly over time across both cultivars (PerMANOVA, P = 0.0001, R^2 = 0.608). VOCs did not change significantly between cultivars (PerMANOVA P = 0.175, R^2 = *0.036*). Samples discriminated over time in a CAP analysis but not between cultivar (Fig. 5.6; Fig. 5.7a & b). Volatiles which varied most significantly between storage times in the warehouse were identified with a random forest classification with a 0% OOB estimate of error (Fig. 5.8a). The seven most significant compounds were included in a second random forest which also had a 0% OOB estimate of error (Fig. 5.8b). The seven compounds are listed in Table 5.1 and consisted of two aldehydes, one organosulphur compound, one alkane, one ketone, one terpene and one aromatic compound.

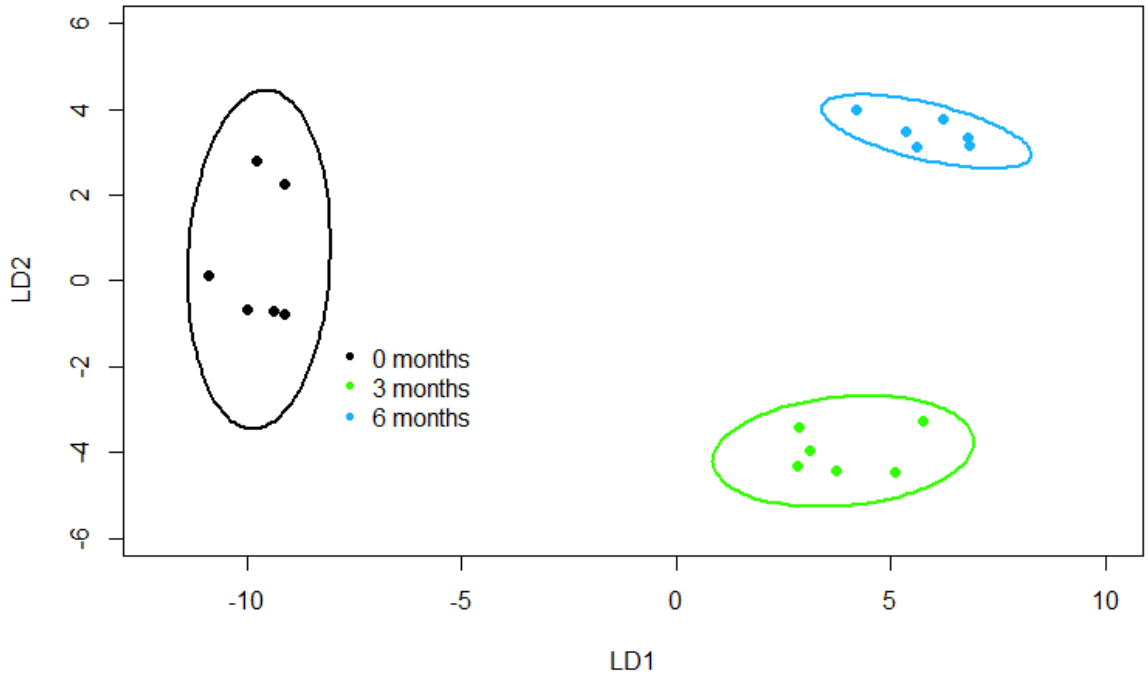


Figure 5.6: Canonical Analysis of Principal Coordinates based on all VOCs from garlic cv. Blanco and Morado using TD-GC-ToFMS. A CAP model was produced for warehouse cold stored (-2 °C) VOCs sampled over time at 0, 3 and 6 months. The plots use the first two linear discriminants of the CAP model and each ellipse represents the 95% confidence interval (SE) and the percentage of correct classification in the CAP model was 100% at a confidence of P = 0.05. N = 3 bulbs.

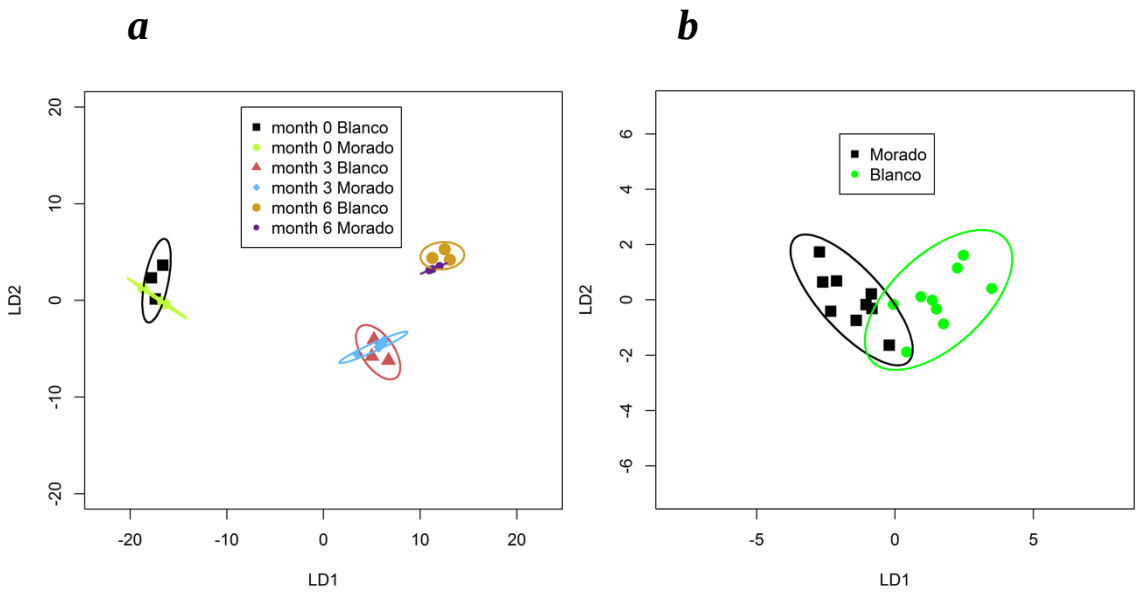


Figure 5.7: Canonical Analysis of Principal Coordinates based on all VOCs from garlic cv. Blanco and Morado using TD-GC-ToF-MS. A CAP model was produced for warehouse stored garlic VOCs, which considered storage time and cultivar **(a)** and another model which only considered cultivar **(b)**. The plots use the first two linear discriminants of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification in the CAP model was 100% at a confidence of P = 0.05, N = 3 bulbs.

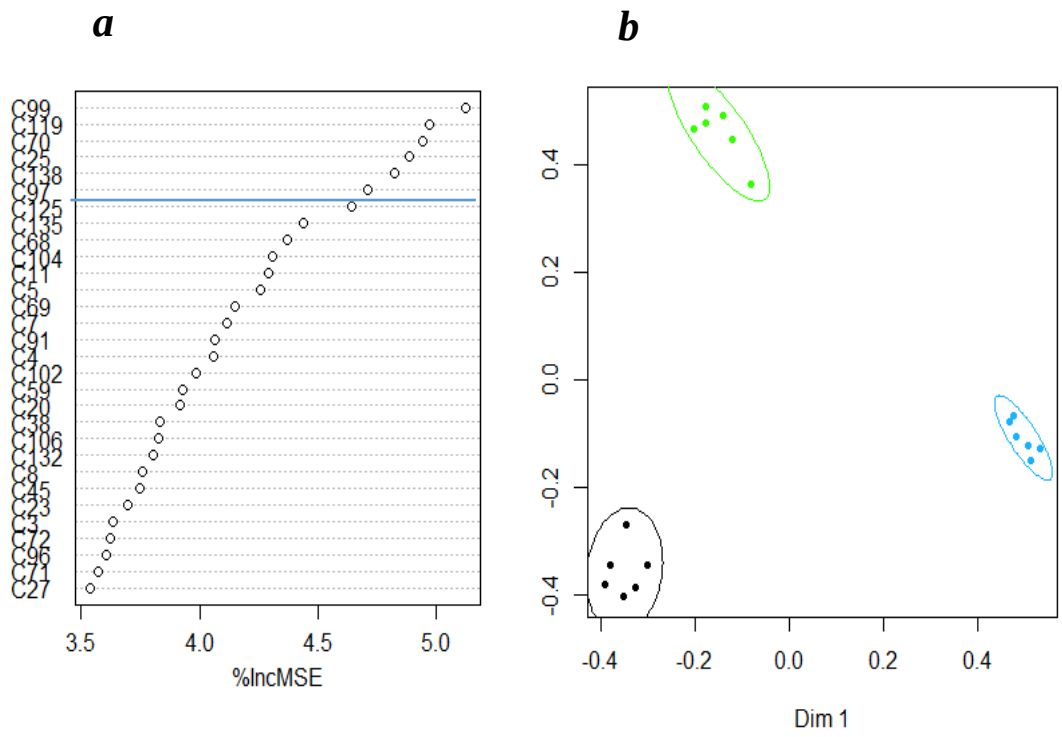


Figure 5.8: **a**) significant features identified by random forest classification to predict storage time in warehouse stored garlic bulbs. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Blue line denotes cut-off for inclusion in subsequent random forest and MDS plot. **b**) Ordiplot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses (SD), where black = 0 months, green = 3 months and blue = 6 months. Garlic bulbs of *Cv*. *Morado* and *Blanco* were cold stored in a commercial warehouse and measured at 0, 3 and 6 months over two consecutive sampling years. *N* = 3 bulbs.

Table 5.1: compounds identified as having a high mean decrease accuracy in the random forest classification algorithm predicting time of storage in warehouse stored garlic headspace samples.

Compound Name	Compound Number
Heptanal	C99
Octane	C119
3-methyl-butanal	C70
2-pentanone	C25
2,4-dimethyl-thiophene	C138
2-pentyl-furan	C97
p-cymene	C125

5.3.3. Results PFPD

A total of 10 peaks were detected from PFPD traces by inspection of chromatograms, compared to 13 reported in Chapter 3 and 27 in Chapter 4 (Fig. 5.9). GCMS analysis of these components yielded putative identification of 5 of the peaks detected with PFPD (Table 5.2). Volatile profiles were significantly different between Spanish garlic cold stored for 9 months and Chilean garlic stored for 3 months at ambient conditions (PerMANOVA, p =0.0495; random forest OOB estimate of error = 33.3%). Selection of the three compounds with highest mean decrease accuracy reduced the OOB estimate of error rate to 11.11% (Fig. 5.10a). When plotted, this algorithm shows partial clustering in Spanish garlic and a very wide spread in Chilean garlic, with overlap in the 95% confidence ellipses (Fig. 5.10b). The compounds found to be most significantly varying across storage time by random forest were diallyl disulphide, di-2-propenyl trisulphide and one unidentified compound.

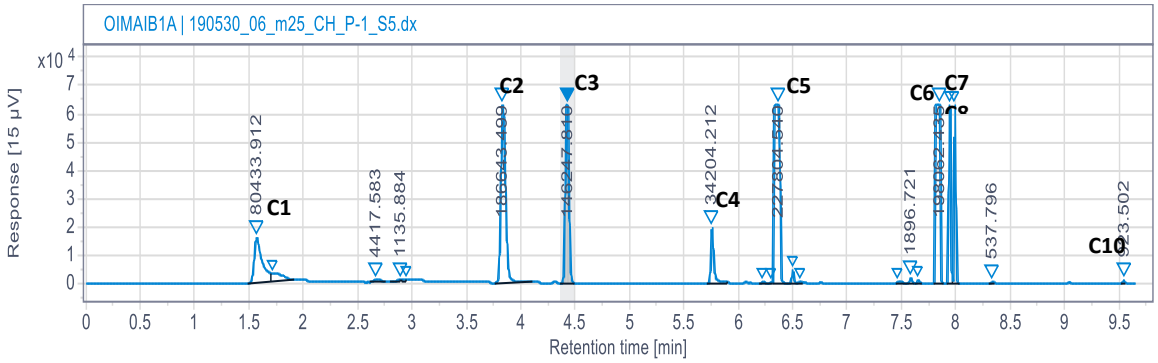


Figure 5.9: Example PFPD chromatogram showing peaks detected and used for analysis. The garlic shown in this chromatogram is a Chilean purple cultivar and C9 was not present in this sample. Compounds are numbered and correspond to the putative identifications, where possible, in Table 5.2.

Table 5.2: Putative identification of PFPD peaks by GCMS.

Compound Number PFPD Detection	Compound Name From MS/NIST	Retention Time (min) PFPD Detection
C1	allyl methyl sulphide	1.60
C2	ND in GCMS	3.80
C3	ND in GCMS	4.40
C4	methyl 2-propenyl disulphide	5.75
C5	diallyl disulphide	6.40
C6	di-2-propenyl trisulphide	7.75
C7	di-2-propenyl tetrasulphide	7.90
C8	ND in GCMS	8.00
C9	ND in GCMS	9.00
C10	ND in GCMS	9.50

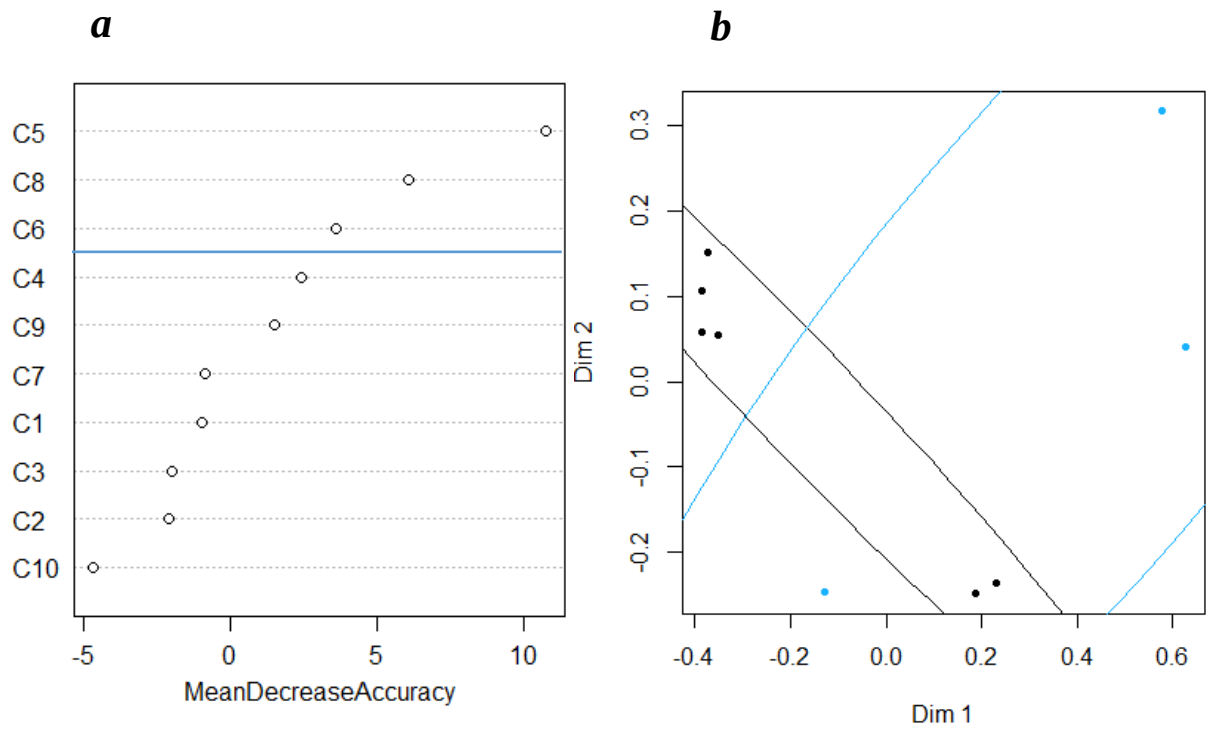


Figure 5.10: Significant features identified by random forest classification to predict storage time in Spanish and Chilean garlic **(a)**. The compounds are ranked by the decrease in classification accuracy according to % increase mean square error (%IncMSE) when OOB during permutation. Blue line denotes cut-off for inclusion in subsequent random forest and MDS plot. **(b)** MDS plot of samples plotted against two dimensions of the random forest, with 95% confidence ellipses, where black = Spanish garlic (cv. 'Morado' and 'Violeta') stored for 9 months, blue = Chilean garlic (unknown purple cv.) stored for 3 months. N = 3 per cultivar.

5.4. Discussion

Three different approaches were used in this Chapter to assess the applicability of garlic VOC profiles to assessing industrial quality.

VOCs were able to differentiate between cold stressed and cold stored garlic. However, no significant change in alliinase activity was noted in this experiment in response to a 96 h period of -5 °C cold stress. As -5 °C is close to the limit of garlic cold tolerance, it is unlikely that a colder temperature would have a more significant impact on alliinase activity, however it is possible that longer periods of -5 °C storage may affect alliinase activity maintenance over time. Therefore, the conclusion is that brief periods of -5 °C do not significantly affect alliinase activity in cold-stored garlic. Previous research has shown that alliinase gene expression may be downregulated in the clove in response to cooler temperatures; however the coldest temperature used in this study was 4 °C (Son *et al.*, 2012). This is considerably higher than the temperatures considered here and moreover, at 4 °C, sprouting occurs much more readily than either -2 or 22 °C as studied here (Mann & Lewis, 1956; Vazquez-Barrios *et al.*, 2006).

Whilst the moisture content for both samples was within the expected range for the moisture content of garlic at 6 months of storage at -2 °C, the data suggests there was a small but significant difference between the treatment groups. Whilst minor in terms of absolute change, this degree of moisture content change from 60.0 % in the control to 63.0 % is similar in magnitude to the difference seen between 6 month old and pre-storage garlic. The moisture content in both samples was in line with what has previously been reported in the literature (Pardo *et al.*, 2007). However, there is no clear explanation for an increase in water content in response to cold stress.

Garlic firmness is a key agronomic trait of relevance to consumers, and a significant reduction is associated with a potential loss in marketability (Vazquez-Barrios *et al.*,

2006; Pardo *et al.,* 2007). In this experiment there was a significant decrease in the firmness of cold stressed cloves. Firmness is very variable in postharvest storage of garlic, with different cultivars responding positively or negatively to low temperature (< / = 0 °C) or ambient temperature storage, however fewer studies compare different sub-zero storage temperatures (Vazquez-Barrios *et al.,* 2006; Park *et al.,* 2000; Bayat *et al.,* 2009; Bayat, 2004; Park & Kim, 2015). One study which did compare sub-zero temperatures compared quality traits of garlic stored at 0 °C and -3 °C for 7 months and reported no difference in firmness, however this experiment was performed qualitatively by a small number of individuals on a taste test panel and the authors acknowledged the limitations of this experimental design (Volk & Rotindo, 2004). The mechanisms behind firmness loss in garlic are not well understood, but in onion, high firmness has been correlated with thick cell wall/middle lamellae, high dry matter and uronic acid contents (Coolong *et al.,* 2008). However, this study was performed over just ~3 months and used a dissimilar storage temperature of 6.6 °C, where freezing damage such as loss of turgor, cell lysis and increase membrane permeability would not occur.

Volatiles in garlic have been shown to change over storage conditions and treatment, but to the best of my knowledge the effect of a short-term cold treatment has not been previously reported. Commonly, such studies in *Alliums* analyse fungal and spoilage-associated VOCs (Li *et al.,* 2011; Vikram *et al.,* 2005). Others have studied volatiles in relation to culinary and agronomic quality traits (Abbey *et al.,* 2004; Abbey *et al.,* 2003). Postharvest treatments such as bulb irradiation have also been shown to change the volatile profile (Wu *et al.,* 1996). Whilst this has not been studied in garlic, temperature abuse in rocket caused a significant decrease in quality in comparison to an isothermally stored control and was accompanied by a change in the VOC profile (Mastrandrea *et al.,* 2017).

The change in volatile profile reported here could provide a useful marker in addition to the loss of firmness to indicate poor storage of garlic cloves, although further work would be required to verify that the effects seen with *cv. Morado*, are reproducible in other cultivars. The change in VOC profile might indicate a change in consumer perception of the smell of the bulb. However, key sulphur compounds, which are the main determinants of the scent of garlic, were not affected (Mazza *et al.*, 1992).

Warehouse trialling of the GCMS based method for volatile analysis successfully discriminated garlic at different storage time points. Both CAP and random forest discriminated the samples based on VOC profile and as these methods use different approaches to analyse VOC profiles, this increases the confidence that a difference exits. However, the VOC profiles did not discriminate between cultivars; this may be due to the sampling method as both cultivars were stored in the same warehouse and the VOCs may have mixed in the air. However, this could be considered a benefit, as the main objectives of such a tool would be to assess freshness, not discriminate between cultivar. The passive sampling method yielded peaks of comparable abundance to the lab based sampling, however fewer compounds were detected. This may be attributable to the lower temperature in the warehouse reducing the vapour pressure of compounds and causing the least volatile compounds to present in the headspace at a concentration below the detection limit of the GCMS (Peñuelas & Llusià, 2001). Further study over multiple seasons would be required in order to fully validate the VOC profiles as a quality control tool to check that these differences are conserved across harvest years. Since garlic is stored for periods of many months, it is viable to analyse VOCs offsite by GCMS, as the delay to receive the results would not cause them to be outdated.

Organosulphur compounds from garlic were successfully detected and quantified in terms of relative abundance with a PFPD. Fewer organosulphur VOCs were detected than in the work with the TD-GC-ToF-MS, which may be due to only 500 mL instead of 7,500

mL headspace being sampled. However, when running samples in duplicate on the GC-MS, only 5 of the 10 peaks seen with the PFPD were detected, suggesting the PFPD was considerably more sensitive than MS. The amount of headspace could not be increased beyond 500 mL on the PFPD due to the most abundant compounds overloading the sensor, so if more compounds were to be detected, samples would have to be run at two split ratios, or sampled twice with different volumes of headspace. One major benefit of the PFPD was the short run times, with each run taking 17 min, instead of the 2 h 30 min per sample run at two split ratios on the TD-GC-ToFMS, allowing a higher rate of sample throughout. Whilst samples could not be fully discriminated on the MDS plot (Fig. 5.9b), the random forest algorithm performed with an error rate of only 11 %, which may be reduced further with replication, as it was generated from only 3 replicated samples. Allyl methyl sulphide, as previously identified in Chapter 4 as being indicative of high alliinase activity, was detected by PFPD. The alliinase activity of samples analysed by PFPD was not measured in this experiment, but warrants further study. PFPD shows potential as a tool for garlic VOC analysis which could offer significant time and money savings to industry.

As well as PFPD, there are other low cost VOC analysis tools available, such as eNoses, which have been used extensively in the food industry for spoilage detection (Casalinuovo *et al.*, 2006). eNoses have been applied to *Alliums* in the past. An eNose was used to successfully discriminate between soil type treatments and nitrogen and sulphur fertilisation regimes in field grown onion but did not correlate with conventional analytical and sensory measures of onion quality (Abbey *et al.*, 2004). VOCs of diced onion have also been analysed by eNoses, where a significant change in the VOC profile was detected after 9 days of storage, but not 3 or 6 days (Abbey & Joyce, 2007). The eNose in this example was able to detect changes in the VOC profile associated with the lachrymatory factor and total pyruvic acid concentration, which is relevant to the quality

of onions. Classification of garlic cultivars was also possible by eNose, detecting compounds initially identified by GC-MS analysis, similarly to work presented here (Trirongjitmoah *et al.*, 2015). VOCs given off by fungal spoilage organisms can also be detected with eNoses, with sufficient sensitivity to detect the VOCs before visible signs of spoilage were apparent and also to discriminate between spoilage organisms to a degree (Keshri *et al.*, 1998). eNose arrays of various complexity and technologies are therefore appropriate low-cost methods for potential volatile based quality control tools of *Alliums*. eNoses would act as a suitable alternative to PFPD if a VOC was identified as being highly predictive of garlic quality that the PFPD could not detect, such as a hydrocarbon, phenol or terpene. However, eNoses have a poorer sensitivity than PFPD, with eNose limits of detection being in the order of parts per million, whereas PFPD operates in the parts per billion range of sensitivity, which may limit the range of compounds detectable by eNose (Chatterjee *et al*., 2013; Catalan *et al*., 2006).

In conclusion, a 96h period of -5 °C cold stress did not affect alliinase activity in stored garlic, but caused a significant increase in water content and loss of firmness. The VOC profile of stressed garlic was altered and 12 compounds were identified that changed significantly after stress, upon which the samples discriminated. Warehouse trials of the TD-GC-ToF-MS sampling and analysis method discriminated the VOC profile over storage time, but did not discriminate between cultivar. Trialling of the PFPD detected 10 VOCs of which 5 were identified, including allyl methyl sulphide. As such, it showed great promise as a tool which could be developed for quality assessment in garlic.

6. Chapter 6- General Discussion

6.1. Synthesis

The work presented here furthers current understanding of the postharvest biology of garlic. For the first time, alliinase activity has been monitored over a time course throughout storage at different temperatures. Potential molecular marker systems have been developed for the postharvest quality assessment of garlic in relation to agronomically important quality parameters as well as alliinase activity which perform across cultivars. VOC sampling methods have been tested for work in industry and furthermore, new analysis methods have been assessed using PFPD, potentially offering significant cost savings over GCMS.

Chapter 3 focussed on one cultivar of garlic, *Altanero*, and studied the effects of cold and ambient temperature storage on physiological and enzymatic quality traits and correlated these changes with VOCs over a 12 month time course. The major finding was that alliinase activity remained higher at ambient storage temperature for up to 6 months compared to storage at -2 °C. Although cold storage has been shown to change the sulphur chemistry of garlic, the negative impact of cold storing garlic on alliinase activity in some garlic cultivars has not previously been reported to my knowledge. Disease incidence was lower in the cold stored samples after extended periods of storage, broadly agreeing with the literature (Llamas *et al.*, 2013). No significant increase in sprouting rate was found between the two temperatures, which goes against the findings of Vazquez-Barrios *et al.*, (2006) but supports the findings of Cantwell *et al.*, (2003) suggesting a cultivar dependant or growing locality response to sprouting at ambient storage (Cantwell, 2004). Two distinct groups of VOCs were identified which correlated with storage time and storage temperature, facilitating a potential quality monitoring system.

Chapter 4 expanded on the previous chapter, with three additional cultivars studied over two experimental years. Cultivar and year of harvest have previously been shown to influence strongly quality characteristics of garlic at harvest and throughout storage (Waterer & Schmitz, 1994; Sharma *et al.*, 2005; Shalaby & El-Ramady, 2014). The degree of intercultivar differences between garlic grown and stored in the same way was further studied and significant differences in alliinase activity were found between cropping year. What has become apparent from this research is the high degree of variability in economically important quality traits even between garlic grown in the same locality under very similar agronomic regimes. It is not apparent what caused this interseasonal variability or how it interacts with cultivar. Studies performed on just one cultivar or over one cropping season need to be interpreted with caution. Furthermore, it adds to the pre-existing limitations of applying the findings of research to other cultivars and the applicability of findings year on year (Volk & Stern, 2009; Waterer & Schmitz, 1994).

Chapter 5 examined applied aspects of the work reported in both previous chapters. Evidence was presented for the existence of a cold-stress response in garlic subjected to a temperature of -5 °C for 96 h during the 6 month storage period at -2 °C. Storage at -4 °C was proposed for the control of *Penicillium hirsutum*, citing no occurrence of freezing injury (Bertolini & Tian, 1996), but firmness was not measured in their study. A VOC marker system was developed which discriminated between cold stressed and unstressed bulbs with a 0 % error rate in all samples tested in this study. Discrimination has been shown between the VOCs of plants under abiotic stress before, but this is the first time in garlic (Jardine *et al.*, 2013). Whilst the literature suggested an increase in total phenolic compounds in the cell would occur, due to their protective effects against cold stress, this was not observed in the volatile profile of garlic that was cold stressed (Christie *et al.*, 1994; Ncube *et al.*, 2012). However, as the control group was stored at -2 °C, phenolic

content may have been high in both samples and may not have increased significantly in response to the cold stress. Furthermore, phenolic compounds are not all volatile, so it is entirely possible that an increase in non-volatile phenolic compounds did occur, however this was not measured. The methodologies designed in previous chapters were adapted to be used in industry, with onsite passive sampling and offsite GCMS analysis. The discrimination between storage time points improved in comparison to the lab experiments, but cultivars could not be separated. Despite the sampling having been performed inside crates containing one cultivar of garlic, diffusion of VOCs from the broader warehouse air into the crate is likely to account for the lack of discrimination between cultivars. Pulsed Flame Photometric Detection showed promise as an alternative detector to the GC-MS approach with significant cost savings, whilst retaining high sensitivity but would require verification with pure standards for peak identification.

6.2. The stability of alliinase in stored garlic

Alliinase is highly sensitive to low pH, freeze-thaw cycles and loses activity even in frozen aqueous solution (Lawson Wang, 2001; Mazelis & Crews, 1968; Jansen *et al.*, 1989). The most stable preparations of alliinase are those that are dehydrated, either by freeze drying, such as the extracts in this study, or by convective drying, or those in stored in glycerol (Mallika *et al.,* 2014; Lagunas & Castaigne, 2008; Mazelis & Crews, 1968). Alliinase has been extensively studied in terms of protein and transcript levels throughout growth, (Rabinkov *et al.,* 1994; Smeets *et al.,* 1997). However, little is known about how alliinase activity changes throughout storage or if storage temperature affects maintenance of alliinase activity postharvest.

Garlic is known to be variable between cropping years in terms of physiological and biochemical characteristics, but much less is known about the yearly variance in alliinase activity (Waterer & Schmitz, 1994; Maaβ & Klaas, 1995). There was a high degree of

variability in the alliinase activity of all cultivars between years of growth, which to my knowledge has not been reported before. Furthermore, maintenance of alliinase activity throughout storage was markedly different between the years. Data presented here suggests that cold storage of garlic may not be optimal for alliinase activity maintenance, however it does keep disease, sprouting and weight loss to a minimum. Whilst there is also a cultivar and season effect, further study into the relationship between storage temperature and alliinase activity maintenance throughout storage is warranted.

6.3. Expression of cysteine synthase in stored garlic

The expression of cysteine synthase was studied, with no clear pattern of expression discerned over storage at either temperature. Cysteine synthase catalyses the terminal step of cysteine biosynthesis, an amino acid that is the essential precursor of cysteine sulphoxides (CSO) in garlic, therefore being implicated in synthesis of sulphur VOCs (Jones *et al.*, 2004; Jones *et al*., 2007). However, there are other downstream enzymes in the CSO pathway which were not studied here. For instance, gamma-glutamyl transpeptidases (GGTs) catalyse the removal of the gamma-glutamyl moiety from the putative biosynthetic intermediate of alliin and flavin-containing monooxygenase (AsFMO1), which is responsible for the S-oxygenation reaction in the biosynthesis of alliin (Yoshimoto *et al.*, 2015a; Yoshimoto *et al.*, 2015b). Future work would consider studying the expression of genes coding for these enzymes and to attempt to correlate changes in VOC profile with changes in gene expression.

6.4. VOC markers for alliinase activity in stored garlic

The results demonstrate that garlic varies significantly between years based on volatiles and alliinase activity. A potential molecular marker system for the post-harvest quality assessment of alliinase activity over time in storage is presented, which used the relative abundance of three compounds (allyl methyl sulphide, 1,4-dicyclohexylbenzene and

methyl propyl sulphide) and functioned across three cultivars of garlic (two Spanish purple, one Chinese white) over two storage seasons and at both ambient and -2 °C storage temperature. To the best of my knowledge, this is the first study that correlates alliinase activity of garlic with the VOCs emitted by bulbs in storage.

This presents an interesting opportunity for biotech firms aiming to extract alliinase from garlic, as VOCs could be used as a proxy for average alliinase activity across a warehouse or storage crate of garlic. It is hoped that these tools can ultimately improve the efficiency and reduce costs of alliinase extractions by directing high-activity garlic to such processes and detecting disease earlier.

Furthermore, a relationship was identified between samples with high alliinase activity and the presence of AMS. This offers the opportunity to detect which samples have a high alliinase activity (> 0.074 umol mg^{-1} min^{-1}, corresponding to the top 20^{th} percentile of samples measured here). AMS is widely reported in the VOC profile of breath from those who have recently consumed garlic (Munch & Barringer, 2014; Van Den Velde *et al.*, 2007; Rosen *et al.*, 2000). It has been shown that AMS in breath is derived from allyl thiosulphinates, predominantly allicin, and furthermore than AMS is the main metabolite of allicin in the human body (Lawson & Wang, 2005). Furthermore, AMS presence in breath has been used to quantify the bioavailability of allyl thiosulphinates, including allicin, from enteric-coated dried fresh garlic and dried aged garlic extract in clinical trials (Lawson & Gardner, 2005). A common cause of poor allyl thiosulphinate bioavailability from such tablets is low alliinase activity, commonly due to the enzymes sensitivities to pH and low stability in solution (Lawson & Wang, 2001). However, this is the first instance to my knowledge where AMS release from stored, intact bulbs of garlic is shown to predict the activity of alliinase extracts from those bulbs.

AMS was detected in the VOC profiles of the warehouse trail and by PFPD, suggesting it is not only a good potential marker for alliinase activity in the lab, but that it can also be applied to industry. The limitation, however, is that AMS abundance does not have a simple relationship with alliinase activity when present and the data here suggests that it cannot be used to predict the precise activity of the sample beyond assigning it to the 20th percentile of highest activity. Furthermore, it is unknown if changing the detector from MS to PFPD or using the warehouse VOC sampling method would alter the sensitivity of the detection, so this method would need refinement and calibration to work in industry. Besides its potential applications for quality control of garlic bulbs for alliinase extraction and similar biotechnological work, it may be able to assist crop breeders in developing high-alliinase activity cultivars. As the sampling method is entirely non-destructive, no seed cloves would need to be sacrificed. Also, the VOC analysis can be completed in less time than an alliinase extraction and activity assay, with a higher degree of automation, saving costs.

6.5. Quality changes in stored garlic

Broadly, the changes noted in physiological quality parameters were in line with the literature. Quality changed more slowly in the first 3 months of storage, consistent with the hypothesis of a dormant period lasting approximately 90 days post-harvest during which garlic stores well and resists sprouting (Pellegrini *et al.*, 1998). However, it is worth noting that the garlic was harvested ~ 2 months prior to the start of the storage experiment, and so would have been part of the way through this 90 day dormant period. The water content of the bulbs was remarkably consistent over storage and in line with values previously reported (Lawson, 1996). The proportion of diseased bulbs was indeed higher in most cultivars when stored at ambient conditions, however only occurred after 6 or 12

months of storage depending on the cultivar, which is in agreement with previous studies (Tyson & Fullerton, 2004; Llamas *et al.*, 2013). However, the proportion of diseased bulbs observed in this study were significantly lower at ambient conditions than previously reported, which may be due to the small number of bulbs stored together, which may reduce the initial disease pressure (Tripathi & Lawande, 2006). Here, only cultivar and storage temperature significantly affected the incidence of sprouting, whereas in other studies, storage time was also a significant factor (Cantwell *et al.*, 2003).

Data presented here show a more complex relationship between garlic cultivar, storage temperature and storage time with respect to clove firmness than the literature suggests (Cantwell *et al.*, 2003; Park *et al.*, 2000). Some cultivars showed a linear decrease of firmness over time as previously reported, while the firmness of others decreased at first and then rose back to harvest levels. Whilst the mechanisms of firmness loss have been studied in onion (Coolong *et al*., 2008), little is known about how firmness is lost in garlic. In the aforementioned study, high dry matter content was associated with firmness in onions; however the data presented here show that dry matter content does not change substantially in stored garlic. This suggests a difference in the mechanism of firmness loss between onions and garlic, however the other factors found to be associated with firmness loss in onion, namely uronic acid content and the thickness of the middle lamellae, have not yet been studied in garlic.

Here, sprouting was determined as when the developing shoot emerged from the tip of the clove. This is inferior to using the internal sprouting index, as described by Pellegrini *et al.*, 2000 and Cantwell *et al*., 2003, by which the length of the developing shoot within the clove is expressed as a proportion of the total length of the clove. The internal sprouting index can be used to estimate shelf-life, with internal sprouting index of < 50 % suggested as a proxy for the end of the effective shelf life of garlic (Vazquez-Barrios *et al.*, 2006). Measuring the internal sprouting is typically a destructive process, which

was not compatible with experiments performed here, however it is possible to visualise the internal structure of the clove with Computed-Tomography (CT) scanning (Comparini *et al.*, 2016). This would allow comparisons to be made between the sprouting index and the VOC analysis methods outlined here.

Likewise, VOCs discriminated between the proportion of disease in samples, which is also believed to be novel in garlic, but studies in onion have identified VOCs associated with specific pathogen inoculation (Wang *et al.,* 2015). Garlic suppliers for the culinary industry could benefit from using the VOC marker system for disease rate as an early warning system for rising infection rates in long-term stored garlic. This would create a greater opportunity for intervention with pesticides or cultural control measures as detailed in Mishra *et al.,* (2014) and El-Marzoky *et al.,* (2013).

An area that merits further study is the use of VOCs for identification of spoilage organisms. This has been performed in onion, where successful identification and discrimination of common storage pathogens was achieved (Vikram *et al.,* 2005; Fischer *et al.,* 1999; Prithiviraj *et al.*, 2004). A similar investigation in garlic would have extensive applications as an early warning system for pathogens. The method would allow a non-intrusive, rapid and non-destructive measurement of VOCs from bulbs even if disease outbreaks occur in inaccessible crates, which could easily be missed by manual checks.

6.6. VOC markers for storage conditions and quality of stored garlic

The VOC profile of garlic changed over time in storage irrespective of the proportion of disease within a sample. This suggests that there are biological processes within the garlic which change over time and lead to a change in the VOC profile throughout storage. Gamma-glutamyl peptides and sulfoxides have been shown to change in concentration throughout storage, suggesting a time dependent modulation of the organosulphur biochemistry of garlic throughout storage (Ichikawa *et al.*, 2006). Furthermore, in nature,

garlic would not be exposed to the environmental conditions of storage for such long periods of time and it is reasonable to expect a stress response to this. Stress response compounds such as allixin, are known to become significantly more abundant throughout long-term storage (Kodera *et al*., 2002). It is possible, therefore, that a broader change in metabolism in response to long term stress causes differential rates of synthesis of volatile compounds, or the synthesis of new volatile compounds in response to the stress. Furthermore, membrane breakdown could occur due to chilling injury (Lyons, 1973), oxidative stress (Hodges *et al.*, 2004) or pathogen attack, allowing alliin and alliinase to interact and generating organosulphur VOCS.

The analysis of garlic 'Altanero' in this thesis showed that garlic bulbs can remain in a saleable condition for 12 months. Therefore, confirming that garlic which is claimed to be fresh, from the new season's harvest, is indeed fresh and not from last season's stock may be a legitimate concern if buying from a seller of unknown reputability. It may be possible to develop a marker system to discriminate between garlic of new and old seasons, with potential commercial applications. However, these differences proved challenging to detect in subsequent work in Chapter 4, suggesting that either cultivar or seasonality is affecting the VOC profile in some way. With further replications over more seasons and possibly considering more cultivars, it would be possible to confirm whether any stable markers for storage time exist which could be applicable to stored garlic more broadly.

The warehouse marker system needs to be trialled *in situ* over several more seasons, to see if the VOCs identified here change consistently and predictably in different harvest years. Expanding the selection of cultivars and working with garlic from different countries would be of interest, as it could validate the global applicability of such a marker system. Further integration of the VOC markers with existing quality control measures in industry would offer exciting new opportunities to correlate VOCs with these traits and

potentially expand the marker system to encompass more factors. For instance, root growth has a negative effect on garlic shelf life (Fraga & Enríquez, 1998). Root growth was not assessed or observed in this thesis, however it is a widely reported issue that occurs throughout storage, occurring in response to high humidity (> 80 % RH) (Hwang & Lee, 1990). Storing garlic at higher humidity to purposely induce root growth may elicit changes in the VOC profile that could be identified using the methods developed in this thesis, allowing the development of a VOC marker tool for the detection of root growth in stored garlic. It is also possible to apply the methods outlined in this thesis for correlating VOC profiles with postharvest quality parameters for other crops which are stored long-term, such as onions, potatoes or apples. This could lead to the marker system acting as an early warning system for garlic stores for end of shelf-life detection. Further development of PFPD-based VOC analysis would also allow for on-site analysis to be performed.

The biosynthetic pathways that produce the VOCs which were identified as being indicative of quality traits in this thesis are to the best of my knowledge unknown. Elucidation of the biosynthetic pathways of these compounds would allow a mechanistic understanding of why these compounds change in abundance. Some VOCs are known to be a response to, or are associated with resistance to abiotic stress, for example, so monitoring this may be indicative of stresses within the stored bulbs (Possell & Loreto, 2013; Šimpraga *et al.*, 2011). This in turn could be used to make informed choices about storage conditions.

To conclude, future work should focus on exploring the applicability of the putative VOC marker systems outlined in this thesis to further seasons and cultivars. Measurement of the length of the growing shoot within the clove may allow VOCs to be associated with the Sprouting Index and provide an early warning system for garlic nearing the end of its shelf life. Identifying the source of the variability in alliinase activity between seasons

could allow for mitigation steps to improve activity in poor years; the data presented here suggests that this process occurs pre-harvest. Therefore, monitoring the growth conditions of the crop or performing a crop trial under controlled conditions may help elucidate the environmental cues or stresses which affect the alliinase activity of the harvested bulb. Specific primers to each of the 9 known alliinase genes would further help to understand how alliinase genes are expressed throughout storage. Further development of the GC-MS and PFPD methodology, particularly *in situ*, is warranted, with the aim of developing a commercially applicable marker system for alliinase activity and broader quality parameters.

7. References

A. Liaw and M. Wiener (2002). Classification and Regression by randomForest. *R News*, 2, 18--22.

Abbey, L., & Joyce, D. C. (2007). Quality assessment of diced onion (*Allium cepa* L.) using an electronic nose. *Ghana Journal of Science*, 47, 83-90.

Abbey, L., Joyce, D. C., Aked, J., & Smith, B. (2003). Electronic Nose-based Discrimination among Spring Onions Grown on Two Different Soils at Three Water-deficit Stress Levels. *Acta horticulturae*, 600, 479-484.

Abbey, L., Joyce, D. C., Aked, J., Smith, B., & Marshall, C. (2004). Electronic nose evaluation of onion headspace volatiles and bulb quality as affected by nitrogen, sulphur and soil type. *Annals of Applied Biology*, 145, 41-50.

Abd-Alla, M. A., El-Mohamedy, R. S. R., & Badeaa, R. I. (2006). Effect of some volatile compounds on black mould disease on onion bulbs during storage. *Research Journal of Agriculture and Biological Sciences*, 2, 384.

Ackermann, R. T., Mulrow, C. D., Ramirez, G., Gardner, C. D., Morbidoni, L., & Lawrence, V. A. (2001). Garlic shows promise for improving some cardiovascular risk factors. *Archives of Internal Medicine*, 161, 813-824.

Adekpe, D. I., Shebayan, J. A. Y., Chiezey, U. F., & Miko, S. (2007). Yield responses of garlic (*Allium sativum* L.) to oxadiazon, date of planting and intra-row spacing under irrigation at Kadawa, Nigeria. *Crop Protection*, 26, 1785-1789.

Agarwal, K. C. (1996). Therapeutic actions of garlic constituents. *Medicinal Research Reviews*, 16, 111-124.

Akan, S., Gunes, N. T., & Yanmaz, R. (2019). Methyl jasmonate and low temperature can help for keeping some physicochemical quality parameters in garlic (*Allium sativum* L.) cloves. *Food Chemistry*, 270, 546-553.

Amagase, H. (2006). Clarifying the real bioactive constituents of garlic. *The Journal of Nutrition*, 136, 716S-725S.

Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S., & Itakura, Y. (2001). Intake of garlic and its bioactive components. *The Journal of Nutrition*, 131, 955S-962S.

Amirav, A., & Jing, H. (1995). Pulsed flame photometer detector for gas chromatography. *Analytical Chemistry*, 67, 3305-3318.

Amirav, A., & Jing, H. (1998). Simultaneous pulsed flame photometric and mass spectrometric detection for enhanced pesticide analysis capabilities. *Journal of Chromatography A*, 814, 133-150.

Anderson, M. J., & Willis, T. J. (2003). Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. *Ecology*, *84*(2), 511-525.

Ankri, S., & Mirelman, D. (1999). Antimicrobial properties of allicin from garlic. *Microbes and infection*, 1, 125-129.

Appleton, M. D., Haab, W., Eisenstadt, M. L., Rodgers, R., & Thoman, C. J. (1981). Incorporation of maleic hydrazide into ribonucleic acid of Saccharomyces cerevisiae. *Journal of Agricultural and Food Chemistry*, 29, 986-989.

Aprea, E., Biasioli, F., Carlin, S., Endrizzi, I., & Gasperi, F. (2009). Investigation of Volatile Compounds in Two Raspberry Cultivars by Two Headspace Techniques: Solid Phase Microextraction/Gas Chromatography− Mass Spectrometry (SPME/GC− MS) and Proton-Transfer Reaction− Mass Spectrometry (PTR− MS). *Journal of agricultural and food chemistry*, 57, 4011-4018.

Argüello, J. A., Ledesma, A., Núñez, S. B., Rodríguez, C. H., & Goldfarb, M. D. C. D. (2006). Vermicompost effects on bulbing dynamics, nonstructural carbohydrate content, yield, and quality ofRosado Paraguayo'garlic bulbs. *HortScience*, 41, 589-592.

Ashraf, R., Aamir, K., Shaikh, A. R., & Ahmed, T. (2005). Effects of garlic on dyslipidemia in patients with type 2 diabetes mellitus. *Journal of Ayub Medical College Abbottabad*, 17, 60-4.

Atashi, S., Akbarpour, V., Mashayekhi, K., & Mousavizadeh, S. J. (2011). Garlic physiological characteristics from harvest to sprouting in response to low temperature. *Journal of Stored Products and Postharvest Research*, 2, 285-291.

Augusti, K. T., Chackery, J., Jacob, J., Kuriakose, S., George, S., & Nair, S. S. (2005). Beneficial effects of a polar fraction of garlic (*Allium sativum* Linn) oil in rats fed with two different high fat diets. *Indian Journal of Experimental Biology*, 43, 76-83.

Baldwin, I. T. (2010). Plant volatiles. *Current Biology*, 20, R392-R397.

Baldwin, I. T., Halitschke, R., Paschold, A., Von Dahl, C. C., & Preston, C. A. (2006). Volatile signaling in plant-plant interactions:" talking trees" in the genomics era. *Science*, 311, 812-815.

Banerjee, S. K., & Maulik, S. K. (2002). Effect of garlic on cardiovascular disorders: a review. *Nutrition Journal*, 1, 4.

Banerjee, S. K., Mukherjee, P. K., & Maulik, S. K. (2003). Garlic as an antioxidant: the good, the bad and the ugly. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 17, 97-106.

Bayat, F. (2004). Effect of storage duration and conditions on the weight loss and quality of the garlic populations of Hamadan province. *Journal of Agricultural Engineering Research*, 5, 49-62.

Bayat, F., & Rezvani, S. M. (2012). Effect of harvesting time and moisture on mechanical properties of garlic (Allium sativum L.) skin. *Agricultural Engineering International: CIGR Journal*, 14, 161-167.

Bayat, F., Rezvani, S., & Nosrati, A. E. (2009, April). Effect of harvesting time and curing temperature on some properties of Iranian white garlic. *In VI International Postharvest Symposium* 877, 869-875.

Beato, V. M., Orgaz, F., Mansilla, F., & Montaño, A. (2011). Changes in phenolic compounds in garlic (*Allium sativum* L.) owing to the cultivar and location of growth. *Plant Foods for Human Nutrition*, 66, 218-223.

Bell, L., Spadafora, N. D., Müller, C. T., Wagstaff, C., & Rogers, H. J. (2016). Use of TD-GC–TOF-MS to assess volatile composition during post-harvest storage in seven accessions of rocket salad (*Eruca sativa*). *Food Chemistry*, 194, 626-636.

Bertolini, P., & Tian, S. P. (1996). Low-temperature biology and pathogenicity of *Penicillium hirsutum* on garlic in storage. *Postharvest Biology and Technology*, 7, 83-89.

Bertoni, G., Morard, P., Soubieille, C., & Llorens, J. M. (1992). Growth and nitrogen nutrition of garlic (*Allium sativum* L.) during bulb development. *Scientia Horticulturae*, 50, 187-195.

Blania, G., & Spangenberg, B. (1991). Formation of allicin from dried garlic (*Allium sativum*): a simple HPTLC method for simultaneous determination of allicin and ajoene in dried garlic and garlic preparations. *Planta Medica*, 57, 371-375.

Block, E. (1985). The chemistry of garlic and onions. *Scientific American*, 252, 114-9.

Block, E. (1992). The organosulfur chemistry of the genus *Allium*–implications for the organic chemistry of sulfur. *Angewandte Chemie International Edition in English*, 31, 1135-1178.

Block, E. (2010). Garlic and Other *Alliums*. Cambridge: RSC publishing.

Bloem, E., Haneklaus, S., & Schnug, E. (2005). Influence of nitrogen and sulfur fertilization on the alliin content of onions and garlic. *Journal of Plant Nutrition*, 27, 1827-1839.

Bloem, E., Haneklaus, S., & Schnug, E. (2011). Storage life of field-grown garlic bulbs (*Allium sativum* L.) as influenced by nitrogen and sulfur fertilization. *Journal of Agricultural and Food Chemistry*, 59, 4442-4447.

Boatright, J., Negre, F., Chen, X., Kish, C. M., Wood, B., Peel, G., ... & Dudareva, N. (2004). Understanding in vivo benzenoid metabolism in petunia petal tissue. *Plant Physiology*, 135, 1993-2011.

Bocchini, P., Andalo, C., Pozzi, R., Galletti, G. C., & Antonelli, A. (2001). Determination of diallyl thiosulfinate (allicin) in garlic (*Allium sativum* L.) by high-performance liquid chromatography with a post-column photochemical reactor. *Analytica Chimica Acta*, 441, 37-43.

Borlinghaus, J., Albrecht, F., Gruhlke, M., Nwachukwu, I., & Slusarenko, A. (2014). Allicin: chemistry and biological properties. *Molecules*, 19, 12591-12618.

Bozin, B., Mimica-Dukic, N., Samojlik, I., Goran, A., & Igic, R. (2008). Phenolics as antioxidants in garlic (*Allium sativum* L., *Alliaceae*). Food Chemistry, 111, 925-929.

Brewester, J.L. 1994. Onion and other vegetable Alliums. UK: *University Press Cambridge*.

Brodmann, J., Twele, R., Francke, W., Hölzler, G., Zhang, Q. H., & Ayasse, M. (2008). Orchids mimic green-leaf volatiles to attract prey-hunting wasps for pollination. *Current Biology*, 18, 740-744.

Brodnitz, M. H., Pascale, J. V., & Van Derslice, L. (1971). Flavor components of garlic extract. *Journal of Agricultural and Food Chemistry*, 19, 273-275.

Buchanan, B. B., Gruissem, W., & Jones, R. L. (Eds.). (2015). Biochemistry and molecular biology of plants. *John Wiley & Sons*.

Calvey, E. M., Matusik, J. E., White, K. D., DeOrazio, R., Sha, D., & Block, E. (1997). Allium Chemistry: Supercritical Fluid Extraction and LC− APCI− MS of Thiosulfinates and Related Compounds from Homogenates of Garlic, Onion, and Ramp. Identification in Garlic and Ramp and Synthesis of 1-Propanesulfinothioic Acid S-Allyl Ester. *Journal of Agricultural and Food Chemistry*, 45, 4406-4413.

Calvo-Gómez, O., Morales-López, J., & López, M. G. (2004). Solid-phase microextraction–gas chromatographic–mass spectrometric analysis of garlic oil obtained by hydrodistillation. *Journal of Chromatography A*, 1036, 91-93.

Cantwell, M. I., Kang, J., & Hong, G. (2003). Heat treatments control sprouting and rooting of garlic cloves. *Postharvest Biology and Technology*, 30, 57-65.

Cantwell, M.I., 2004. Garlic. Recommendations for maintaining postharvest quality. http:/postharvest.ucdavis.edu/Produce/ProduceFacts.

Caporaso, N., Smith, S. M., & Eng, R. H. (1983). Antifungal activity in human urine and serum after ingestion of garlic (*Allium sativum*). *Antimicrobial Agents and Chemotherapy*, 23, 700-702.

Casalinuovo, I., Di Pierro, D., Coletta, M., & Di Francesco, P. (2006). Application of electronic noses for disease diagnosis and food spoilage detection. *Sensors*, 6, 1428-1439.

Catalan, L. J., Liang, V., & Jia, C. Q. (2006). Comparison of various detection limit estimates for volatile sulphur compounds by gas chromatography with pulsed flame photometric detection. *Journal of Chromatography A*, 1136, 89-98.

Cavagnaro, P., Masuelli, R., & Simon, P. W. (2003). Molecular data suggest multiple members comprising the alliinase gene family in garlic. *Hort Science*, 38, 804-5.

Cavallito, C. J., & Bailey, J. H. (1944). Allicin, the antibacterial principle of *Allium sativum*. I. Isolation, physical properties and antibacterial action. *Journal of the American Chemical Society*, 66, 1950-1951.

Chatterjee, S., Castro, M., & Feller, J. F. (2013). An e-nose made of carbon nanotube based quantum resistive sensors for the detection of eighteen polar/nonpolar VOC biomarkers of lung cancer. *Journal of Materials Chemistry B*, 1, 4563-4575.

Chen, T. H., & Murata, N. (2002). Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Current Opinion in Plant Biology*, 5, 250-257.

Cho, J., Park, M., Choi, D., & Lee, S. K. (2011). Cloning and expression of γ-glutamyl transpeptidase and its relationship to greening in crushed garlic (*Allium sativum*) cloves. *Journal of the Science of Food and Agriculture*, 92, 253-257.

Choi, D. J., Lee, S. J., Kang, M. J., Cho, H. S., Sung, N. J., & Shin, J. H. (2008). Physicochemical characteristics of black garlic (*Allium sativum* L.). *Journal of the Korean Society of Food Science and Nutrition*, 37, 465-471.

Chong, K., Zamora, M. P., Tilakawardane, D. A., Buckley, N. E., Rego, J. A., & Liu, Y. (2014). Investigation of Allicin Stability in Aqueous Garlic Extract by High Performance Liquid Chromatography Method.

Christie, P. J., Alfenito, M. R., & Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, 194, 541-549.

Chung, L. Y. (2006). The antioxidant properties of garlic compounds: allyl cysteine, alliin, allicin, and allyl disulfide. *Journal of Medicinal Food*, 9, 205-213.

Ciesa, F., Dalla Via, J., Wisthaler, A., Zanella, A., Guerra, W., Mikoviny, T., ... & Oberhuber, M. (2013). Discrimination of four different postharvest treatments of 'Red Delicious' apples based on their volatile organic compound (VOC) emissions during shelf-life measured by proton transfer reaction mass spectrometry (PTR-MS). *Postharvest Biology and Technology*, 86, 329-336.

Comparini, D., Kihara, T., & Kawano, T. (2016). Uses of X-ray 3D-computed-tomography to monitor the development of garlic shooting inside the intact cloves. *Environmental Control in Biology*, 54, 39-44.

Coolong, T. W., Randle, W. M., & Wicker, L. (2008). Structural and chemical differences in the cell wall regions in relation to scale firmness of three onion (*Allium cepa* L.) selections at harvest and during storage. *Journal of the Science of Food and Agriculture*, 88, 1277-1286.

Costa, G., Noferini, M., Fiori, G., & Spinelli, F. (2007). Innovative application of non-destructive techniques for fruit quality and disease diagnosis. *Acta Horticultura*e, 753, 275.

CREA (2016). SIAR Integral Advisory Service to the Irrigator of Castilla La-Mancha, SPAIN. Ministry of Agriculture of the Government of Castilla-La Mancha. C / Pintor Matías Moreno, 4 CP 45071 TOLEDO. http://crea.uclm.es/siar/recomendaciones/ Accessed: 14th Aug. 2019

Croci, C. A., & Curzio, O. A. (1983). The influence of gamma-irradiation on the storage life of "red" variety garlic. *Journal of Food Processing and Preservation*, 7, 179-183.

Curzio, O. A., & Urioste, A. M. (1994). Sensory quality of irradiated onion and garlic bulbs. *Journal of Food Processing and Preservation*, 18, 149-158.

Del Rio, D., Costa, L. G., Lean, M. E. J., & Crozier, A. (2010). Polyphenols and health: what compounds are involved? *Nutrition, Metabolism and Cardiovascular Diseases*, 20, 1-6.

Dicke, M., & Baldwin, I. T. (2010). The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. *Trends in Plant Science*, 15, 167-175.

Diriba-Shiferaw, G., Nigussie-Dechassa, R., Kebede, W., Getachew, T., & Sharma, J. J. (2013). Growth and nutrients content and uptake of garlic (*Allium sativum* L.) as influenced by different types of fertilizers and soils. *Science, Technology and Arts Research Journal*, 2, 35-50.

Dorant, E., van den Brandt, P. A., Goldbohm, R. A., Hermus, R. J., & Sturmans, F. (1993). Garlic and its significance for the prevention of cancer in humans: a critical view. *British Journal of Cancer*, 67, 424.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, 88, 308-316.

Du, X., Song, M., Baldwin, E., & Rouseff, R. (2015). Identification of sulphur volatiles and GC-olfactometry aroma profiling in two fresh tomato cultivars. *Food Chemistry*, 171, 306-314.

Dufoo-Hurtado, M. D., Zavala-Gutiérrez, K. G., Cao, C. M., Cisneros-Zevallos, L., Guevara-González, R. G., Torres-Pacheco, I., Vázquez-Barrios, M. E., Rivera-Pastrana, D. M. & Mercado-Silva, E. M. (2013). Low-temperature conditioning of "seed" cloves

enhances the expression of phenolic metabolism related genes and anthocyanin content in ‘coreano’ garlic (*Allium sativum*) during plant development. *Journal of Agricultural and Food Chemistry*, 61, 10439-10446.

Effmert, U., Kalderás, J., Warnke, R., & Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. *Journal of Chemical Ecology*, 38, 665-703.

El Ariebi, N., Hiscox, J., Scriven, S. A., Müller, C. T., & Boddy, L. (2016). Production and effects of volatile organic compounds during interspecific interactions. *Fungal Ecology*, 20, 144-154.

Ellmore GS, Feldberg RS (1994) Alliin lyase localization in bundle sheaths of the garlic clove (*Allium sativum*). *American Journal of Botany*, 81, 89-94

El-Marzoky, H. A., & Shaban, W. I. (2013). Studies on some Garlic Diseases during Storage in Egypt. *Journal of Applied Plant Protection*, 2, 15-20.

El-Mogy, M. M., Ludlow, R. A., Roberts, C., Müller, C. T., & Rogers, H. J. Postharvest exogenous melatonin treatment of strawberry reduces postharvest spoilage but affects components of the aroma profile. *Journal of Berry Research*, (Preprint), 1-11.

Endo, A., Imai, Y., Nakamura, M., Yanagisawa, E., Taguchi, T., Torii, K. & Ichinose, K. (2014). Distinct intraspecific variations of garlic (*Allium sativum* L.) revealed by the exon–intron sequences of the alliinase gene. *Journal of Natural Medicines*, 68, 442-447.

Engeland, L. (1991). Growing great garlic: The definitive guide for organic gardeners and small farmers. Filaree farms. Okanogan, WA. 226p.

Enüstün, B. V., Şentürk, H. S., & Yurdakul, O. (1978). Capillary freezing and melting. *Journal of Colloid and Interface Science*, 65, 509-516.

ETOH, T. (1985). Studies on the Sterility in Garlic, *Allium sativum* L. *Memoirs of the Faculty of Agriculture, Kagoshima University*, 21, 77-132.

Etoh, T., Noma, Y., Nishitarumizu, Y., & Wakamoto, T. (1988). Seed productivity and germinability of various garlic [*Allium sativum* L.] clones collected in Soviet Central Asia. *Memoirs of the Faculty of Agriculture-Kagoshima University* (Japan).

Evans, J. A., Eyre, C. A., Rogers, H. J., Boddy, L., & Müller, C. T. (2008). Changes in volatile production during interspecific interactions between four wood rotting fungi growing in artificial media. *Fungal Ecology*, 1, 57-68.

Fanaei, H., Narouirad, M., Farzanjo, M., & Ghasemi, M. (2014). Evaluation of yield and some agronomical traits in garlic genotypes (*Allium sativum* L.). *Annu. Res. Rev. Biol*, 4, 3386-3391.

FAO (2017). FAOSTAT. Food and Agriculture Organization of the United Nations, Rome, Italy.

Figliuolo, G., Candido, V., Logozzo, G., Miccolis, V., & Zeuli, P. S. (2001). Genetic evaluation of cultivated garlic germplasm (*Allium sativum* L. and *A. ampeloprasum* L.). *Euphytica*, 121, 325-334.

Fischer, G., Schwalbe, R., Möller, M., Ostrowski, R., & Dott, W. (1999). Species-specific production of microbial volatile organic compounds (MVOC) by airborne fungi from a compost facility. *Chemosphere*, 39, 795-810.

Fleischauer, A. T., Poole, C., & Arab, L. (2000). Garlic consumption and cancer prevention: meta-analyses of colorectal and stomach cancers. *The American Journal of Clinical Nutrition*, 72, 1047-1052.

Fonollosa, J., Halford, B., Fonseca, L., Santander, J., Udina, S., Moreno, M., ... & Marco, S. (2009). Ethylene optical spectrometer for apple ripening monitoring in controlled atmosphere store-houses. *Sensors and Actuators B: Chemical*, 136, 546-554.

Fraga, R. (1998). Conservación postcosecha de nuevas variedades de ajo irradiadas y sin irradiar. *Alimentaria: Revista de Tecnología e Higiene de Los Alimentos*, 295, 85-89.

Fraga, R., & Enríquez, I. I. (1998). Envase y forma de almacenamiento adecuado para la coservación postcosecha del ajo irradiado y sin irradiar. *Alimentaria: Revista de Tecnología e Higiene de Los Alimentos*, 295, 91-96.

Fratianni, F., Riccardi, R., Spigno, P., Ombra, M. N., Cozzolino, A., Tremonte, P., ... & Nazzaro, F. (2016). Biochemical characterization and antimicrobial and antifungal activity of two endemic varieties of garlic (*Allium sativum* L.) of the campania region, southern Italy. *Journal of Medicinal Food*, 19, 686-691.

Freeman, F., & Kodera, Y. (1995). Garlic chemistry: stability of S-(2-propenyl)-2-propene-1-sulfinothioate (allicin) in blood, solvents, and simulated physiological fluids. *Journal of Agricultural and Food Chemistry*, 43, 2332-2338.

Gallego, E., Roca, F. J., Perales, J. F., Sánchez, G., & Esplugas, P. (2012). Characterization and determination of the odorous charge in the indoor air of a waste treatment facility through the evaluation of volatile organic compounds (VOCs) using TD–GC/MS. *Waste Management*, 32, 2469-2481.

Gaquerel, E., Weinhold, A., & Baldwin, I. T. (2009). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphigidae) and its natural host *Nicotiana attenuata.* VIII. An unbiased GCxGC-ToFMS analysis of the plant's elicited volatile emissions. *Plant Physiology*, 149, 1408-1423.

Gast, K.L.B., 2001. Storage conditions: fruits and vegetables. In: Postharvest Management of Commercial Horticultural Crops, Bulletin, no. 4135. The University of Maine, USA

Gebhardt, R. (1993). Multiple inhibitory effects of garlic extracts on cholesterol biosynthesis in hepatocytes. *Lipids*, 28, 613-619.

Goff, S. A., & Klee, H. J. (2006). Plant volatile compounds: sensory cues for health and nutritional value? *Science*, 311, 815-819.

Granroth, B. (1970). Biosynthesis and decomposition of cysteine derivatives in onion and other *Allium* species. Biosynthesis and decomposition of cysteine derivatives in onion and other *Allium* species. Annales Academiae, Scientiarum Fennicae, 154, 71.

Guevara-Figueroa, T., López-Hernández, L., Lopez, M. G., Hurtado, M. D., Vázquez-Barrios, M. E., Guevara-Olvera, L., ... & Mercado-Silva, E. M. (2015). Conditioning garlic "seed" cloves at low temperature modifies plant growth, sugar, fructan content, and sucrose sucrose fructosyl transferase (1-SST) expression. *Scientia Horticulturae*, 189, 150-158.

Guoyi, C. Z. L. (1992). Effects of Seed Clove Storage Temperatures and Humidty on the Physiology and Secondary Growth of Garlic [J]. *Journal of Northwest Sci-Tech University of Agriculture and Forestry*, 4.

Gupta, N., & Porter, T. D. (2001). Garlic and garlic-derived compounds inhibit human squalene monooxygenase. *The Journal of Nutrition*, 131, 1662-1667.

Hahn, G. 1996. Botanical characterization and cultivation of garlic. In: H.P. Kock and L.D. Lawson (eds.). Garlic: The science and therapeutic application of *Allium sativum* L. and related species. Williams and Wilkins, Baltimore.

Halitschke, R., Stenberg, J. A., Kessler, D., Kessler, A., & Baldwin, I. T. (2008). Shared signals–'alarm calls' from plants increase apparency to herbivores and their enemies in nature. *Ecology Letters*, 11, 24-34.

Hanson, B., May, D., Voss, R., Cantwell, M., & Rice, R. (2003). Response of garlic to irrigation water. *Agricultural Water Management*, 58, 29-43.

Hanum T, Sinha NK, Cash JN (1995) Characteristics of gamma-glutamyl-transpeptidase and alliinase of onion and their effects on the enhancement of pyruvate formation in onion macerates. *Journal of Food Biochemistry*, 19, 51–65.

Haque, M. S., Sattar, A., & Pramanik, H. R. (2002). Land configuration and varietal effects on yield contributing traits and yield of garlic. *Pakistan Journal of Biological Sciences*, 5, 1024-1027.

Harauma, A., & Moriguchi, T. (2006). Aged garlic extract improves blood pressure in spontaneously hypertensive rats more safely than raw garlic. *The Journal of Nutrition*, 136, 769S-773S.

Harenberg, J., Giese, C., & Zimmermann, R. (1988). Effect of dried garlic on blood coagulation, fibrinolysis, platelet aggregation and serum cholesterol levels in patients with hyperlipoproteinemia. *Atherosclerosis*, 74, 247-249.

Hariprakash, C. S., & Nambisan, B. (1996). Carbohydrate metabolism during dormancy and sprouting in yam (*Dioscorea*) tubers: changes in carbohydrate constituents in yam (*Dioscorea*) tubers during dormancy and sprouting. *Journal of Agricultural and Food Chemistry*, 44, 3066-3069.

Harris, J. C., Cottrell, S., Plummer, S., & Lloyd, D. (2001). Antimicrobial properties of *Allium sativum* (garlic). *Applied Microbiology and Biotechnology*, 57, 282-286.

Hartmann, J., Auge, J., & Hauptmann, P. (1994). Using the quartz crystal microbalance principles for gas detection with reversible and irreversible sensors. *Sensors and Actuators B: Chemical*, 19, 429-433.

Hayashi, S., Yagi, K., Ishikawa, T., Kawasaki, M., Asai, T., Picone, J., ... & Ogawa, K. (2004). Emission of 2-phenylethanol from its β-D-glucopyranoside and the biogenesis of these compounds from [2H8] L-phenylalanine in rose flowers. *Tetrahedron*, 60, 7005-7013.

Hlusek, J., Richter, R., & Rigerová, L. (2002). Sulphur in the nutrition and fertilization of vegetables. *Chemia i Inżynieria Ekologiczna*, 9, 1383-1390.

Hodges, D. M., Lester, G. E., Munro, K. D., & Toivonen, P. M. (2004). Oxidative stress: importance for postharvest quality. *HortScience*, 39, 924-929.

Höfgen, R., Kreft, O., Willmitzer, L., & Hesse, H. (2001). Manipulation of thiol contents in plants. *Amino Acids*, 20, 291-299.

Hong, C. J., & Etoh, T. (1996). Fertile clones of garlic (*Allium sativum* L.) abundant around the Tien Shan mountains. *Japanese Journal of Breeding*, 46, 349-353.

Hong, G., Kang, J., Nie, X., & Cantwell, M. I. (2001). Controlled atmospheres retard sprout growth, affect compositional changes, and maintain visual quality attributes of garlic. *In VIII International Controlled Atmosphere Research Conference 600* (pp. 791-794).

Hosseini, A., & Hosseinzadeh, H. (2015). A review on the effects of Allium sativum (Garlic) in metabolic syndrome. *Journal of Endocrinological Investigation*, 38, 1147-1157.

Huchette, O., Kahane, R., Auger, J., Arnault, I., & Bellamy, C. (2004, April). Influence of environmental and genetic factors on the alliin content of garlic bulbs. *In IV International Symposium on Edible Alliaceae* 688 (pp. 93-100).

Hughes, J., Collin, H. A., Tregova, A., Tomsett, A. B., Cosstick, R., & Jones, M. G. (2006). Effect of low storage temperature on some of the flavour precursors in garlic (*Allium sativum*). *Plant Foods for Human Nutrition*, 61, 78-82.

Hwang, J. M., & Lee, B. Y. (1990). The effect of temperature and humidity conditions on rooting and sprouting of garlic. *Journal of the Korean Society for Horticultural Science*, 31, 15-21.

Ichikawa, M., Ide, N., & Ono, K. (2006). Changes in organosulfur compounds in garlic cloves during storage. *Journal of agricultural and food chemistry*, 54(13), 4849-4854.

Ide, N., & Lau, B. H. S. (1999). Aged garlic extract attenuates intracellular oxidative stress. *Phytomedicine*, 6, 125-131.

Ilić, D. P., Nikolić, V. D., Nikolić, L. B., Stanković, M. Z., Stanojević, L. P., & Cakić, M. D. (2011). Allicin and related compounds: Biosynthesis, synthesis and pharmacological activity. *Facta universitatis-series: Physics, Chemistry and Technology*, 9, 9-20.

Imai, J., Ide, N., Nagae, S., Moriguchi, T., Matsuura, H., & Itakura, Y. (1994). Antioxidant and radical scavenging effects of aged garlic extract and its constituents. *Planta Medica*, 60, 417-420.

Ipek, M., Ipek, A., & Simon, P. W. (2008). Genetic characterization of *Allium* tuncelianum: An endemic edible *Allium* species with garlic odor. *Scientia Horticulturae*, 115, 409-415.

Isenberg, F. M. R., Thomas, T. H., Pendergrass, M., & Abdel-Rahman, M. (1973). Hormone and histological differences between normal and maleic hydrazide treated onions stored over winter. *In Symposium on Vegetable Storage* 38 (pp. 95-126).

Ivanov, P., Llobet, E., Vergara, A., Stankova, M., Vilanova, X., Hubalek, J., ... & Correig, X. (2005). Towards a micro-system for monitoring ethylene in warehouses. *Sensors and Actuators B: Chemical*, 111, 63-70.

Jabalpurwala, F., Gurbuz, O., & Rouseff, R. (2010). Analysis of grapefruit sulphur volatiles using SPME and pulsed flame photometric detection. *Food Chemistry*, 120, 296-303.

James, C., Seignemartin, V., & James, S. J. (2009). The freezing and supercooling of garlic (*Allium sativum* L.). *International Journal of Refrigeration*, 32, 253-260.

Jansen, H., Müller, B., & Knobloch, K. (1989). Characterization of an alliin lyase preparation from garlic (*Allium sativum*). *Planta Medica*, 55, 434-439.

Jardine, K. J., Meyers, K., Abrell, L., Alves, E. G., Serrano, A. M. Y., Kesselmeier, J., ... & Chambers, J. Q. (2013). Emissions of putative isoprene oxidation products from mango branches under abiotic stress. *Journal of Experimental Botany*, 64, 3669.

Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner (2019). vegan: Community Ecology Package. R package version 2.5-5. https://CRAN.R-project.org/package=vegan

Jenderek, M. M., & Hannan, R. M. (2004). Variation in reproductive characteristics and seed production in the USDA garlic germplasm collection. *HortScience*, 39, 485-488.

Jenderek, M. M., & Zewdie, Y. (2005). Within-and between-family variability for important bulb and plant traits among sexually derived progenies of garlic. *HortScience*, 40, 1234-1236.

Jones, H. A., & Mann, L. K. (1963). Onions and their allies. Onions and their allies. Leonard Hill, London, 286.

Jones, M. G., Collin, H. A., Tregova, A., Trueman, L., Brown, L., Cosstick, R., ... & Thomas, B. (2007). The biochemical and physiological genesis of alliin in garlic. *Medicinal and Aromatic Plant Science and Biotechnology*, 1, 21-24.

Jones, M. G., Hughes, J., Tregova, A., Milne, J., Tomsett, A. B., & Collin, H. A. (2004). Biosynthesis of the flavour precursors of onion and garlic. *Journal of Experimental Botany*, 55, 1903-1918.

Jordon-Thaden, I. E., Chanderbali, A. S., Gitzendanner, M. A., & Soltis, D. E. (2015). Modified CTAB and TRIzol protocols improve RNA extraction from chemically complex Embryophyta. *Applications in Plant Sciences*, 3, 1400105.

Kader, A. A. (2008). Flavor quality of fruits and vegetables. *Journal of the Science of Food and Agriculture*, 88, 1863-1868.

Kamenetsky, R., & Rabinowitch, H. D. (2001). Floral development in bolting garlic. *Sexual Plant Reproduction*, 13, 235-241.

Kamenetsky, R., Faigenboim, A., Mayer, E. S., Michael, T. B., Gershberg, C., Kimhi, S., ... & Sherman, A. (2015). Integrated transcriptome catalogue and organ-specific profiling of gene expression in fertile garlic (*Allium sativum L.*). *BMC Genomics*, 16, 12.

Kamenetsky, R., Shafir, I. L., Zemah, H., Barzilay, A., & Rabinowitch, H. D. (2004). Environmental control of garlic growth and florigenesis. *Journal of the American Society for Horticultural Science*, 129, 144-151.

Kaplan, F., & Guy, C. L. (2005). RNA interference of Arabidopsis beta-amylase8 prevents maltose accumulation upon cold shock and increases sensitivity of PSII photochemical efficiency to freezing stress. *The Plant Journal*, 44, 730-743.

Kaplan, F., Kopka, J., Haskell, D. W., Zhao, W., Schiller, K. C., Gatzke, N., ... & Guy, C. L. (2004). Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiology*, *136*, 4159-4168.

Keller, E. J., Zanke, C. D., Senula, A., Breuing, A., Hardeweg, B., & Winkelmann, T. (2013). Comparing costs for different conservation strategies of garlic (*Allium sativum* L.) germplasm in genebanks. *Genetic Resources and Crop Evolution*, 60, 913-926.

Keshri, G., Magan, N., & Voysey, P. (1998). Use of an electronic nose for the early detection and differentiation between spoilage fungi. *Letters in Applied Microbiology*, 27, 261-264.

Kim, J. S., Kang, O. J., & Gweon, O. C. (2013). Comparison of phenolic acids and flavonoids in black garlic at different thermal processing steps. *Journal of Functional Foods*, 5(1), 80-86.

Kim, K. H. (2005). Performance characterization of the GC/PFPD for H2S, CH3SH, DMS, and DMDS in air. *Atmospheric Environment*, 39, 2235-2242.

Koch, M., & Salomon, R. (1993, February). Improvement of garlic via somaclonal variation and virus elimination. *In International Symposium on Alliums for the Tropics* 358 (pp. 211-214).

Kodera, Y., Ayabe, M., Ogasawara, K., Yoshida, S., Hayashi, N., & Ono, K. (2002). Allixin accumulation with long-term storage of garlic. *Chemical and Pharmaceutical Bulletin*, 50, 405-407.

Kononkow, P.F. (1953) The question of obtaining garlic seed (Russian) *Allium Newsletter* 1:1-17

Krest, I., Glodek, J., & Keusgen, M. (2000). 2367801. Cysteine sulfoxides and alliinase activity of some *Allium* species. *Journal of Agricultural and Food Chemistry*, 48, 3753-3760.

Kwon, J. H., Byun, M. W., & Cho, H. O. (1985). Effects of gamma irradiation dose and timing of treatment after harvest on the storeability of garlic bulbs. *Journal of Food Science*, 50, 379-381.

Lagunas, L. L. M., & Castaigne, F. (2008). Effect of temperature cycling on alliinase activity in garlic. *Food Chemistry*, 111, 56-60.

Lancaster, J. E., & Shaw, M. L. (1989). γ-Glutamyl peptides in the biosynthesis of S-alk (en) yl-L-cysteine sulphoxides (flavour precursors) in *Allium*. *Phytochemistry*, 28, 455-460.

Lang, C., & Hübert, T. (2012). A colour ripeness indicator for apples. *Food and Bioprocess Technology*, 5, 3244-3249.

Langfelder, P., & Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, 9, 559.

Lanzotti, V. (2006). The analysis of onion and garlic. *Journal of Chromatography A*, 1112, 3-22.

Lawson, L. D. (1996). The composition and chemistry of garlic cloves and processed garlic. Garlic: The Science and Therapeutic Applications of *Allium sativum* L. and Related Species, 37-109. Williams & Wilkins, Philadelphia USA.

Lawson, L. D. (1998). Garlic: a review of its medicinal effects and indicated active compounds.

Lawson, L. D., & Gardner, C. D. (2005). Composition, stability, and bioavailability of garlic products used in a clinical trial. *Journal of Agricultural and Food Chemistry*, 53, 6254-6261.

Lawson, L. D., & Wang, Z. J. (1993). Pre-hepatic fate of the organosulfur compounds derived from garlic (*Allium sativum*). *Planta Medica*, 59, A688-A689.

Lawson, L. D., & Wang, Z. J. (2001). Low allicin release from garlic supplements: a major problem due to the sensitivities of alliinase activity. *Journal of Agricultural and Food Chemistry*, 49, 2592-2599.

Lawson, L. D., & Wang, Z. J. (2005). Allicin and allicin-derived garlic compounds increase breath acetone through allyl methyl sulfide: use in measuring allicin bioavailability. *Journal of Agricultural and Food Chemistry*, 53, 1974-1983.

Lawson, L. D., Ransom, D. K., & Hughes, B. G. (1992). Inhibition of whole blood platelet-aggregation by compounds in garlic clove extracts and commercial garlic products. *Thrombosis Research*, 65, 141-156.

Lawson, L. D., Wang, Z. Y. J., & Hughes, B. G. (1991b). γ-Glutamyl-S-alkylcysteines in garlic and other *Allium* spp.: Precursors of age-dependent trans-1-propenyl thiosulfinates. *Journal of Natural Products*, 54, 436-444.

Lawson, L. D., Wood, S. G., & Hughes, B. G. (1991a). HPLC analysis of allicin and other thiosulfinates in garlic clove homogenates. *Planta Medica*, 57, 263-270.

Ledesma, A., Nunez, S. B., & Argüello, J. A. (1994, March). Bulbing physiology in garlic (*Allium sativum* L.) cv." Rosado Paraguayo" II. Characterization of ontogenic stages by shoot growth dynamics and its relation to bulbing. *In I International Symposium on Edible Alliaceae* 433 (pp. 405-416).

Ledezma, E., & Apitz-Castro, R. (2006). Ajoene the main active compound of garlic (*Allium sativum*): a new antifungal agent. *Revista Iberoamericana de Micologia*, 23, 75-80.

Lee, S. N., Kim, N. S., & Lee, D. S. (2003). Comparative study of extraction techniques for determination of garlic flavor components by gas chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, 377, 749-756.

Legendre, P., & Anderson, M. J. (1999). Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. *Ecological Monographs*, 69, 1-24.

Lelièvre, J. M., Latchè, A., Jones, B., Bouzayen, M., & Pech, J. C. (1997). Ethylene and fruit ripening. *Physiologia Plantarum*, 101, 727-739.

Li, C., Schmidt, N. E., & Gitaitis, R. (2011). Detection of onion postharvest diseases by analyses of headspace volatiles using a gas sensor array and GC-MS. *LWT-Food Science and Technology*, 44, 1019-1025.

Liaw, A. and M. Wiener (2002). Classification and Regression by randomForest. *R News* 2, 18--22.

Liu, P., Weng, R., Sheng, X., Wang, X., Zhang, W., Qian, Y., & Qiu, J. (2019). Profiling of organosulfur compounds and amino acids in garlic from different regions of China. *Food Chemistry*, 125, 499.

Livak KJ, Schmittgen TD. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, 25, 402-408.

Llamas, D. P., Patón, L. G., Díaz, M. G., Serna, J. G., & Sáez, S. B. (2013). The effects of storage duration, temperature and cultivar on the severity of garlic clove rot caused by *Fusarium proliferatum*. *Postharvest Biology and Technology*, 78, 34-39.

Lu, X., Li, N., Qiao, X., Qiu, Z., & Liu, P. (2017). Composition analysis and antioxidant properties of black garlic extract. *Journal of Food and Drug Snalysis*, 25, 340-349.

Luo, R., Fang, D. Hang, H. & Tang, Z. (2016) The Mechanism in Gastric Cancer Chemoprevention by Allicin. *Anti-Cancer Agents in Medicinal Chemistry*, 16, 802-809.

Lyons, J. M. (1973). Chilling injury in plants. *Annual Review of Plant Physiology*, 24, 445-466.

Maaß, H. I., & Klaas, M. (1995). Intraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. *Theoretical and Applied Genetics*, 91, 89-97.

Magan, N., & Evans, P. (2000). Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of grain spoilage. *Journal of Stored Products Research*, 36, 319-340.

Mahattanatawee, K., & Rouseff, R. L. (2014). Comparison of aroma active and sulfur volatiles in three fragrant rice cultivars using GC–Olfactometry and GC–PFPD. *Food Chemistry*, 154, 1-6.

Mallika, T., Omer, E., & Lianfu, Z. (2014). Separation and purification of alliinase and alliin from garlic (*Allium sativum*). *Journal of Academia and Industrial Research*, 2, 599-605.

Mann, L. K., & Minges, P. A. (1958). Growth and bulbing of garlic (allium sativum in response to storage temperature of planting stocks, day length, and planting date. *Hilgardia*, 27, 385-419

Mann, L., & Lewis, D. (1956). Rest and dormancy in garlic. *Hilgardia*, 26, 161-189.

Martin-Lagos, R. A., Serrano, M. O., & Lopez, M. R. (1995). Determination of organic sulphur compounds in garlic extracts by gas chromatography and mass spectrometry. *Food Chemistry*, 53, 91-93.

Martins, N., Petropoulos, S., & Ferreira, I. C. (2016). Chemical composition and bioactive compounds of garlic (*Allium sativum* L.) as affected by pre-and post-harvest conditions: A review. *Food Chemistry*, 211, 41-50.

Mastrandrea, L., Amodio, M. L., Pati, S., & Colelli, G. (2017). Effect of modified atmosphere packaging and temperature abuse on flavor related volatile compounds of rocket leaves (*Diplotaxis tenuifolia* L.). *Journal of Food Science and Technology*, 54, 2433-2442.

Mathur, P. B. (1963). Extension of storage life of garlic bulbs by gamma-irradiation. *The International Journal of Applied Radiation and Isotopes*, 14.

Maude, R. B. (2018). Leaf diseases of onions. *Onions and Allied Crops: Volume II: Agronomy Biotic Interactions*.

Mazelis, M., & Crews, L. (1968). Purification of the alliin lyase of garlic, *Allium sativum* L. *Biochemical Journal*, 108, 725-730.

Mazza, G., Ciaravolo, S., Chiricosta, G., & Celli, S. (1992). Volatile flavour components from ripening and mature garlic bulbs. *Flavour and fragrance journal*, 7, 111-116.

McDonald, M. R., de los Angeles Jaime, M., & Hovius, M. H. (2004). Management of diseases of onions and garlic. In Diseases of Fruits and Vegetables: Volume II (pp. 149-200). Springer, Dordrecht.

Miron, T., Listowsky, I., & Wilchek, M. (2010). Reaction mechanisms of allicin and allyl-mixed disulfides with proteins and small thiol molecules. *European Journal of Medicinal Chemistry*, 45, 1912-1918.

Miron, T., Shin, I., Feigenblat, G., Weiner, L., Mirelman, D., Wilchek, M., & Rabinkov, A. (2002). A spectrophotometric assay for allicin, alliin, and alliinase (alliin lyase) with

a chromogenic thiol: reaction of 4-mercaptopyridine with thiosulfinates. *Analytical Biochemistry*, 307, 76-83.

Mishra, R. K., Jaiswal, R. K., Kumar, D., Saabale, P. R., & Singh, A. (2014). Management of major diseases and insect pests of onion and garlic: A comprehensive review. *Journal of Plant Breeding and Crop Science*, 6, 160-170.

Mitrova, K., Svoboda, P., Milella, L., & Ovesná, J. (2018). Alliinase and cysteine synthase transcription in developing garlic (*Allium sativum* L.) over time. *Food Chemistry*, 251, 103-109.

Moalemiyan, M., Vikram, A., & Kushalappa, A. C. (2007). Detection and discrimination of two fungal diseases of mango (cv. Keitt) fruits based on volatile metabolite profiles using GC/MS. *Postharvest Biology and Technology*, 45, 117-125.

Molina-Calle, M., Priego-Capote, F., & de Castro, M. D. L. (2016). HS–GC/MS volatile profile of different varieties of garlic and their behavior under heating. *Analytical and Bioanalytical Chemistry*, 408, 3843-3852.

Mondy, N., Naudin, A., Christides, J. P., Mandon, N., & Auger, J. (2001). Comparison of GC-MS and HPLC for the analysis of *Allium* volatiles. *Chromatographia*, 53(1), S356-S360.

Montaño, A., Beato, V. M., Mansilla, F., & Orgaz, F. (2011). Effect of genetic characteristics and environmental factors on organosulfur compounds in garlic (*Allium sativum* L.) grown in Andalusia, Spain. *Journal of Agricultural and Food Chemistry*, 59, 1301-1307.

Moreno, C. M., Stadler, T., da Silva, A. A., Barbosa, L. C., & de Queiroz, M. E. L. (2012). Determination of maleic hydrazide residues in garlic bulbs by HPLC. *Talanta*, 89, 369-376.

Moriguchi, T., Takashina, K., Chu, P., Saito, H., & Nishiyama, N. (1994). Prolongation of life span and improved learning in the senescence accelerated mouse produced by aged garlic extract. *Biological and Pharmaceutical Bulletin*, 17, 1589-1594.

Muldrew, K., & McGann, L. E. (1990). Mechanisms of intracellular ice formation. *Biophysical Journal*, 57, 525-532.

Mulrow, C., Lawrence, V., Ackerman, R., Ramirez, G., Morbidoni, L., Aguilar, C., ... & Harris, M. (2000). Garlic: effects on cardiovascular risks and disease, protective effects against cancer, and clinical adverse effects. In Database of Abstracts of Reviews of Effects (DARE): Quality-assessed Reviews. *Centre for Reviews and Dissemination* (UK).

Munch, R., & Barringer, S. A. (2014). Deodorization of garlic breath volatiles by food and food components. Journal of Food Science, 79, 526-533.

Musa. S. K. (1972). M.Sc. thesis, Univeristy of Khartoum.

Musini, V. M., & Wright, J. M. (2009). Factors affecting blood pressure variability: lessons learned from two systematic reviews of randomized controlled trials. *PLoS One*, 4, e5673.

Ncube, B., Finnie, J. F., & Van Staden, J. (2012). Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. *South African Journal of Botany*, 82, 11-20.

Nencini, C., Menchiari, A., Franchi, G. G., & Micheli, L. (2011). In vitro antioxidant activity of aged extracts of some Italian *Allium* species. *Plant Foods for Human Nutrition*, 66, 11-16.

Nian, H., Delage, B., Pinto, J. T., & Dashwood, R. H. (2008). Allyl mercaptan, a garlic-derived organosulfur compound, inhibits histone deacetylase and enhances Sp3 binding on the P21WAF1 promoter. *Carcinogenesis*, 29, 1816-1824.

Nordberg, Å., Hansson, M., Sundh, I., Nordkvist, E., Carlsson, H., & Mathisen, B. (2000). Monitoring of a biogas process using electronic gas sensors and near-infrared spectroscopy (NIR). *Water Science and Technology*, 41, 1-8.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., & Suggests, M. A. S. S. (2019). *The vegan package. Community ecology package*, 10, 631-637.

Oms-Oliu, G., Odriozola-Serrano, I., & Martín-Belloso, O. (2013). Metabolomics for assessing safety and quality of plant-derived food. *Food Research International*, 54, 1172-1183.

Ovesná, J., Kučera, L., Horníčková, J., Svobodová, L., Stavělíková, H., Velíšek, J., & Milella, L. (2011). Diversity of S-alk (en) yl cysteine sulphoxide content within a collection of garlic (*Allium sativum* L.) and its association with the morphological and genetic background assessed by AFLP. *Scientia Horticulturae*, 129, 541-547.

Ovesná, J., Mitrová, K., & Kučera, L. (2015). Garlic (*A. sativum* L.) alliinase gene family polymorphism reflects bolting types and cysteine sulphoxides content. *BMC Genetics*, 16, 53.

Panthee, D. R., Kc, R. B., Regmi, H. N., Subedi, P. P., Bhattarai, S., & Dhakal, J. (2006). Diversity analysis of garlic (*Allium sativum* L.) germplasms available in Nepal based on morphological characters. *Genetic Resources and Crop Evolution*, 53, 205-212.

Pardo, J. E., Escribano, J., Gomez, R., & Alvarruiz, A. (2007). Physical–chemical and sensory quality evaluation of garlic cultivars. *Journal of Food Quality*, 30, 60

Park, M. H., & Kim, J. G. (2015). Low-dose UV-C irradiation reduces the microbial population and preserves antioxidant levels in peeled garlic (*Allium sativum* L.) during storage. *Postharvest Biology and Technology*, 100, 109-112.

Park, Y., Hwang, J., & Ha, H. (2000). Storability of garlic bulbs as influenced by postharvest clipping treatments and storage temperature. *Journal of the Korean Society for Horticultural Science*, 41, 315-318.

Pearce, R. S. (2001). Plant freezing and damage. *Annals of Botany*, 87, 417-424.

Pellegrini, C. N., Croci, C. A., & Orioli, G. A. (2000). Morphological changes induced by different doses of gamma irradiation in garlic sprouts. *Radiation Physics and Chemistry*, 57, 315-318.

Pellegrini, C. N., Orioli, G. A., & Croci, C. A. (1998). Identification of the method used to inhibit sprouting in garlic. *In XXV International Horticultural Congress, Part 8: Quality of Horticultural Products*, 518 (pp. 55-62).

Peñuelas, J., & Llusià, J. (2001). The complexity of factors driving volatile organic compound emissions by plants. *Biologia Plantarum*, 44, 481-487.

Pichersky, E., & Gershenzon, J. (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology*, 5, 237-243.

Pichersky, E., Noel, J. P., & Dudareva, N. (2006). Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science*, 311, 808-811.

Pino, J. A. (1992). Headspace sampling methods for the volatile components of garlic (*Allium sativum*). *Journal of the Science of Food and Agriculture*, 59, 131-133.

Platenius, H. (1942). Effect of temperature on the respiration rate and the respiratory quotient of some vegetables. *Plant Physiology*, 17, 179.

Pooler, M. R., & Simon, P. W. (1993a). Characterization and classification of isozyme and morphological variation in a diverse collection of garlic clones. *Euphytica*, 68, 121-130.

Pooler, M. R., & Simon, P. W. (1993b). Garlic flowering in response to clone, photoperiod, growth temperature, and cold storage. *HortScience*, 28, 1085-1086.

Possell, M., & Loreto, F. (2013). The role of volatile organic compounds in plant resistance to abiotic stresses: responses and mechanisms. In Biology, controls and models of tree volatile organic compound emissions (pp. 209-235). Springer, Dordrecht.

Prager-Khoutorsky, M., Goncharov, I., Rabinkov, A., Mirelman, D., Geiger, B., & Bershadsky, A. D. (2007). Allicin inhibits cell polarization, migration and division via its direct effect on microtubules. *Cell Motility and the Cytoskeleton*, 64, 321-337.

Prithiviraj, B., Vikram, A., Kushalappa, A. C., & Yaylayan, V. (2004). Volatile metabolite profiling for the discrimination of onion bulbs infected by E*rwinia carotovora* ssp. *Carotovora*, *Fusariumoxysporum* and *Botrytis allii*. *European Journal of Plant Pathology*, 110, 371-377.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rabinkov, A., Zhu, X. Z., Grafi, G., Galili, G., & Mirelman, D. (1994). Alliin lyase (Alliinase) from garlic (*Allium sativum*). Biochemical characterization and cDNA cloning. *Applied Biochemistry and Biotechnology*, 48, 149-171.

Rahim, M. A., & Fordham, R. (1988). Effect of storage temperature on the initiation and development of garlic cloves (*Allium sativum* L.). *Scientia Horticulturae*, 37, 25-38.

Rahim, M. A., & Fordham, R. (1990). Effect of shade and environmental conditions on the initiation and development of garlic cloves (*Allium sativum* L.). *Scientia Horticulturae*, 45, 21-30.

Rahman, K., & Lowe, G. M. (2006). Garlic and cardiovascular disease: a critical review. *The Journal of Nutrition*, 136, 736S-740S.

Rahman, K., & Lowe, G. M. (2006). Significance of garlic and its constituents in cancer and cardiovascular disease. *Journal of Nutrition*, 136, 736S-740S.

Reiter, J., Levina, N., van der Linden, M., Gruhlke, M., Martin, C., & Slusarenko, A. J. (2017). Diallylthiosulfinate (Allicin), a Volatile Antimicrobial from Garlic (*Allium sativum*), Kills Human Lung Pathogenic Bacteria, Including MDR Strains, as a Vapor. *Molecules*, 22, 1711.

Rengwalska, M. M., & Simon, P. W. (1986). Laboratory evaluation of pink root and fusarium basal rot resistance in garlic. *Plant Disease*, 70, 670-672.

Ricco, A. J., Crooks, R. M., & Osbourn, G. C. (1998). Surface acoustic wave chemical sensor arrays: new chemically sensitive interfaces combined with novel cluster analysis to detect volatile organic compounds and mixtures. *Accounts of Chemical Research*, 31, 289-296.

Rivlin, R. S. (2001). Historical perspective on the use of garlic. *The Journal of Nutrition*, 131, 951S-954S.

Rosen, C. J., & Tongn, C. B. (2001). Yield, dry matter partitioning, and storage quality of hardneck garlic as affected by soil amendments and scape removal. *HortScience*, 36, 1235-1239.

Rosen, R. T., Hiserodt, R. D., Fukuda, E. K., Ruiz, R. J., Zhou, Z., Lech, J., ... & Hartman, T. G. (2000). The determination of metabolites of garlic preparations in breath and human plasma. Biofactors, 13, 241-249.

Rosen, R. T., Hiserodt, R. D., Fukuda, E. K., Ruiz, R. J., Zhou, Z., Lech, J., ... & Hartman, T. G. (2001). Determination of allicin, S-allylcysteine and volatile metabolites of garlic in breath, plasma or simulated gastric fluids. *The Journal of Nutrition*, 13, 968S-971S.Rosier, E., Cuypers, E., Dekens, M., Verplaetse, R., Develter, W., Van de Voorde,

W., ... & Tytgat, J. (2014). Development and validation of a new TD-GC/MS method and its applicability in the search for human and animal decomposition products. *Analytical and Bioanalytical Chemistry*, 406, 3611-3619.

Ruelland, E., Vaultier, M. N., Zachowski, A., & Hurry, V. (2009). Cold signalling and cold acclimation in plants. *Advances in Botanical Research*, 49, 35-150.

Sadaria, S. G., Malavia, D. D., Khanpara, V. D., Dudhatra, M. G., Vyas, M. N., & Mathukia, R. K. (1997). Effect of irrigation, nitrogen and phosphorus on garlic (*Allium sativum*) and their residual effect on groundnut (*Arachis hypogaea*). *Indian Journal of Agronomy*, 42, 653-656.

Salama, A. M., Hicks, J. R., & Nock, J. F. (1990). Sugar and organic acid changes in stored onion bulbs treated with maleic hydrazide. *HortScience*, 25, 1625-1628.

Salman, H., Bergman, M., Bessler, H., Punsky, I., & Djaldetti, M. (1999). Effect of a garlic derivative (alliin) on peripheral blood cell immune responses. *International Journal of Immunopharmacology*, 21, 589-597.

Salvador, I. M., Frenich, A. G., González, F. E., & Vidal, J. M. (2006). Determination of organophosphorus pesticides in vegetables by GC with pulsed flame-photometric detection, and confirmation by MS. *Chromatographia*, 64, 667-672.

Santhosha, S. G., Jamuna, P., & Prabhavathi, S. N. (2013). Bioactive components of garlic and their physiological role in health maintenance: A review. *Food Bioscience*, 3, 59-74.

Shalaby, T. A ., & El-Ramady, H. (2014). Effect of foliar application of bio-stimulants on growth, yield, components, and storability of garlic (*Allium sativum* L.). *Australian Journal of Crop Science*, 8, 271.

Sharma, M. D., Gautam, D. M., & Panthee, D. R. (2004). Characterization and Evaluation of Indigeneous Garlic (*Alium sativum* L.) Germplasms of Nepal. *Agricultural research for enhancing livelihood of Nepalese people*, 30, 196.

Shemesh, E., Scholten, O., Rabinowitch, H. D., & Kamenetsky, R. (2008). Unlocking variability: inherent variation and developmental traits of garlic plants originated from sexual reproduction. *Planta*, 227, 1013-1024.

Shemesh-Mayer, E., Ben-Michael, T., Rotem, N., Rabinowitch, H. D., Doron-Faigenboim, A., Kosmala, A., ... & Kamenetsky, R. (2015). Garlic (*Allium sativum* L.) fertility: transcriptome and proteome analyses provide insight into flower and pollen development. *Frontiers in Plant Science*, 6, 271.

Shiina, T., Umehara, H., Miyatake, F., & Nakamura, N. (2004, June). The mechanism controlling maintenance of produce initial water content during long-term storage: exemplification by using garlic bulb. *In V International Postharvest Symposium* 682 (pp. 879-886).

Siddique, M. A., & Rabbani, M. G. (1985). Growth and bulbing of garlic in response to low temperature treatment of bulb and planting date. *Bangladesh Journal of Botany*, 14(1), 41-46.

Silagy, C. A., & Neil, H. A. (1994). A meta-analysis of the effect of garlic on blood pressure. *Journal of Hypertension*, 12, 463-468.

Šimpraga, M., Verbeeck, H., Demarcke, M., Joó, É., Pokorska, O., Amelynck, C., ... & Aubinet, M. (2011). Clear link between drought stress, photosynthesis and biogenic volatile organic compounds in *Fagus sylvatica* L. *Atmospheric Environment*, 45, 5254-5259.

Sisk, B. C., & Lewis, N. S. (2003). Estimation of chemical and physical characteristics of analyte vapors through analysis of the response data of arrays of polymer-carbon black composite vapor detectors. *Sensors and Actuators B: Chemical*, 96, 268-282.

Smeets, K., Van Damme, E. J., & Peumans, W. J. (1997). Developmental regulation of lectin and alliinase synthesis in garlic bulbs and leaves. *Plant Physiology*, 113, 765-771.

Sobenin, I. A., Pryanishnikov, V. V., Kunnova, L. M., Rabinovich, Y. A., Martirosyan, D. M., & Orekhov, A. N. (2010). The effects of time-released garlic powder tablets on multifunctional cardiovascular risk in patients with coronary artery disease. *Lipids in health and Disease*, 9, 119.

Sommer, N. F. (1985). Role of controlled environments in suppression of postharvest diseases. *Canadian Journal of Plant Pathology*, 7, 331-339.

Son, J. H., Park, K. C., Lee, S. I., Kim, H. H., Kim, J. H., Kim, S. H., & Kim, N. S. (2012). Isolation of cold-responsive genes from garlic, *Allium sativum*. *Genes & Genomics*, 34, 93-101.

Starks, C. M., Back, K., Chappell, J., & Noel, J. P. (1997). Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. *Science*, 277, 1815-1820.

Steel, R.; Torri,J; Dickey, D.(1997) Principles and Procedures of Statistics A Biometrical Approach. pp 178

Stein, S. E., Mikaia, A., White, E., Zaikin, V., Zhu, D., Milman, B., ... & Tchekhovskoi, D. (2008). NIST Standard Reference Database 1A NIST. EPA/NIH Mass Spectral Library (NIST 11) and NIST Mass Spectral Search Program (Version 2.2). The NIST Mass Spectrometry Data Center, USA.

Stitt, M., & Hurry, V. (2002). A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. *Current Opinion in Plant Biology*, 5, 199-206.

Stoll, A., & Seebeck, E. (1947). Über alliin, die genuine muttersubstanz des knoblauchöls. *Experientia*, 3, 114-115.

Stoll, A., & Seebeck, E. (1951). Chemical investigations on alliin, the specific principle of garlic. *Advances in Enzymology and Related Areas of Molecular Biology*, 11, 377-400.

Stoll, V. A., & Seebeck, E. (1949). Über den enzymatischen Abbau des Alliins und die Eigenschaften der Alliinase. 2. *Mitteilung über Allium-Substanzen. Helvetica Chimica Acta*, 32, 197-205.

Stow, J. R. (1975a). Effects of humidity on losses of bulb onions (*Allium cepa*) stored at high temperature. *Experimental Agriculture*, 11, 81-87.

Stow, J. R. (1975b). Resistance of 20 cultivars of onions (*Allium cepa*) to rotting and sprouting during high-temperature storage. *Experimental Agriculture*, 11, 201-207.

Stow, J. R., Tucker, W. G., & Ward, C. M. (1973). High temperature storage of onions. *Report of the National Vegetable Research Station*. 1972, 69.

Takagi, H., & Qu, Y. (1994). Effects of light quality, photoperiod and cold treatment on in vitro bulbing of garlic shoot tip. *Environmental Effects and their Control in Plant Tissue Culture* 393, 181-188.

Takagi, H., 1990. Garlic *Allium sativum* L. In: Brewster, J.L., Rabinowitch, H.D. (Eds.), Onions and Allied Crops. Biochemistry, Food Science and Minor Crops, vol. III. CRC Press, Boca Raton, pp. 109/146.

Tanaka, F., Tatsuki, M., Matsubara, K., Okazaki, K., Yoshimura, M., & Kasai, S. (2018). Methyl ester generation associated with flesh browning in 'Fuji'apples after long storage under repressed ethylene function. *Postharvest Biology and Technology*, 145, 53-60.

Tekalign, T., Abdissa, Y., & Pant, L. M. (2012). Growth, bulb yield and quality of onion (*Allium cepa* L.) as influenced by nitrogen and phosphorus fertilization on vertisol. II: Bulb quality and storability. *African Journal of Agricultural Research*, 7, 5980-5985.

Thomashow, M. F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Biology*, 50, 571-599.

Tian, S. P., & Bertolini, P. (1995). Effects of low temperature on mycelial growth and spore germination of *Botrytis allii* in culture and on its pathogenicity to stored garlic bulbs. *Plant Pathology*, 44, 1008-1015.

Tournas, V. H. (2005). Spoilage of vegetable crops by bacteria and fungi and related health hazards. *Critical Reviews in Microbiology*, 31, 33-44.

Tripathi, P., & Lawande, K. E. (2006). Cold storage of onion and garlic. *DOGR Technical Bulletin*, 15, 1-8.

Trirongjitmoah, S., Juengmunkong, Z., Srikulnath, K., & Somboon, P. (2015). Classification of garlic cultivars using an electronic nose. *Computers and Electronics in Agriculture*, 113, 148-153.

Tyson, J. L., & Fullerton, R. A. (2004). Effect of soilborne inoculum on incidence of onion black mould (*Aspergillus niger*). *New Zealand Plant Protection*, 57, 138-141.

Uddin, M. M., & MacTavish, H. S. (2003). Controlled atmosphere and regular storage-induced changes in S-alk (en) yl-l-cysteine sulfoxides and alliinase activity in onion bulbs (*Allium cepa* L. cv. Hysam). *Postharvest Biology and Technology*, 28, 239-245.

Ueno, E., Oshima, H., Saito, I., Matsumoto, H., & Nakazawa, H. (2003). Determination of organophosphorus pesticide residues in onion and welsh onion by gas chromatography with pulsed flame photometric detector. *Journal of Pesticide Science*, 28, 422-428.

Unsicker, S. B., Kunert, G., & Gershenzon, J. (2009). Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. *Current opinion in plant biology*, 12, 479-485.

Van Den Velde, S., Quirynen, M., & van Steenberghe, D. (2007). Halitosis associated volatiles in breath of healthy subjects. Journal of Chromatography B, 853, 54-61.

Varga, J., Juhász, Á., Kevei, F., & Kozakiewicz, Z. (2004). Molecular diversity of agriculturally important *Aspergillus* species. In Molecular Diversity and PCR-detection of Toxigenic *Fusarium* Species and Ochratoxigenic Fungi (pp. 627-640). Springer, Dordrecht.

Vazquez-Barrios, M. E., Lopez-Echevarria, G., Mercado-Silva, E., Castano-Tostado, E., & Leon-Gonzalez, F. (2006). Study and prediction of quality changes in garlic cv. Perla (*Allium sativum* L.) stored at different temperatures. *Scientia Horticulturae*, 108, 127-132.

Vernin, G., Metzger, J., Fraisse, D., & Scharff, C. (1986). GC-MS (EI, PCI, NCI) Computer Analysis of Volatile Sulfur Compounds in Garlic Essential Oils. Application of the Mass Fragmentometry SIM Technique1. *Planta Medica*, 52, 96-101.

Vickers, C. E., Gershenzon, J., Lerdau, M. T., & Loreto, F. (2009). A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nature Chemical Biology*, 5, 283.

Vikram, A., Hamzehzarghani, H., & Kushalappa, A. C. (2005). Volatile metabolites from the headspace of onion bulbs inoculated with postharvest pathogens as a tool for disease discrimination. *Canadian Journal of Plant Pathology*, 27, 194-203.

Vikram, A., Prithiviraj, B., Hamzehzarghani, H., & Kushalappa, A. C. (2004). Volatile metabolite profiling to discriminate diseases of McIntosh apple inoculated with fungal pathogens. *Journal of the Science of Food and Agriculture*, 84, 1333-1340.

Volk, G. M., & Stern, D. (2009). Phenotypic characteristics of ten garlic cultivars grown at different North American locations. *HortScience*, 44, 1238-1247.

Volk, G. M., Rotindo, K. E., & Lyons, W. (2004). Low-temperature storage of garlic for spring planting. *HortScience*, 39, 571-573.

Walcott, R. R., Gitaitis, R. D., Castro, A. C., Sanders Jr, F. H., & Diaz-Perez, J. C. (2002). Natural infestation of onion seed by *Pantoea ananatis*, causal agent of center rot. *Plant Disease*, 86, 106-111.

Walkey, D. G. A., & Antill, D. N. (1989). Agronomic evaluation of virus-free and virus-infected garlic (*Allium sativum* L.). *Journal of Horticultural Science*, 64, 53-60.

Walkey, D. G. A., Webb, M. J. W., Bolland, C. J., & Miller, A. (1987). Production of virus-free garlic (*Allium sativum* L.) and shallot (*A. ascalonicum* L.) by meristem-tip culture. *Journal of Horticultural Science*, 62, 211-220.

Wang, A., Casadei, F., Johansen, A., Bukman, H., & Edelenbos, M. (2015, June). Emission of volatile organic compounds from healthy and diseased onions. In III International Symposium on Postharvest Pathology: Using Science to Increase Food Availability 1144 (pp. 333-340).

Wang, L., Baldwin, E. A., & Bai, J. (2016). Recent advance in aromatic volatile research in tomato fruit: the metabolisms and regulations. *Food and Bioprocess Technology*, 9, 203-216.

Ward, C. M. (1976). The influence of temperature on weight loss from stored onion bulbs due to desiccation, respiration and sprouting. *Annals of Applied Biology*, 83, 149-155.

Ward, C. M., & Tucker, W. G. (1976). Respiration of maleic hydrazide treated and untreated onion bulbs during storage. *Annals of Applied Biology*, 82, 135-141.

Warren, J. M., Parkinson, D. R., & Pawliszyn, J. (2013). Assessment of thiol compounds from garlic by automated headspace derivatized in-needle-NTD-GC-MS and derivatized in-fiber-SPME-GC-MS. *Journal of Agricultural and Food Chemistry*, 61, 492-500.

Waterer, D., & Schmitz, D. (1994). Influence of variety and cultural practices on garlic yields in Saskatchewan. *Canadian Journal of Plant Science*, 74, 611-614.

Wenke, K., Kai, M., & Piechulla, B. (2010). Belowground volatiles facilitate interactions between plant roots and soil organisms. *Planta*, 231, 499-506.

Wheatley, R., Hackett, C., Bruce, A., & Kundzewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. *International Biodeterioration & Biodegradation*, 39, 199-205.

Woolfenden, E. (2010). Sorbent-based sampling methods for volatile and semi-volatile organic compounds in air: Part 1: Sorbent-based air monitoring options. *Journal of Chromatography A*, 1217, 2674-2684.

WordWeatherOnline historical average weather charts.
https://www.worldweatheronline.com

Wu, J. J., Yang, J. S., & Liu, M. S. (1996). Effects of Irradiation on the Volatile Compounds of Garlic (*Allium sativum* L). *Journal of the Science of Food and Agriculture*, 70, 506-508.

Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research*, 43, W251-W257.

Yamazaki, M., Sugiyama, M., & Saito, K. (2002). Intercellular localization of cysteine synthase and alliinase in bundle sheaths of *Allium* plants. *Plant Biotechnology*, 19, 7-10.

Yang, F. J., Liu, S. Q., Wang, X. F., & Zang, J. B. (2005). The effect of zinc on physiological-biochemical indexes and nutrition quality of garlic [J]. *Soils and Fertilizers*, 2005.

Yang, F., Liu, S., Wang, X., Zhang, Y., & Zang, J. (2004). Effects of sulfur on physiological and biochemical indices and nutrition quality of garlic. *The Journal of Applied Ecology*, 15, 2095-2098.

Yeh, Y. Y., & Liu, L. (2001). Cholesterol-lowering effect of garlic extracts and organosulfur compounds: human and animal studies. *The Journal of Nutrition*, 131, 989S-993S.

Yoshimoto, N., Onuma, M., Mizuno, S., Sugino, Y., Nakabayashi, R., Imai, S., Tsuneyoshi, T., Sumi, S.I. and Saito, K., (2015a). Identification of a flavin-containing S-oxygenating monooxygenase involved in alliin biosynthesis in garlic. *The Plant Journal*, 83, 941-951.

Yoshimoto, N., Onuma, M., Mizuno, S., Sugino, Y., Nakabayashi, R., Imai, S. & Saito, K. (2015). Identification of a flavin-containing S-oxygenating monooxygenase involved in alliin biosynthesis in garlic. *The Plant Journal*, 83, 941-951.

Yoshimoto, N., Yabe, A., Sugino, Y., Murakami, S., Sai-ngam, N., Sumi, S.I., Tsuneyoshi, T. and Saito, K., (2015b). Garlic γ-glutamyl transpeptidases that catalyze deglutamylation of biosynthetic intermediate of alliin. *Frontiers in plant science*, 5, p.758.

Yu, T. H., Wu, C. M., & Liou, Y. C. (1989). Volatile compounds from garlic. *Journal of Agricultural and Food Chemistry*, 37, 725-730.

Zheng, L., Lv, R., Huang, J., Jiang, D., Liu, X., & Hsiang, T. (2010). Integrated control of garlic leaf blight caused by *Stemphylium solani* in China. *Canadian Journal of Plant Pathology*, 32, 135-145.

Zhu, J., Dong, C. H., & Zhu, J. K. (2007). Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Current Opinion in Plant Biology*, 10, 290-295.

Zizina, S. I. (1956). Study of wild *Alliums* from Kazakhstan under cultivation. Doctoral dissertation, PhD thesis. Almaty, Kazakhstan.

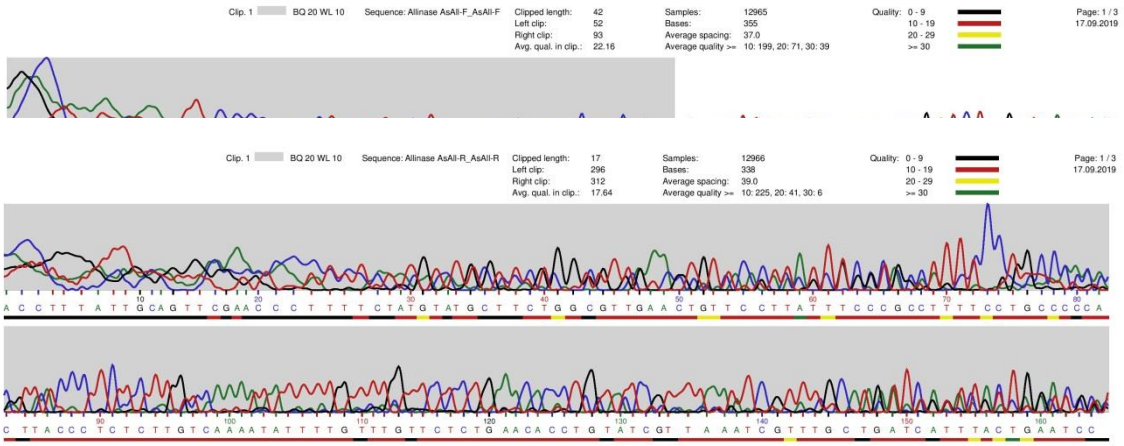
8. Supplementary Material

8.1. Table S3.1: PCR product sequencing.

Primer	PCR Product Sequence
Allinase AsAll-F	CAAATTTCCTCTGGCCTTGCTCTGGGTTATAATCTCCTCCGT
Allinase AsAll-R	TGCCTTCCCCAATACCG
Cysteine AsCysK-F	GCTCATTTAGTCTTGAAGAAGGATCATCATAAAAGCCTTTGGT GGTCAGTTGGTTCTTACCGATCCATTGTTGGGAATCAAAGGTTG CATTCAAAAGGCGGAGGAAATAACCCTCCT
Cysteine AsCysK-R	TCGGTAAGAACCAACTGACCACCAAAGGCTTTTATGATGATCC TTCTTTCAAGACTAAAT
Ubiquitin UBQ-F	AGCGCCTTATATTTGCGGGTAAACAACTGGAAGACGGGAGAAC GCTAGCAGACTATAATATTCAGAAAGAATCAACCCTCCACCTG GTGCTGAGATTGAGAGGTGGTATGCATTGCCTTT
Ubiquitin UBQ-R	AGTGATTCTTTCTGATATTATAGTCTGCTAGCGTTCTCCCGTCTT CCAGTTGTTTACCCGCAAATATAAGGCGCTGCTGATCAGGTGG GATTCCCTCCTTGTCCTGTATCTTGGCTTATACCCTGACA

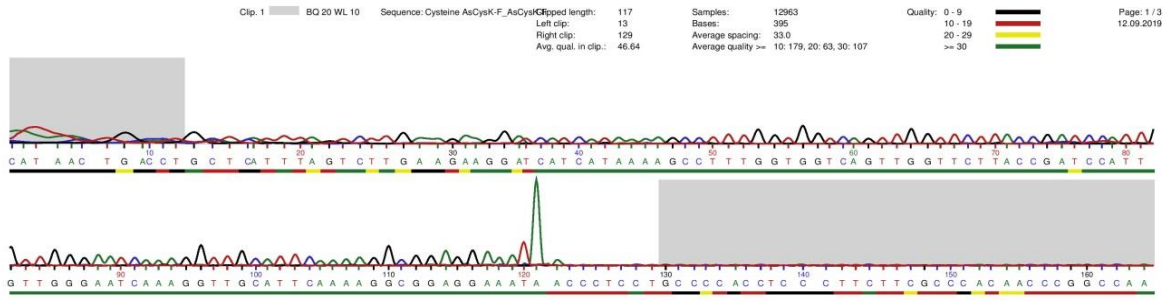
8.2. Sequencing Chromatograms

Alliinase F:

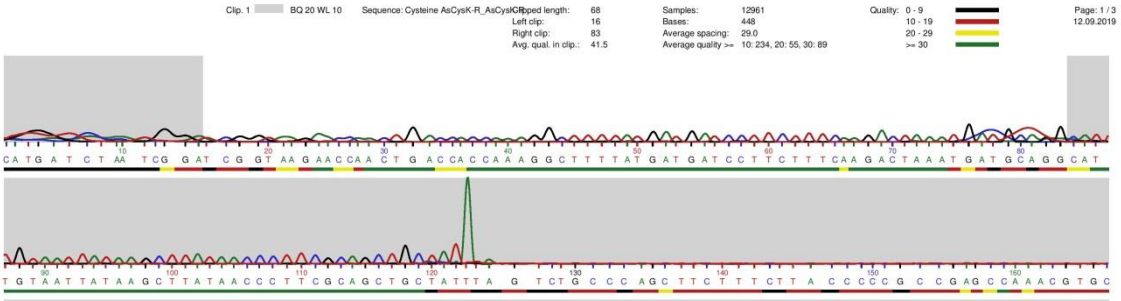


Alliinase R:

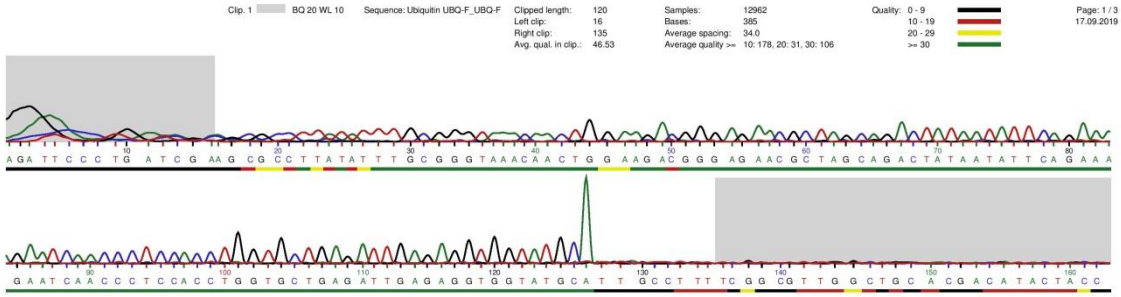
Cysteine F:



Cysteine R:



Ubiquitin F:



Ubiquitin R:

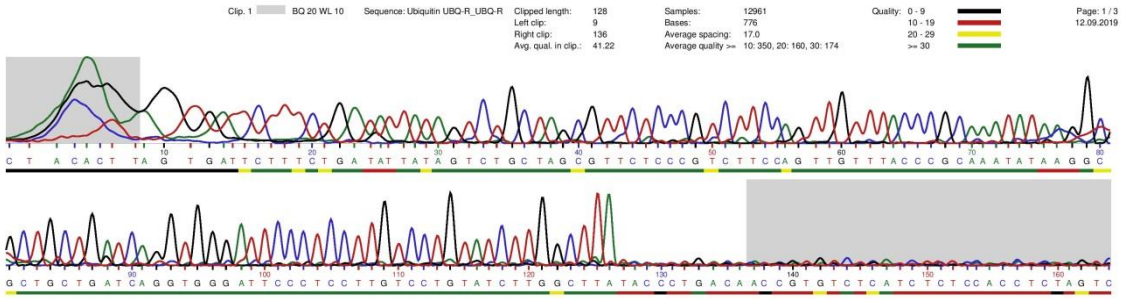


Figure S3.1: Sequencing data of PCR products of the alliinase (AsAll), cysteine synthase (AsCysK) and ubiquitin (AsUBQ) primers. PCR products sequenced by Eurofins Genomics, Germany.

8.3. BLAST results

Table S3.2: BLAST results of PCR product sequencing.

Primer Pair	Hits
Cysteine Synthase (AsCysK R)	1 of 1: Allium sativum cytosolic cysteine synthase GCS3 (gcs3) mRNA, complete cds. 100% match
Alliinase (AsAll F + R)	NA
Ubiquitin (AsUBQ R)	1 of 1: Polyubiquitin-like transcript variant Ziziphus jujuba (LOC107412950) 82 % match

8.4. Melting peaks for qPCR

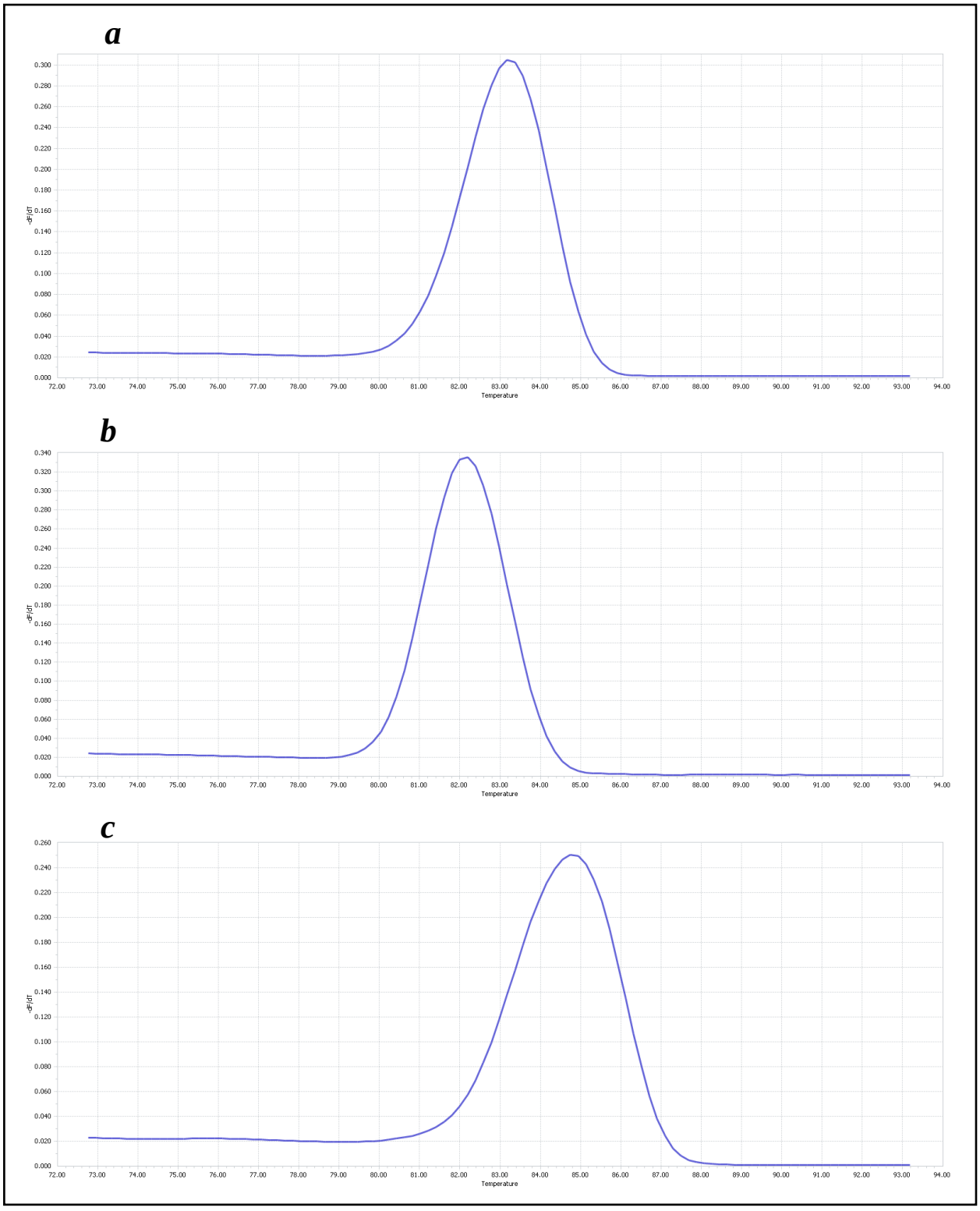


Figure S3.2: melting peaks for alliinase (***a***), cysteine synthase (***b***) and ubiquitin (***c***) primer products in qPCR.

8.5.Sprouting Cloves

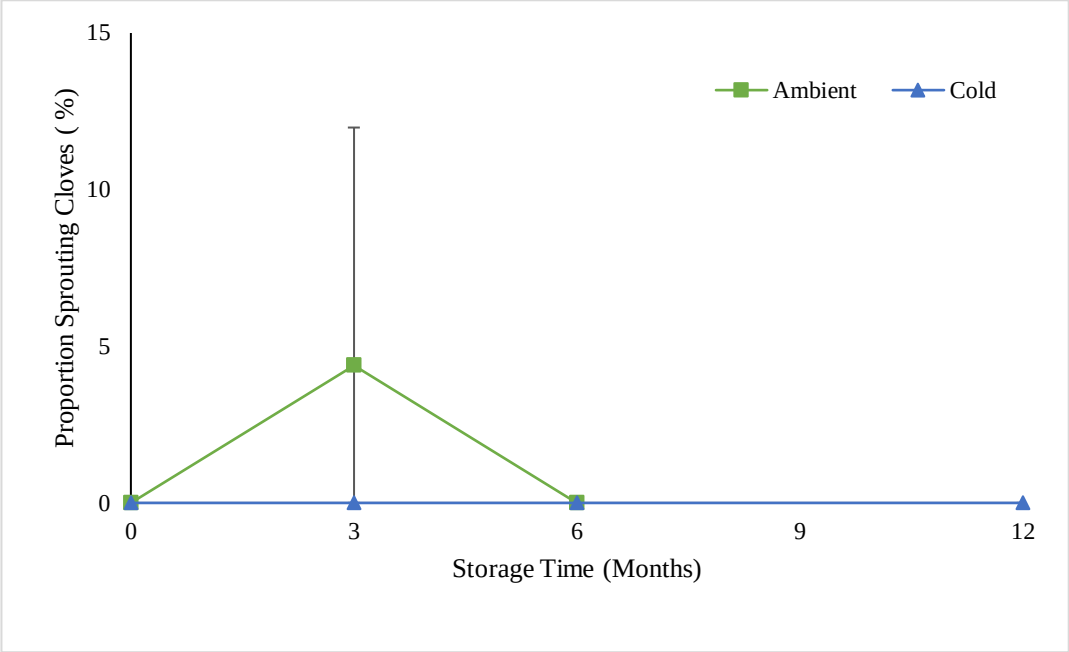


Figure S3.3: The proportion of sprouting cloves in 'Altanero' garlic cloves stored at ambient (■) and cold (▲) temperatures for 0, 3, 6 and 12 months. No significant differences between data points were detected. *N* =3 bulbs, error bars = SD.

8.6.Alliinase gene expression

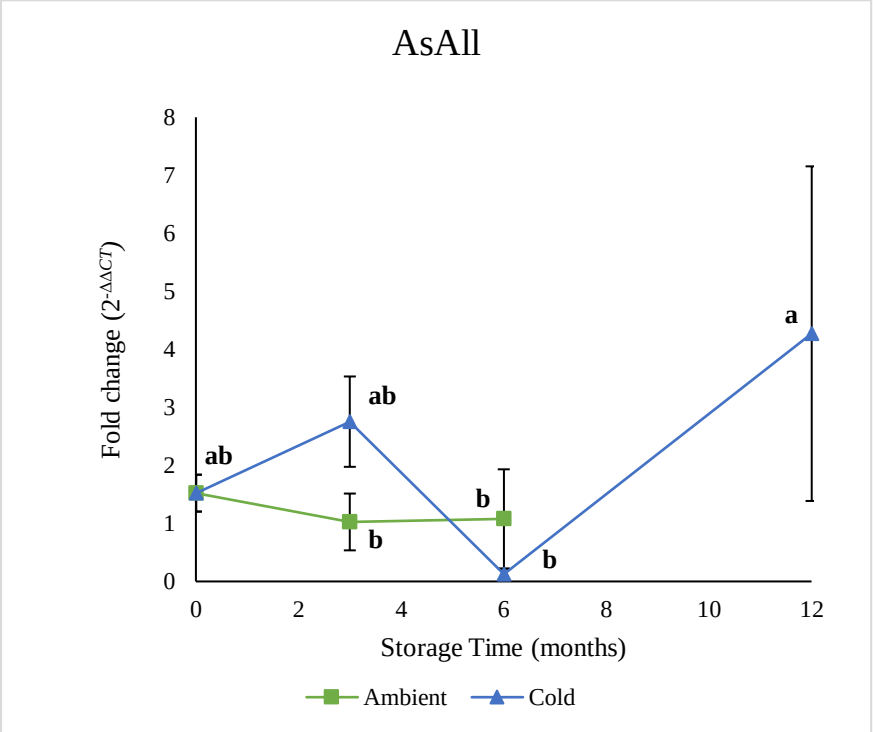


Figure S3.4: Expression of Alliinase (AsAll) normalised to Ubiquitin (UBQ). Letters indicate statistically different values between the different temperatures and time points, based on GLM and LSD, p = 0.05, N =3, error bars = SD.

8.7. 'Blanco' VOCs over time

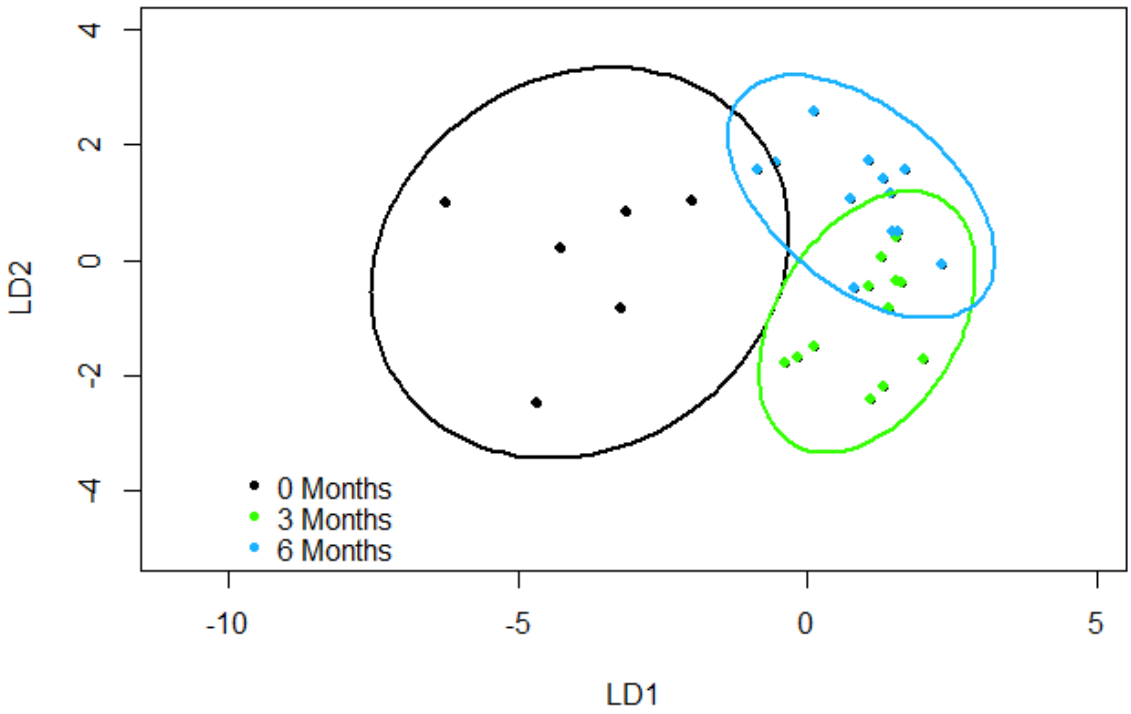


Figure S4.1: Linear discriminant plot from a CAP analysis of **'Blanco'** VOCs over time. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 53.3 % at p = 0.05 (percent successful classification by group: 0 months = 83.3 %, 3 months = 16.7 %, 6 months = 75%). N = 6 replicates of 3 bulbs for 0 months, N = 12 for 3 and 6 months.

8.8. 'Blanco' VOCs between temperatures

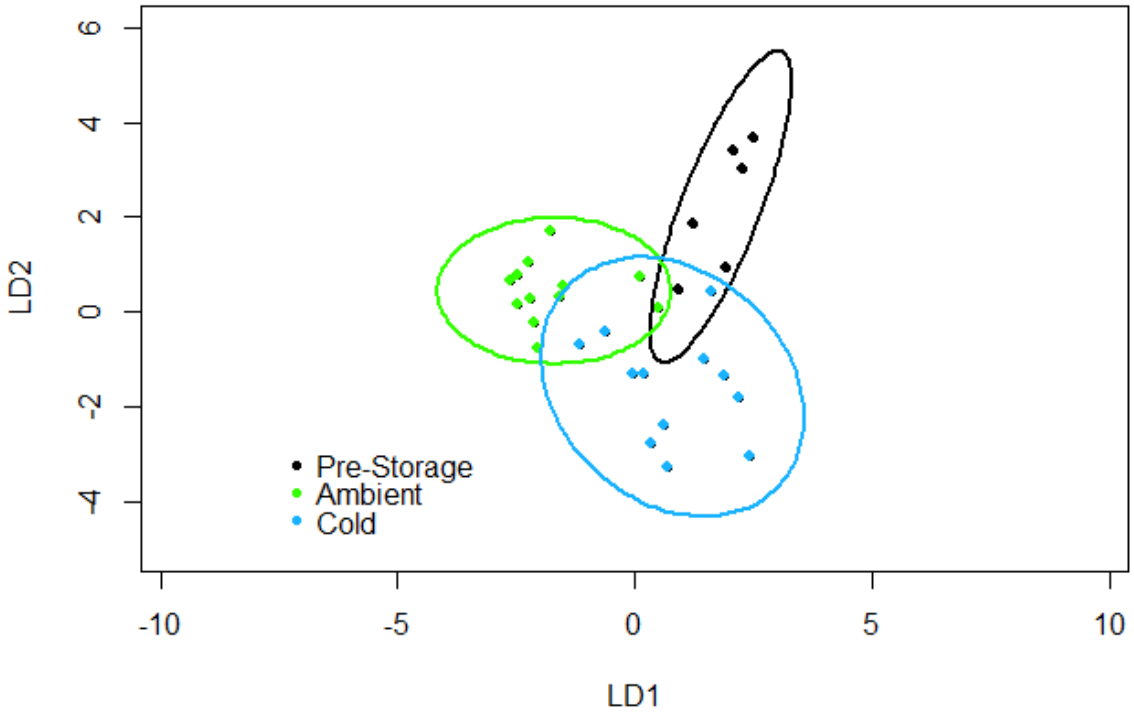


Figure S4.2: Linear discriminant plot from a CAP analysis of **'Blanco'** VOCs between storage temperatures. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 76.7 % at $p = 0.05$ (percent successful classification by group: pre-storage = 83.3 %, ambient = 75 %, cold = 75 %). $N = 6$ replicates of 3 bulbs for prestorage, $N = 12$ for ambient and cold.

8.9.'Morado' VOCs over time

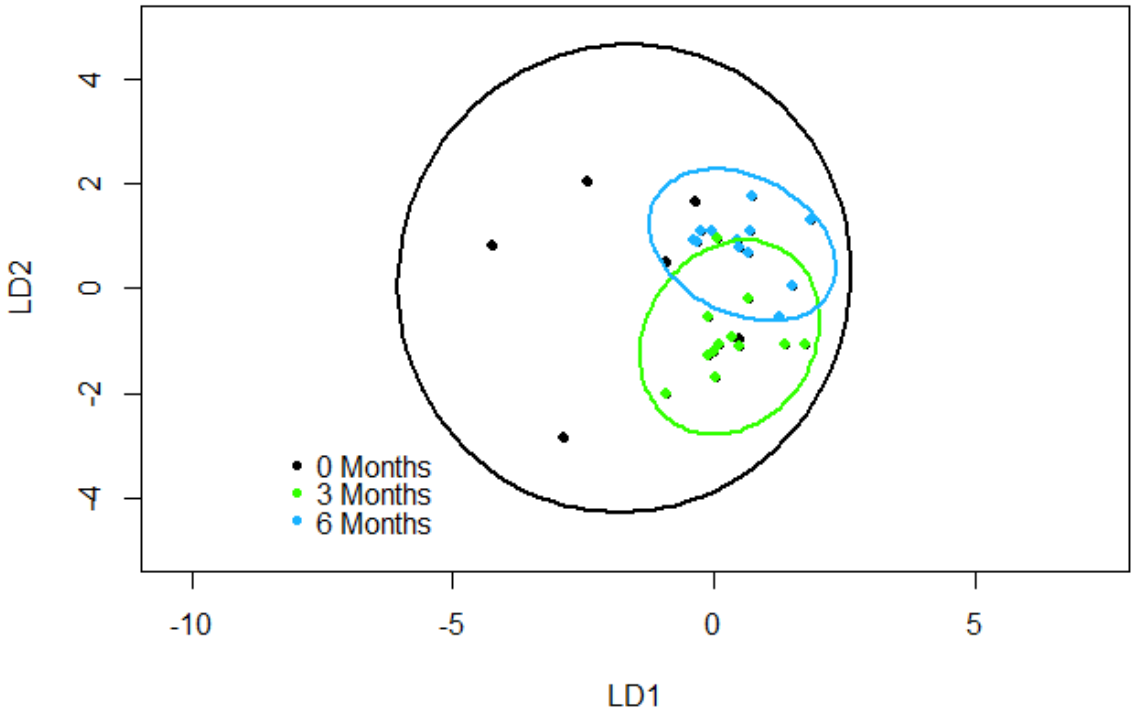


Figure S4.3: Linear discriminant plot from a CAP analysis of **'Morado'** VOCs over time. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 73.3 % at p = 0.05 (percent successful classification by group: 0 months = 16.7 %, 3 months = 83.3 %, 6 months = 91.7 %). N = 6 replicates of 3 bulbs for 0 months, N = 12 for 3 and 6 months.

8.10. Morado VOCs between temperatures

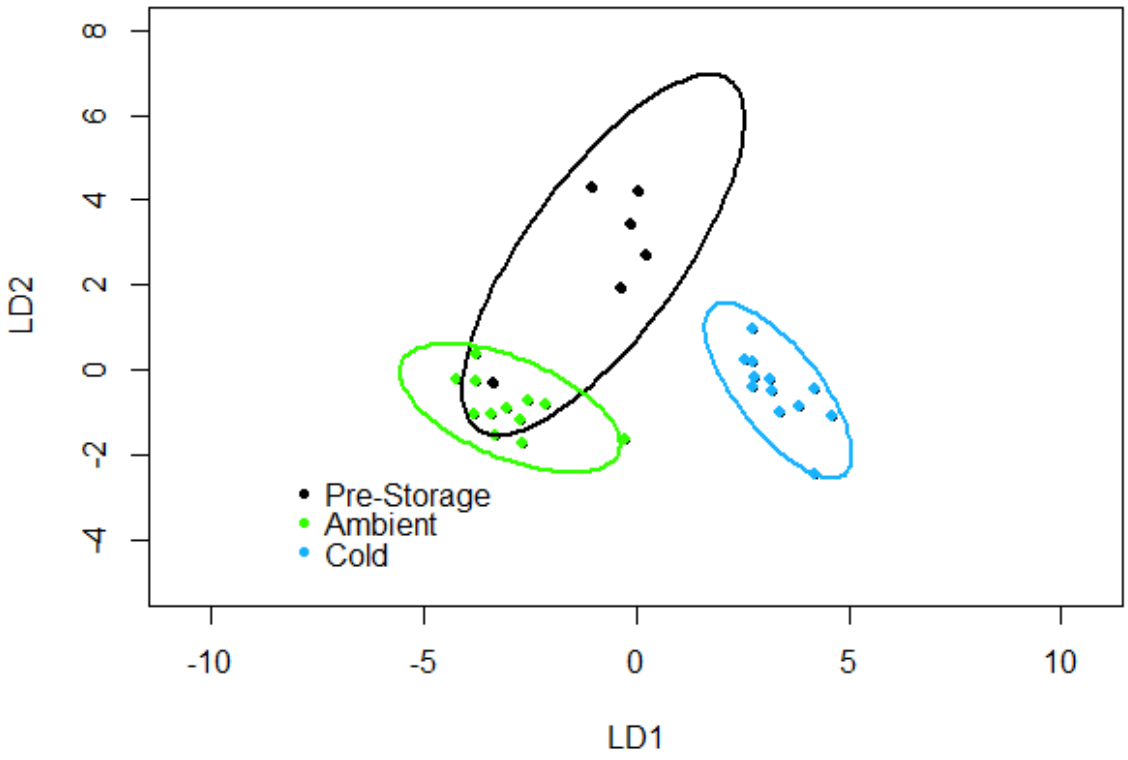


Figure S4.4: Linear discriminant plot from a CAP analysis of **'Morado'** VOCs between storage temperatures. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 96.7 % at $p = 0.05$ (percent successful classification by group: pre-storage = 83.3 %, ambient = 100 %, cold = 100 %). $N = 6$ replicates of 3 bulbs for prestorage, $N = 12$ for ambient and cold.

8.11. 'Violeta' VOCs over time

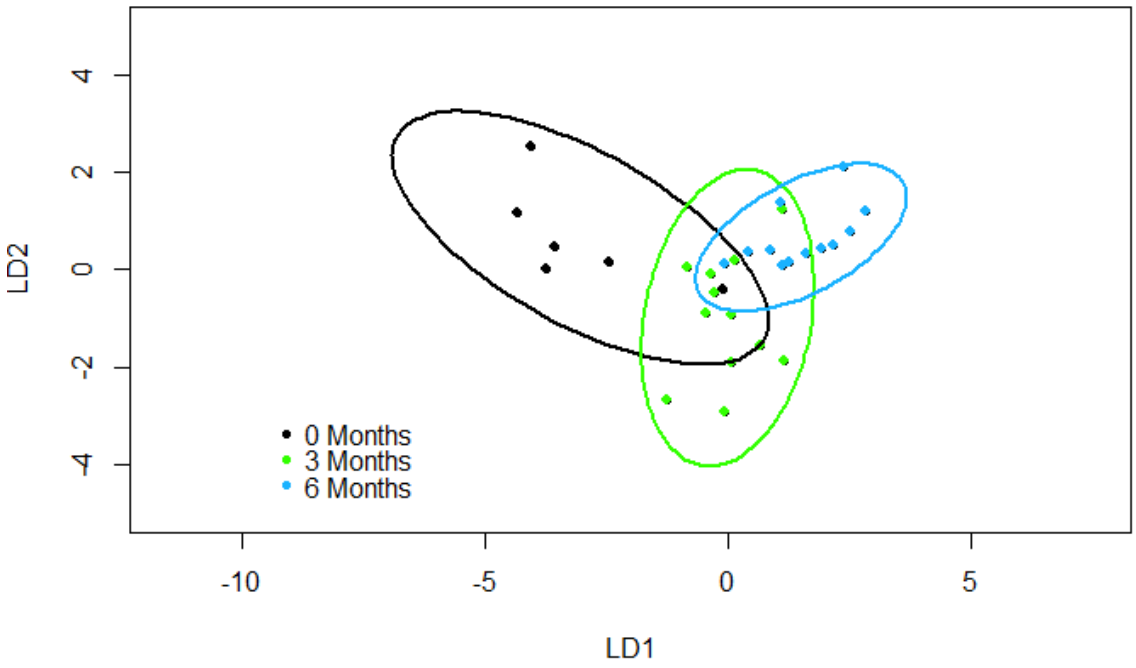


Figure S4.5: Linear discriminant plot from a CAP analysis of **'Violeta'** VOCs over time. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 70 % at $p = 0.05$ (percent successful classification by group: 0 months = 50 %, 3 months = 83.3 %, 6 months = 66.7 %). $N = 6$ replicates of 3 bulbs for 0 months, $N = 12$ for 3 and 6 months.

8.12. 'Violeta' VOCS between temperatures

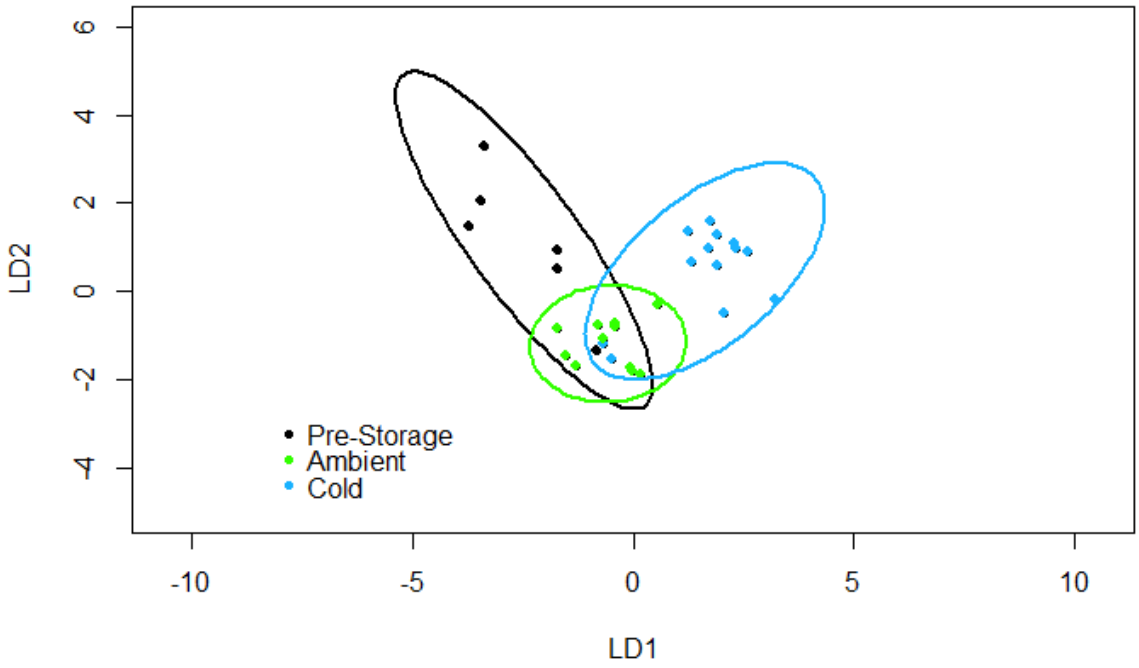


Figure S4.6: Linear discriminant plot from a CAP analysis of **'Violeta'** VOCs between storage temperatures. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 80 % at p = 0.05 (percent successful classification by group: pre-storage = 66.7 %, ambient = 90.9 %, cold = 76.9 %). N = 6 replicates of 3 bulbs for prestorage, N = 12 for ambient and cold.

8.13. Season one VOCs over time

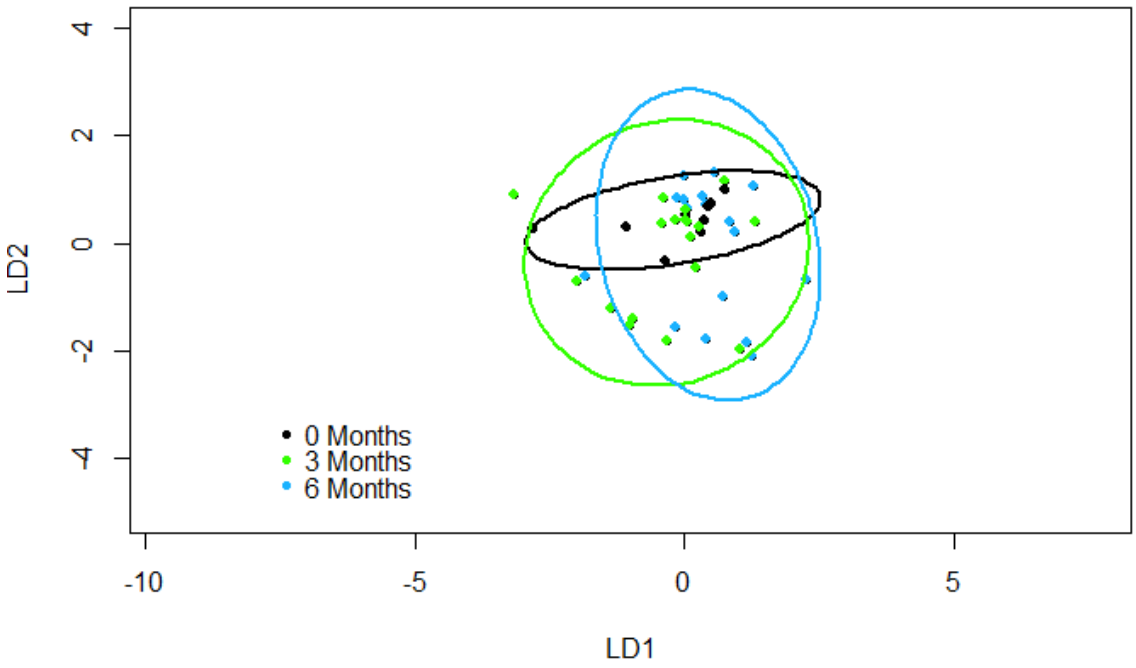


Figure S4.7: Linear discriminant plot from a CAP analysis of VOCs from season one over time. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 48.9 % at p = 0.05 (percent successful classification by group: 0 months = 0 %, 3 months = 61.1 %, 6 months = 61.1 %). N = 9 replicates of 3 bulbs for 0 months, N = 18 for 3 and 6 months.

8.14. Season one VOCs between temperatures

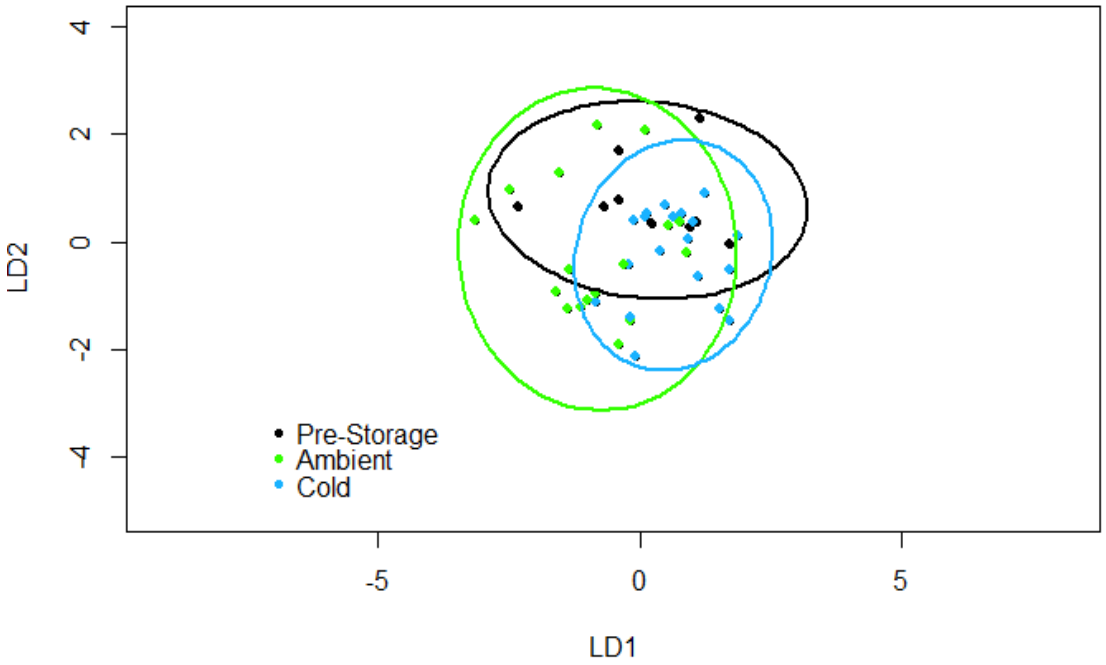


Figure S4.8: Linear discriminant plot from a CAP analysis of VOCs from season one between storage temperatures. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 44.4 % at p = 0.05 (percent successful classification by group: pre-storage = 0 %, ambient = 52.9 %, cold = 57.9 %). N = 9 replicates of 3 bulbs for prestorage, N = 18 for ambient and cold.

8.15. Season one VOCs between cultivars

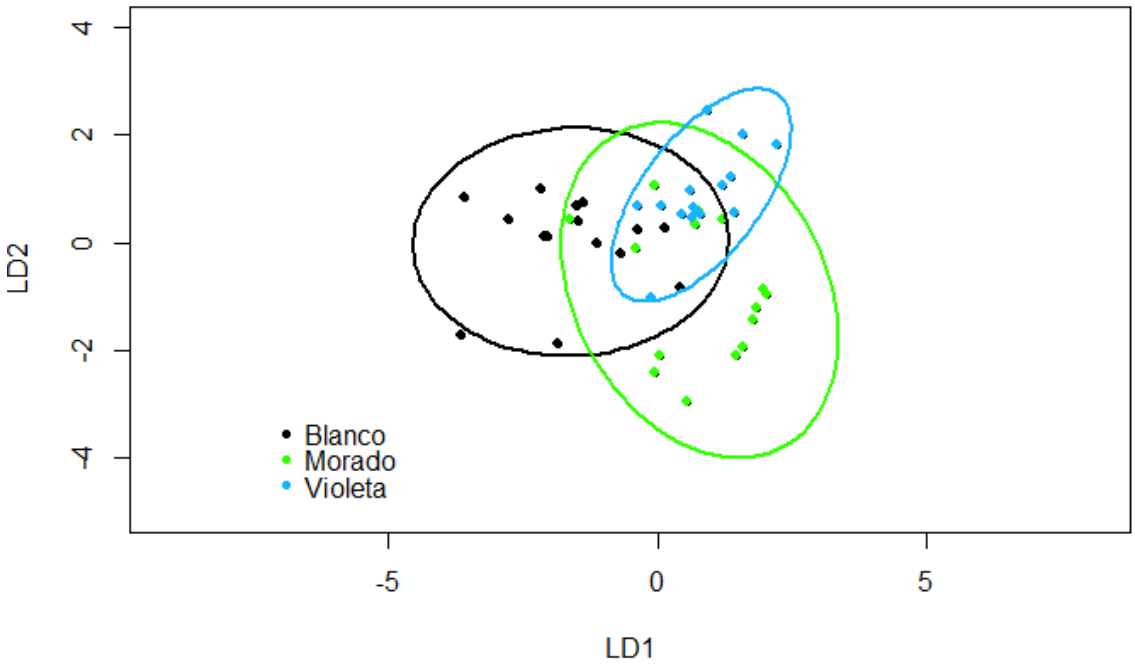


Figure S4.9: Linear discriminant plot from a CAP analysis of VOCs from season one between cultivars. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 75.6 % at p = 0.05 (percent successful classification by group: 'Morado' = 80 %, 'Blanco' = 60 %, 'Violeta' = 86.7 %). N = 12 replicates of 3 bulbs.

8.16. Season two VOCs over time

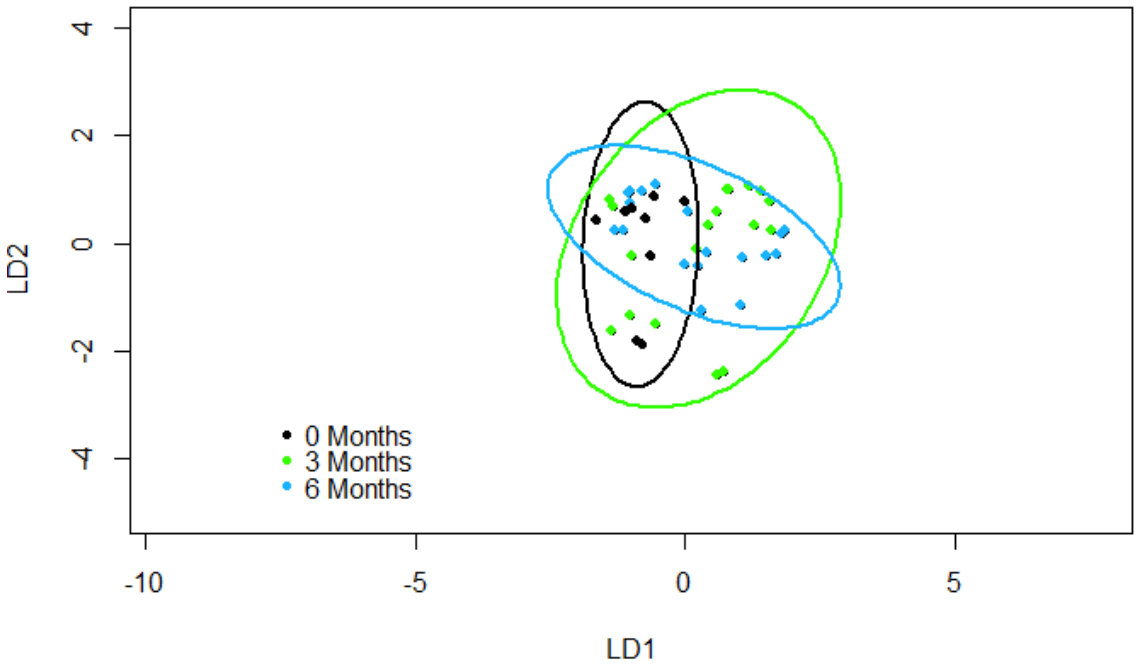


Figure S4.10: Linear discriminant plot from a CAP analysis of VOCs from season two over time. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 11.1 % at $p = 0.05$ (percent successful classification by group: 0 months = 11.1 %, 3 months = 5.6 %, 6 months = 16.7 %). $N = 9$ replicates of 3 bulbs for 0 months, $N = 18$ for 3 and 6 months.

8.17. Season two VOCs between temperatures

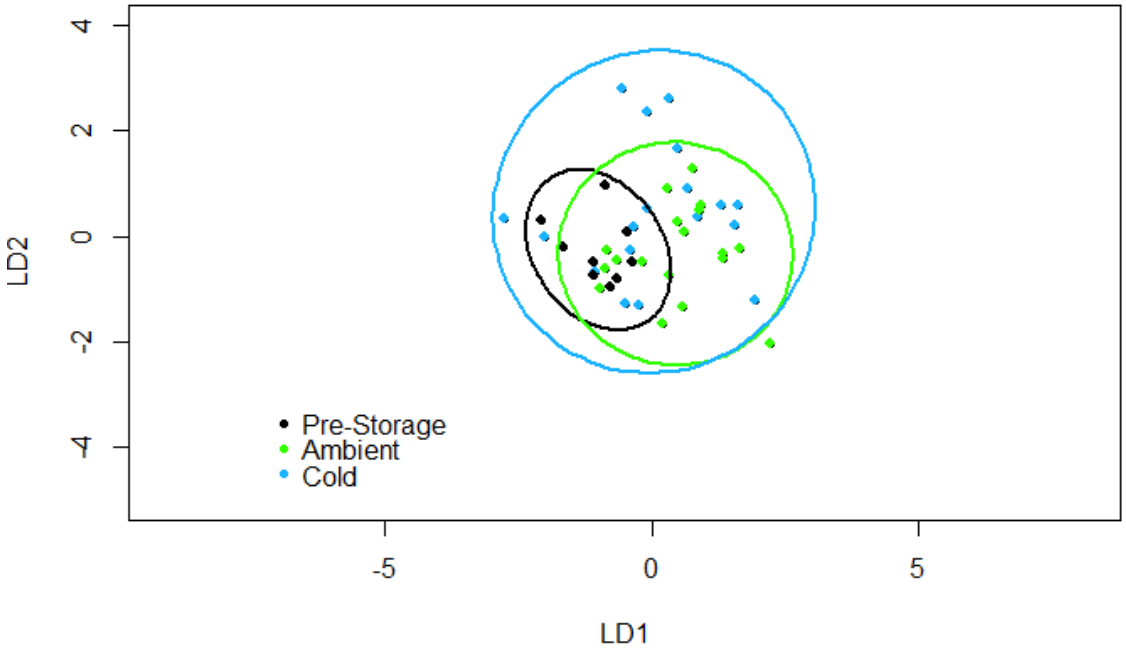


Figure S4.11: Linear discriminant plot from a CAP analysis of VOCs from season two between storage temperatures. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 31.1 % at p = 0.05 (percent successful classification by group: pre-storage = 22.2 %, ambient = 38.9 %, cold = 27.8 %). N = 9 replicates of 3 bulbs for prestorage, N = 18 for ambient and cold.

8.18. Season two VOCs between cultivars

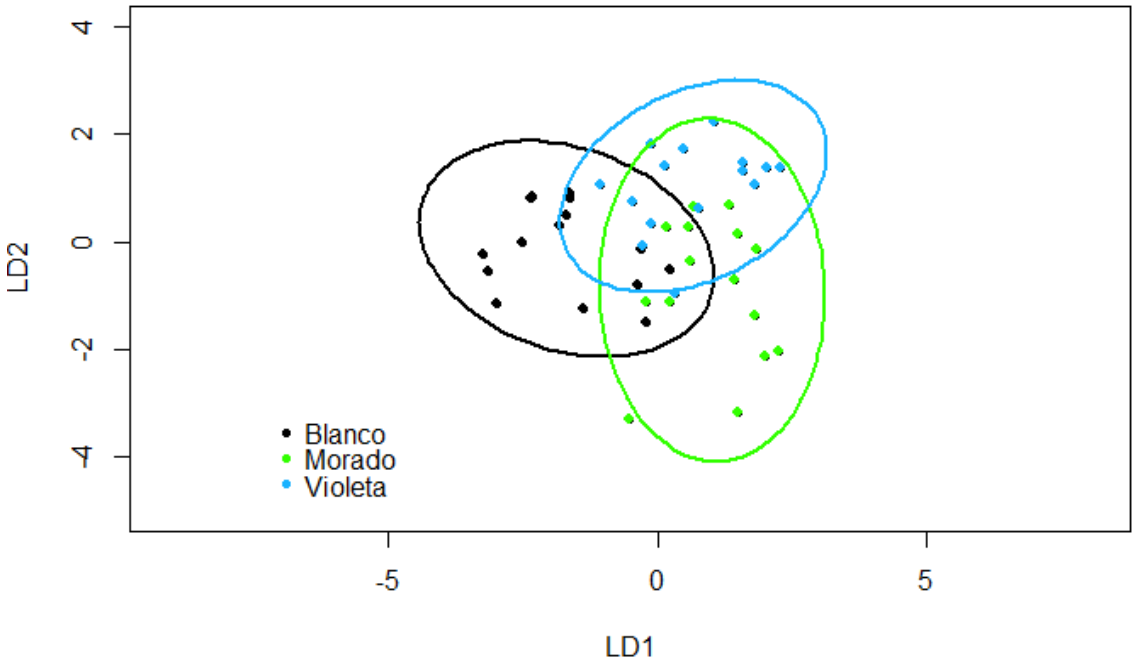


Figure S4.12: Linear discriminant plot from a CAP analysis of VOCs from season two between cultivars. The plots use the first two linear discriminants (LD) of the CAP model and each ellipse represents the 95% confidence interval and the percentage of correct classification was 68.9 % at p = 0.05 (percent successful classification by group: 'Morado' = 73.3 %, 'Blanco' = 60 %, 'Violeta' = 73.3 %). N = 12 replicates of 3 bulbs.

8.19. Average rainfall

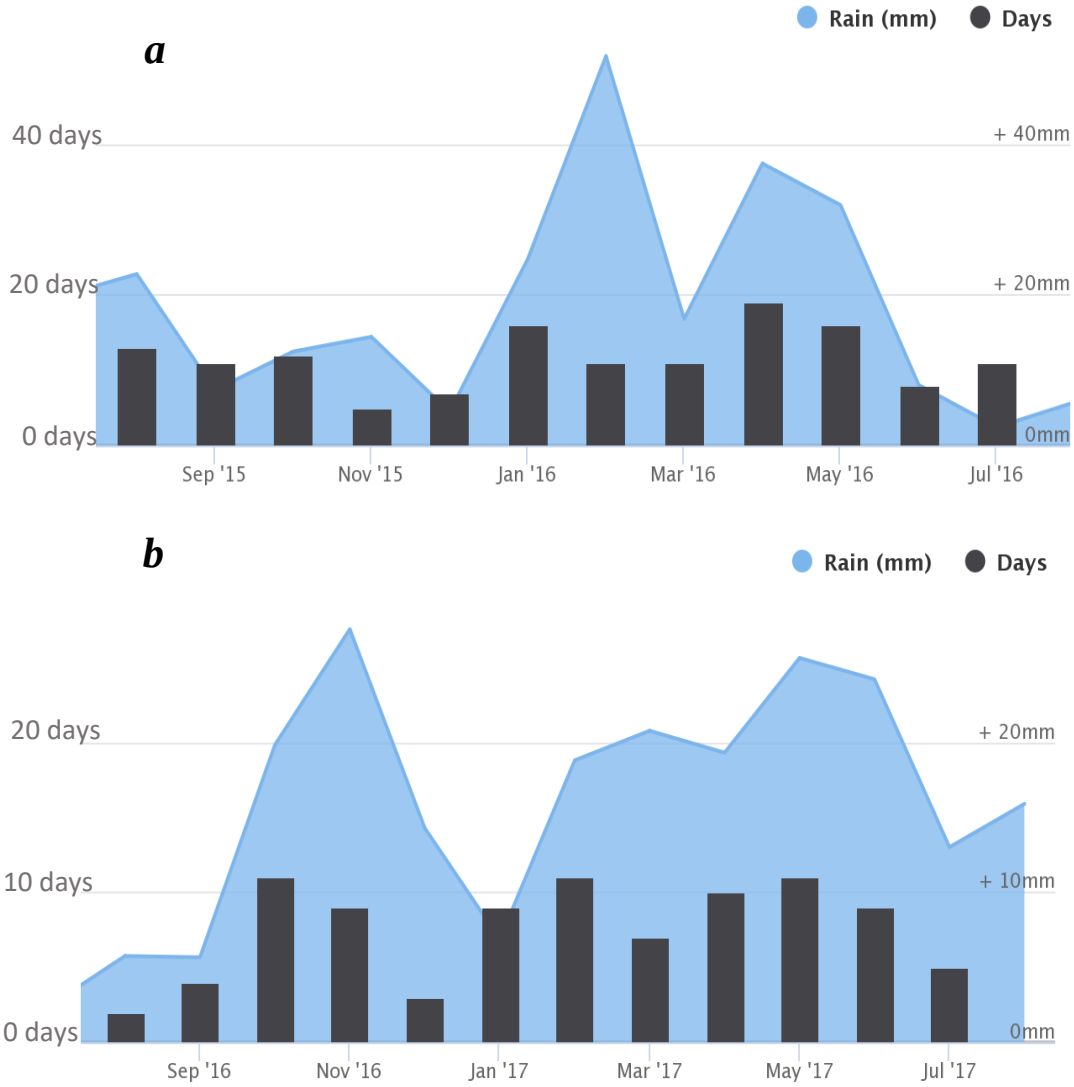


Figure S4.13: Average rainfall in Cuenca, Las Pedroneras, Spain. Season one spans August 2015 to July 2016 (***a***) and season two spans August 2016 to July 2017 (***b***). Light blue indicates total rainfall per month and dark grey bars indicate total number of days with precipitation. Data and graphs adapted sourced from WorldWeatherOnline.com

8.20. Average growing temperature

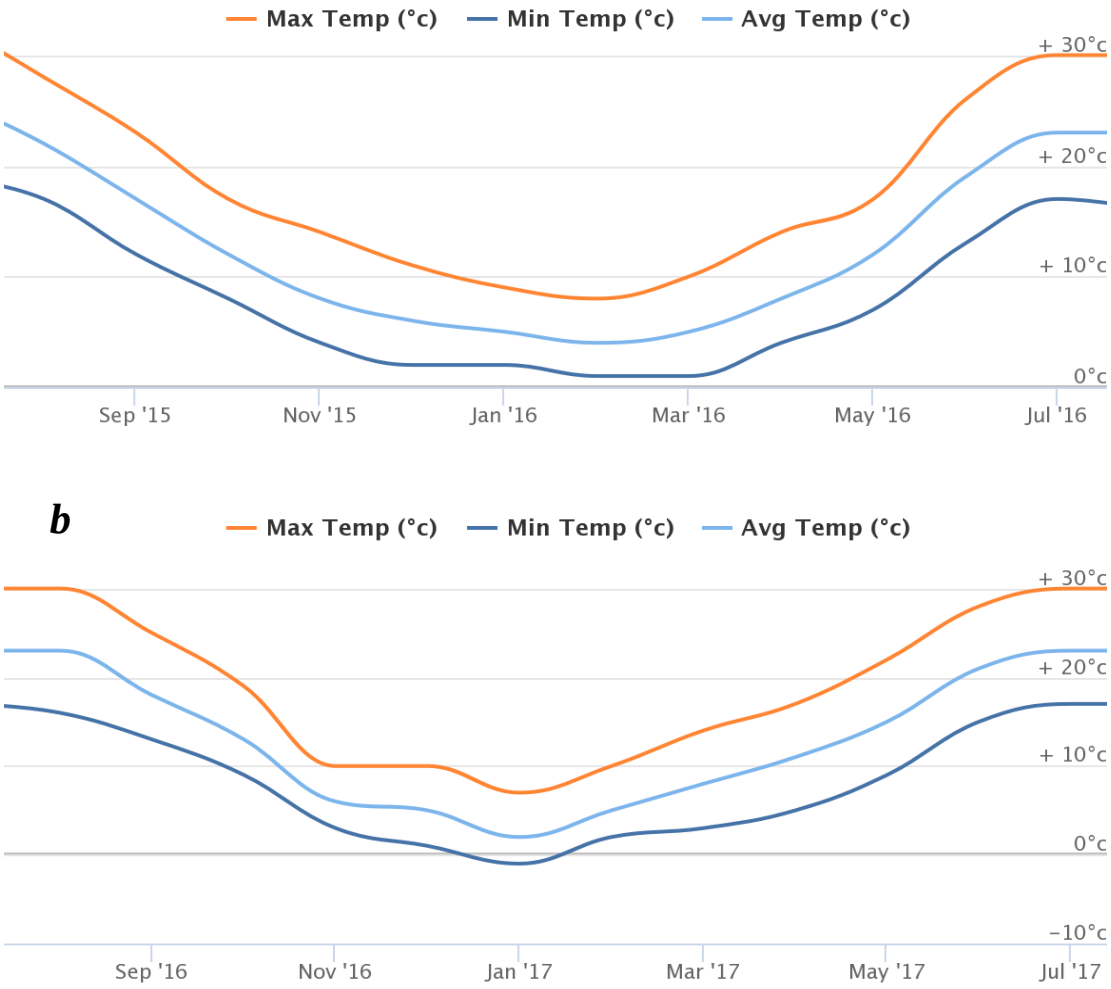


Figure S4.14: Daily average, minimum and maximum temperatures in Cuenca, Las Pedroñeras, Spain. Season one spans August 2015 to July 2016 (***a***) and season two spans August 2016 to July 2017 (***b***). Orange line indicates daily maximum temperature, light blue line indicates daily average temperature and purple line indicates daily minimum temperature. Data and graphs adapted sourced from WorldWeatherOnline.com

8.21. VOCs identified in lab based experiments

Table S3.3: Compounds identified by TD-GC-ToFMS across all samples studied in Chapter 3 and 4. Compound numbers are applicable across both Chapter 3 and 4. Nist Name = name originally assigned to the compound after identification by comparison of spectra and RI to NIST 2011 mass spectral database. IUPAC name = International Union of Pure and Applied Chemistry systematic name. CAS number = Chemical Abstracts Service registry number. RI = mean retention index as measured across all samples for a compound, calculated with reference to a linear alkane standard (C8-C20). Compound class = grouping of compounds according to functional group. Chapter 3 and Chapter 4: 1 indicates presence in either of these chapters, 0 indicates absence. Likewise, 1 indicates that the compound has been reported by the five studies referenced in the right hand columns and 0 indicates the compound was not (Left to right: Vernin et al. 1985, Calvo-Gómez et al. 2004, Yu et al. 1989, Lee et al. 2003, Molina-Calle et al. 2016).

Compound No.	NIST Name	IUPAC Name	CAS No.	Retention Index	Functional Group	Chapter 3	Chapter 4	Vernin et al. 1985	Calvo-Gómez et al. 2004	Yu et al. 1989	Lee et al. 2003	Molina-Calle *et al.* 2016
C1	∝-Copaene	(1*R*)-1,3-dimethyl-8-propan-2-yltricyclo[4.4.0.02,7]dec-3-ene	3856-25-5	1404	terpene	1	1	0	0	0	0	0
C2	1,2,4-Methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1à,2à,3aá,4à,8aá,9R	1,2,4-methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1α,2α,3aβ,4α,8aβ,9R*)]-	1137-12-8	1400	hydrocarbon	0	1	0	0	0	0	0
C3	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	84-69-5	1915	ester	1	1	0	0	0	0	0
C4	1,3,6-Octatriene, 3,7-dimethyl-, (Z)-	1,3,6-octatriene, 3,7-dimethyl-, (Z)-	3338-55-4	1056	hydrocarbon	0	0	0	0	0	0	0
C5	1,3-Cyclopentadiene, 5-(1-methylethylidene)-	1,3-cyclopentadiene, 5-(1-methylethylidene)-	2175-91-9	904	hydrocarbon	1	1	0	0	0	0	0
C6	1,3-Pentadiene, (E)-	1,3-pentadiene, (E)-	2004-70-8	617	hydrocarbon	0	1	0	0	0	0	0
C7	1,4-Methanobenzocyclodecene, 1,2,3,4,4a,5,8,9,12,12a-decahydro-	(4*Z*,8*Z*)-tricyclo[10.2.1.02,11]pentadeca-4,8-diene	74708-73-9	1682	hydrocarbon	0	1	0	0	0	0	0
C8	1,5-Hexadiene	1,5-hexadiene	41919-05-5	635	hydrocarbon	1	1	0	0	0	0	0
C9	1-Allyl-2-isopropyl disulfane	1-allyl-2-isopropyl disulfane	67421-85-6	1105	organosulphur	1	1	0	0	0	0	0
C10	1-Butanol	1-butanol	71-36-3	683	alcohol	1	1	0	0	0	0	0
C11	1-Butanol, 2-methyl-	1-butanol, 2-methyl-	137-32-6	747	alcohol	1	1	0	0	0	0	0
C12	1-Butanol, 3-methyl-	1-butanol, 3-methyl-	123-51-3	747	alcohol	1	1	0	0	0	0	0
C13	1-Butanol, 3-methyl-, acetate	1-butanol, 3-methyl-, acetate	123-92-2	881	alcohol	0	1	0	0	0	0	0
C14	1-Ethyl-4-methylcyclohexane	1-ethyl-4-methylcyclohexane	3728-56-1	890	hydrocarbon	1	1	0	0	0	0	0
C15	1H-Cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-m	1H-cyclopenta[1,3]cyclopropa[1,2]benzene, octahydro-7-methyl-3-methylene-4-(1-m	13744-15-5	1461	hydrocarbon	0	1	0	0	0	0	0
C16	1-Hexanol	1-hexanol	111-27-3	881	alcohol	1	1	0	0	1	0	0
C17	1-Hexanol, 2-ethyl-	1-hexanol, 2-ethyl-	104-76-7	1044	alcohol	1	1	0	0	0	0	0
C18	1-Methoxy-2-propyl acetate	1-methoxy-2-propyl acetate	108-65-6	885	ester	0	1	0	0	0	0	0
C19	1-Nonene	1-nonene	124-11-8	892	hydrocarbon	1	1	0	0	0	0	0
C20	1-Octanol, 2-butyl-	1-octanol, 2-butyl-	08-02-3913	1333	alcohol	0	1	0	0	0	0	0
C21	1-Octene	1-octene	111-66-0	793	hydrocarbon	0	1	0	0	0	0	0

C22	1-Pentanol	1-pentanol	71-41-0	778	alcohol	0	1	0	0	0	0	0
C23	1-Propanol, 2-methyl-	1-propanol, 2-methyl-	78-83-1	660	alcohol	1	1	0	0	0	0	0
C24	1-Propene, 1-(methylthio)-, (E)-	methanethiol	74-93-1	608	organosulphur	0	1	0	0	0	0	0
C25	1-Propene, 1-(methylthio)-, (Z)-	methylsulfanylmethane	75-18-3	617	organosulphur	1	1	0	0	0	0	0
C26	2(3H)-Furanone, 5-ethyldihydro-	prop-2-ene-1-thiol	870-23-5	644	organosulphur	1	1	0	0	0	0	0
C27	2(3H)-Furanone, dihydro-5-methyl-	3-methylsulfanylprop-1-ene	10152-76-8	705	organosulphur	0	1	0	0	0	0	0
C28	2,3-Butanediol	2,3-butanediol	513-85-9	800	alcohol	0	1	0	0	0	0	0
C29	2,3-Butanediol, [R-(R*,R*)]-	(2*R*,3*R*)-butane-2,3-diol	24347-58-8	799	alcohol	1	1	0	0	0	0	0
C30	2,3-Butanediol, [S-(R*,R*)]-	2*S*,3*S*)-butane-2,3-diol	19132-06-0	806	alcohol	0	1	0	0	0	0	0
C31	2,3-Diphenylmaleic anhydride	3,4-diphenylfuran-2,5-dione	4808-48-4	2189	ketone	0	1	0	0	0	0	0
C32	2,4-Dimethyl-1-heptene	2,4-dimethyl-1-heptene	19549-87-2	844	hydrocarbon	1	1	0	0	0	0	0
C33	2,4-Dimethylfuran	2,4-dimethylfuran	3710-43-8	721	furan	1	1	0	0	1	0	0
C34	2,4-Dithiapentane	1-(methylsulfanyl)propane	3877-15-4	717	organosulphur	0	1	0	0	0	0	0
C35	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	2,5-cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	719-22-2	1491	ketone	1	1	0	0	0	0	0
C36	2,6-Diisopropylnaphthalene	2,6-di(propan-2-yl)naphthalene	24157-81-1	1765	hydrocarbon	1	1	0	0	0	0	0
C37	2,6-Dimethyldecane	2,6-dimethyldecane	13150-81-7	1106	hydrocarbon	0	1	0	0	0	0	0
C38	2-Decen-1-ol, (E)-	(*E*)-dec-2-en-1-ol	18409-18-2	1181	ketone	1	1	0	0	0	0	0
C39	2-Ethylhexyl trans-4-methoxycinnamate	2-ethylhexyl 3-(4-methoxyphenyl)prop-2-enoate	5466-77-3	2209	ester	0	1	0	0	0	0	0
C40	2-Heptanone	heptan-2-one	110-43-0	897	ketone	0	1	0	0	0	0	0
C41	2-n-Butyl furan	2-butylfuran	4466-24-4	901	furan	0	1	0	0	0	0	0
C42	2-Nonen-1-ol	non-2-en-1-ol	22104-79-6	1074	alcohol	1	1	0	0	0	0	0
C43	2-Pentanone	pentan-2-one	107-87-9	695	ketone	0	1	0	0	0	0	0
C44	2-Pentanone, 3-methyl-	3-methylpentan-2-one	565-61-7	755	ketone	0	0	0	0	0	0	0
C45	2-Propanol, 1-(2-butoxy-1-methylethoxy)-	1-(1-butoxypropan-2-yloxy)propan-2-ol	29911-28-2	1261	alcohol	1	0	0	0	0	0	0
C46	2-Propanol, 1-(2-methoxy-1-methylethoxy)-	1-(1-methoxypropan-2-yloxy)propan-2-ol	20324-32-7	1017	alcohol	0	1	0	0	0	0	0
C47	2-Propanol, 1-(2-methoxypropoxy)-	1-(2-methoxypropoxy)propan-2-ol	13429-07-7	1029	alcohol	0	1	0	0	0	0	0
C48	2-Propanol, 1-methoxy-	1-methoxypropan-2-ol	107-98-2	692	alcohol	1	1	0	0	0	0	0
C49	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	2-ethylhexyl (*Z*)-3-(4-methoxyphenyl)prop-2-enoate	177352-99-7	2344	ester	1	1	0	0	0	0	0
C50	2-Thiophenecarboxaldehyde	thiophene-2-carbaldehyde	98-03-3	886	aldehyde	0	0	0	1	0	0	0
C51	2-Undecanone	undecan-2-one	112-12-9	1305	ketone	0	1	0	0	0	0	0

C52	3-Carene	3,7,7-trimethylbicyclo[4.1.0]hept-3-ene	13466-78-9	1022	terpene	1	1	0	0	0	0	0
C53	3-Heptanone	heptan-3-one	106-35-4	892	ketone	0	1	0	0	0	0	0
C54	3-Octanone	octan-3-one	106-68-3	991	ketone	0	1	0	0	0	0	0
C55	3-Tetradecene, (Z)-	(*Z*)-tetradec-3-ene	41446-67-7	1393	hydrocarbon	0	1	0	0	0	0	0
C56	3-Vinyl-1,2-dithiacyclohex-4-ene	1-propene, 1-(methylthio)-, (E)-	10152-77-9	728	organosulphur	1	1	0	1	0	0	1
C57	3-Vinyl-1,2-dithiacyclohex-5-ene	1-propene, 1-(methylthio)-, (Z)-	52195-40-1	737	organosulphur	1	1	0	1	1	1	1
C58	4-Heptanone	heptan-4-one	123-19-3	875	ketone	0	0	0	0	0	0	0
C59	4-Heptenal	hept-4-enal	929-22-6	849	aldehyde	1	1	0	0	0	1	1
C60	4-Methylenecyclopentene	4-methylidenecyclopentene	14548-32-4	666	hydrocarbon	0	1	0	0	0	0	0
C61	4-Penten-1-ol, 2-methyl-	2-methylpent-4-en-1-ol	5673-98-3	833	alcohol	1	1	0	0	0	0	0
C62	4-Pentenal, 2-methyl-	2-methylpent-4-enal	5187-71-3	750	aldehyde	0	0	0	1	0	0	1
C63	4-Pentenal, 2-methylene-	2-methylidenepent-4-enal	17854-46-5	761	aldehyde	0	1	0	0	0	0	1
C64	4-tert-Butylcyclohexyl acetate	(4-*tert*-butylcyclohexyl) acetate	32210-23-4	1316	ester	0	1	0	0	0	0	0
C65	5,5-Diethyltridecane	5,5-diethyltridecane	-	1617	hydrocarbon	1	1	0	0	0	0	0
C66	5-Ethyl-1-nonene	5-ethylnon-1-ene	-	1084	hydrocarbon	1	1	0	0	0	0	0
C67	5-Hepten-2-one, 6-methyl-	6-methylhept-5-en-2-one	110-93-0	997	ketone	1	1	0	0	0	0	0
C68	5-Hexen-1-ol	hex-5-en-1-ol	821-41-0	873	alcohol	0	1	0	0	0	0	0
C69	5-Hexen-2-one	hex-5-en-2-one	109-49-9	784	ketone	1	1	0	0	0	0	1
C70	5-Hexenal	hex-5-enal	764-59-0	793	aldehyde	1	1	0	0	0	0	1
C71	7-Octen-2-ol, 2,6-dimethyl-	2,6-Dimethyloct-7-en-2-ol	18479-58-8	1083	alcohol	0	1	0	0	0	0	0
C72	à-Calacorene	(1*S*)-4,7-dimethyl-1-propan-2-yl-1,2-dihydronaphthalene	21391-99-1	1585	terpene	0	0	0	0	0	0	0
C73	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	(1,7,7-trimethyl-2-bicyclo[2.2.1]heptanyl) acetate	92618-89-8	1311	ester	0	1	0	0	0	0	0
C74	Acetophenone	1-phenylethanone	98-86-2	1088	ketone	0	1	0	0	0	0	0
C75	à-Cubebene	4,10-dimethyl-7-propan-2-yltricyclo[4.4.0.$0^{1,5}$]dec-3-ene	17699-14-8	1384	terpene	0	0	1	0	0	0	0
C76	à-Guaiene	(1*S*,4*S*,7*R*)-1,4-dimethyl-7-prop-1-en-2-yl-1,2,3,4,5,6,7,8-octahydroazulene	3691-12-1	1474	terpene	0	1	0	0	0	0	0
C77	Alloaromadendrene	(1aR,4aS,7R,7aR,7bS)-1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa[e]azulene	25246-27-9	1509	hydrocarbon	0	1	0	0	0	0	0
C78	Allyl mercaptan	(methyldisulfanyl) methane	624-92-0	752	organosulphur	0	1	0	1	0	0	1
C79	Allyl n-propyl sulphide	*S*-methyl prop-2-enethioate	5883-16-9	810	organosulphur	0	1	0	0	0	0	1
C80	á-Myrcene	2-methyl-6-methylene-1,7-octadiene	1686-30-2	998	terpene	0	1	0	0	0	0	0

C81	à-Phellandrene	2-methyl-5-propan-2-ylcyclohexa-1,3-diene	99-83-2	938	terpene	0	1	0	0	0	0	0
C82	á-Pinene	2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	80-56-8	987	terpene	0	1	1	0	0	0	0
C83	Azulene	azulene	275-51-4	1219	hydrocarbon	1	1	0	0	0	0	0
C84	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1à,	(3*S*,3*aS*,5*R*)-3,8-dimethyl-5-prop-1-en-2-yl-1,2,3,3*a*,4,5,6,7-octahydroazulene	3691-11-0	1538	hydrocarbon	0	0	0	0	0	0	0
C85	Benzaldehyde	benzaldehyde	100-52-7	984	aldehyde	1	1	0	0	0	0	1
C86	Benzene	benzene	71-43-2	689	hydrocarbon	1	1	0	0	0	0	0
C87	Benzene, (1-butylheptyl)-	decan-5-ylbenzene	4537-11-5	1659	hydrocarbon	0	1	0	0	0	0	0
C88	Benzene, 1,2,3,5-tetramethyl-	1,2,3,5-tetramethylbenzene	527-53-7	1136	hydrocarbon	1	1	0	0	0	0	0
C89	Benzene, 1,2,4-trimethyl-	1,2,4-trimethylbenzene	95-63-6	986	hydrocarbon	1	1	0	0	0	0	0
C90	Benzene, 1,2-dimethoxy-	1,2-dimethoxybenzene	91-16-7	1175	hydrocarbon	0	1	0	0	0	0	0
C91	Benzene, 1,3-bis(1,1-dimethylethyl)-	1,3-di*tert*-butylbenzene	1014-60-4	1273	hydrocarbon	1	1	0	0	0	0	0
C92	Benzene, 1,3-dimethyl-	1,3-dimethylbenzene	108-38-3	886	hydrocarbon	0	1	0	0	0	0	0
C93	Benzene, 1-ethyl-2,4-dimethyl-	1-ethyl-2,4-dimethylbenzene	874-41-9	1074	hydrocarbon	1	1	0	0	0	0	0
C94	Benzene, 1-ethyl-2-methyl-	1-ethyl-2-methylbenzene	25550-14-5	994	hydrocarbon	1	1	0	0	0	0	0
C95	Benzene, 1-ethyl-4-methyl-	1-ethyl-4-methylbenzene	622-96-8	975	hydrocarbon	1	1	0	0	0	0	0
C96	Benzene, 1-methyl-2-propyl-	1-methyl-2-propylbenzene	1074-17-5	1080	hydrocarbon	0	1	0	0	0	0	0
C97	Benzene, 1-methyl-4-propyl-	1-methyl-4-propylbenzene	1074-55-1	1069	hydrocarbon	0	1	0	0	0	0	0
C98	Benzene, propyl-	propylbenzene	103-65-1	967	hydrocarbon	1	1	0	0	0	0	0
C99	Benzeneacetaldehyde	2-phenylacetaldehyde	122-78-1	1063	aldehyde	0	1	0	0	0	0	0
C100	Benzoic acid	benzoic acid	65-85-0	1210	carboxylic acid	0	1	0	0	0	0	0
C101	Benzoic acid, pentadecyl ester	pentadecyl benzoate	68411-27-8	2482	ester	0	1	0	0	0	0	0
C102	Berkheyaradulene	2,5,6,8-tetramethyltricyclo[6.3.0.0^{1,5}]undec-6-ene	65372-78-3	1412	terpene	1	1	0	0	0	0	0
C103	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	4-methyl-1-propan-2-ylbicyclo[3.1.0]hex-2-ene	28634-89-1	987	hydrocarbon	0	1	0	0	0	0	0
C104	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-	(1*R*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	87-44-5	1442	hydrocarbon	0	1	0	0	0	0	0
C105	Butanal, 2-ethyl-3-methyl-	2-ethyl-3-methylbutanal	26254-92-2	839	aldehyde	1	1	0	0	0	0	0
C106	Butanal, 3-methyl-	3-methylbutanal	590-86-3	678	aldehyde	1	1	0	0	0	0	1
C107	Butane, 2,3-dimethyl-	2,3-dimethylbutane	79-29-8	635	hydrocarbon	0	1	0	0	0	0	0
C108	Butane, 2-methyl-	2-methylbutane	78-78-4	615	hydrocarbon	0	1	0	0	0	0	0
C109	Butanenitrile, 2-methyl-	2-methylbutanenitrile	18936-17-9	732	nitrogenous	0	1	0	0	0	0	0
C110	Butanenitrile, 3-methyl-	3-methylbutanenitrile	625-28-5	734	nitrogenous	0	1	0	0	0	0	0
C111	Butylated Hydroxytoluene	2,6-di*tert*-butyl-4-methylphenol	128-37-0	1543	phenol	0	1	0	0	0	0	0

C112	Caryophyllene	(1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene	87-44-5	1454	terpene	1	1	0	0	0	0	0
C113	Cyclohexane, (2-methylpropyl)-	2-methylpropylcyclohexane	1678-98-4	988	hydrocarbon	0	0	0	0	0	0	0
C114	Cyclohexane, 1,3,5-trimethyl-	1,3,5-trimethylcyclohexane	1839-63-0	852	hydrocarbon	0	0	0	0	0	0	0
C115	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1à,2á,4á)]-	1-ethenyl-1-methyl-2,4-bis(prop-1-en-2-yl)cyclohexane	33880-83-0	1419	hydrocarbon	1	1	1	0	0	0	0
C116	Cyclohexane, 1-ethyl-2-methyl-, cis-	(1*S*,2*R*)-1-ethyl-2-methylcyclohexane	4923-77-7	911	hydrocarbon	0	0	0	0	0	0	0
C117	Cyclohexane, 1-ethyl-4-methyl-, trans-	trans-1-Ethyl-4-methylcyclohexane	6236-88-0	897	hydrocarbon	0	1	0	0	0	0	0
C118	Cyclohexane, 1-methyl-3-propyl-	1-methyl-3-propylcyclohexane	4291-80-9	979	hydrocarbon	1	1	1	0	0	0	0
C119	Cyclohexane, 1-methyl-4-(1-methylethyl)-, trans-	trans-1-isopropyl-4-methylcyclohexane	1678-82-6	981	hydrocarbon	0	1	0	0	0	0	0
C120	Cyclohexane, 2-butyl-1,1,3-trimethyl-	2-butyl-1,1,3-trimethylcyclohexane	54676-39-0	1237	hydrocarbon	0	1	0	0	0	0	0
C121	Cyclohexane, butyl-	butylcyclohexane	1678-93-9	1037	hydrocarbon	1	1	0	0	0	0	0
C122	Cyclohexane, methyl-	methylcyclohexane	108-87-2	725	hydrocarbon	1	1	0	0	0	0	0
C123	Cyclohexane, octyl-	octylcyclohexane	1795-15-9	1459	hydrocarbon	1	1	0	0	0	0	0
C124	Cyclohexane, pentyl-	pentylcyclohexane	4292-92-6	1142	hydrocarbon	1	1	1	0	0	0	0
C125	Cyclohexane, propyl-	propylcyclohexane	1678-92-8	933	hydrocarbon	1	1	0	0	0	0	0
C126	Cyclohexanol, 4-(1,1-dimethylethyl)-, acetate, trans-	(trans-4-tert-butylcyclohexyl) acetate	1900-69-2	1392	alcohol	0	1	0	0	0	0	0
C127	Cyclopropane, ethylidene-	ethylidenecyclopropane	18631-83-9	622	hydrocarbon	0	1	0	0	0	0	0
C128	Decanal	decanal	112-31-2	1217	aldehyde	1	1	0	0	0	0	0
C129	Decane	decane	124-18-5	1001	hydrocarbon	1	1	1	0	0	0	0
C130	Decane, 2-methyl-	2-methyldecane	6975-98-0	1063	hydrocarbon	0	1	0	0	0	0	0
C131	Decane, 3-methyl-	3-methyldecane	13151-34-3	1071	hydrocarbon	1	1	0	0	0	0	0
C132	Decane, 4-methyl-	4-methyldecane	2847-72-5	1026	hydrocarbon	1	1	0	0	0	0	0
C133	Decane, 5-methyl-	5-methyldecane	13151-35-4	1055	hydrocarbon	1	1	0	0	0	0	0
C134	Diallyl disulphide	3-[(Prop-2-en-1-yl)disulfanyl]prop-1-ene	2179-57-9	849	organosulphur	1	1	0	1	1	1	1
C135	Diallyl sulfide	3-prop-2-enylsulfanylprop-1-ene	592-88-1	867	organosulphur	1	1	0	1	1	1	1
C136	Dimethyl sulfide	1-prop-2-enylsulfanylpropane	27817-67-0	877	organosulphur	0	1	0	0	0	0	1
C137	Dimethyl sulfone	2,4-dithiapentane	1618-26-4	898	organosulphur	0	1	0	0	0	0	0
C138	Dimethyl Sulfoxide	2,4-dimethylthiophene	638-00-6	914	organosulphur	0	1	0	0	0	0	0
C139	Dimethyl trisulfide	3-(methyldisulfanyl)prop-1-ene	2179-58-0	927	organosulphur	0	1	0	1	1	0	1

C140	Disulfide, dimethyl	1-(methyldisulfanyl)propane	2179-60-4	938	organosulphur	1	1	0	1	1	1	1
C141	Disulfide, methyl 2-propenyl	methylsulfonylmethane	67-71-0	950	organosulphur	1	1	0	1	1	1	1
C142	Disulfide, methyl propyl	5-methyl-1,3-dihydro-2H-benzimidazol-2-one	5400-75-9	970	organosulphur	0	0	0	1	1	1	0
C143	D-Limonene	(4*R*)-1-methyl-4-prop-1-en-2-ylcyclohexene	5989-27-5	1039	terpene	1	1	0	0	0	0	0
C144	Dodecane	dodecane	112-40-3	1201	hydrocarbon	1	1	0	0	0	0	0
C145	Dodecane, 2,6,11-trimethyl-	2,6,11-trimethyldodecane	31295-56-4	1338	hydrocarbon	1	1	0	0	0	0	0
C146	Dodecane, 2,7,10-trimethyl-	2,7,10-trimethyldodecane	74645-98-0	1293	hydrocarbon	1	1	0	0	0	0	0
C147	Dodecane, 2-methyl-	2-methyldodecane	1560-97-0	1266	hydrocarbon	1	1	0	0	0	0	0
C148	Dodecane, 4-methyl-	4-methyldodecane	6117-97-1	1262	hydrocarbon	0	1	0	0	0	0	0
C149	E 1-Allyl-2-(prop-1-en-1-yl)disulfane	(methyltrisulfanyl)methane	3658-80-8	984	organosulphur	1	1	0	0	0	0	0
C150	Eicosane	icosane	112-95-8	2000	hydrocarbon	1	1	0	0	0	0	0
C151	Epizonarene	1,6-dimethyl-4-propan-2-yl-1,2,3,7,8,8*a*-hexahydronaphthalene	41702-63-0	1529	terpene	0	1	0	0	0	0	0
C152	Ethanol, 2-ethoxy-	2-ethoxyethanol	110-80-5	743	alcohol	0	1	0	0	0	0	0
C153	Ethanone, 1-(1-methylcyclohexyl)-	1-(1-methylcyclopentyl)ethanone	13388-93-7	987	ketone	1	1	0	0	0	0	0
C154	Ethyl Acetate	ethyl acetate	141-78-6	661	ester	0	1	0	0	0	0	0
C155	Furan, 2-ethyl-	2-ethylfuran	3208-16-0	714	furan	1	1	0	0	0	0	0
C156	Furan, 2-ethyl-5-methyl-	2-ethyl-5-methylfuran	1703-52-2	807	furan	0	0	0	0	0	0	0
C157	Furan, 2-pentyl-	2-pentylfuran	3777-69-3	1002	furan	1	1	0	0	0	0	0
C158	Furan, 3-methyl-	3-methylfuran	930-27-8	657	furan	0	1	0	0	0	0	0
C159	Heptanal	heptanal	111-71-7	905	aldehyde	1	1	0	0	0	0	0
C160	Heptane, 2,2,4,6,6-pentamethyl-	2,2,4,6,6-pentamethylheptane	13475-82-6	998	hydrocarbon	0	1	0	0	0	0	0
C161	Heptane, 2,4-dimethyl-	2,4-dimethylheptane	2213-23-2	860	hydrocarbon	0	1	0	0	0	0	0
C162	Heptane, 2,5,5-trimethyl-	2,5,5-trimethylheptane	1189-99-7	1013	hydrocarbon	0	1	0	0	0	0	0
C163	Heptane, 3-methyl-	3-methylheptane	589-81-1	771	hydrocarbon	1	1	0	0	0	0	0
C164	Hexadecane, 2,6,10,14-tetramethyl-	2,6,10,14-tetramethylhexadecane	638-36-8	1812	hydrocarbon	0	1	0	0	0	0	0
C165	Hexadecane, 3-methyl-	3-methylhexadecane	6418-43-5	1673	hydrocarbon	1	1	0	0	0	0	0
C166	Hexanal	hexanal	66-25-1	806	aldehyde	1	1	0	0	0	0	0
C167	Hexane, 2,3-dimethyl-	2,3-dimethylhexane	584-94-1	763	hydrocarbon	1	1	0	0	0	0	0
C168	Hexanoic acid	hexanoic acid	142-62-1	1006	carboxylic acid	0	1	0	0	0	0	0
C169	Hexanoic acid, methyl ester	methyl hexanoate	106-70-7	934	carboxylic acid	0	1	0	0	0	0	0
C170	Indane	2,3-dihydro-1*H*-indene	496-11-7	1052	hydrocarbon	1	1	0	0	0	0	0
C171	Isobutyronitrile	2-methylpropanenitrile	78-82-0	658	nitrogenous	0	0	0	0	0	0	0
C172	Isopropyl acetate	propan-2-yl acetate	108-21-4	678	ester	1	0	0	0	0	0	0

C173	Isopropyl myristate	propan-2-yl tetradecanoate	110-27-0	1839	ester	1	1	0	0	0	0	0
C174	L-Lactic acid	(2*S*)-2-hydroxypropanoic acid	79-33-4	811	organic acid	0	1	0	0	0	0	0
C175	Longifolene	3,3,7-trimethyl-8-methylidenetricyclo [5.4.0.02,9]undecane	475-20-7	1442	terpene	1	1	0	0	0	0	0
C176	Mesitylene	1,3,5-trimethylbenzene	108-67-8	1005	terpene	1	1	0	0	0	0	0
C177	Methanethiol	5-ethyldihydro-2(3H)-thiophenone	570-03-6	1071	organosulphur	0	1	0	0	0	0	0
C178	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalene	473-13-2	1524	terpene	1	1	0	0	0	0	0
C179	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)	(1R,4aS,8aS)-7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1H-naphthalene	39029-41-9	1551	terpene	1	1	0	0	0	0	0
C180	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	(1*S*,4*S*)-1,6-dimethyl-4-propan-2-yl-1,2,3,4-tetrahydronaphthalene	72937-55-4	1560	terpene	1	1	0	0	0	0	0
C181	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	(1S-cis)-4,7-dimethyl-1,2,3,5,6,8-hexahydro-1-(1-methylethyl)naphthalene	483-76-1	1556	terpene	1	1	0	0	0	0	0
C182	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1à,4	4,7-dimethyl-1-propan-2-yl-1,2,4*a*,5,8,8*a*-hexahydronaphthalene	29350-73-0	1574	terpene	0	1	0	0	0	0	0
C183	Naphthalene, decahydro-, cis-	1,2,3,4,4*a*,5,6,7,8,8*a*-decahydronaphthalene	493-01-6	1111	terpene	1	1	0	0	0	0	0
C184	Naphthalene, decahydro-, trans-	trans-Decahydronaphthalene	493-02-7	1064	terpene	1	1	0	0	0	0	0
C185	Naphthalene, decahydro-2-methyl-	2-methyl-1,2,3,4,4*a*,5,6,7,8,8*a*-decahydronaphthalene	2958-76-1	1122	terpene	0	0	0	0	0	0	0
C186	n-Hexadecanoic acid	hexadecanoic acid	57-10-3	2007	organic acid	0	1	0	0	0	0	0
C187	Nonadecane	nonadecane	629-92-5	1902	hydrocarbon	1	1	0	0	0	0	0
C188	Nonanal	nonanal	124-19-6	1111	aldehyde	1	1	0	0	0	0	0
C189	Nonane	nonane	111-84-2	900	hydrocarbon	1	1	0	0	0	0	0
C190	Nonane, 2,2,4,4,6,8,8-heptamethyl-	2,2,4,4,6,8,8-heptamethylnonane	4390-04-9	1341	hydrocarbon	0	0	0	0	0	0	0
C191	Nonane, 2,6-dimethyl-	2,6-dimethylnonane	17302-28-2	1058	hydrocarbon	1	1	0	0	0	0	0
C192	Nonane, 2-methyl-	2-methylnonane	871-83-0	965	hydrocarbon	1	1	0	0	0	0	0
C193	Nonane, 3-methyl-	3-methylnonane	5911-04-6	973	hydrocarbon	1	1	0	0	0	0	0
C194	Nonane, 4-methyl-	4-methylnonane	17301-94-9	964	hydrocarbon	1	1	0	0	0	0	0
C195	n-Propyl acetate	propyl acetate	109-60-4	724	ester	0	1	0	0	0	0	0

C196	Octadecane	octadecane	593-45-3	1802	hydrocarbon	1	1	0	0	0	0	0
C197	Octanal	octanal	124-13-0	1009	aldehyde	1	1	0	0	0	0	0
C198	Octane	octane	111-65-9	800	hydrocarbon	1	1	0	0	0	0	0
C199	Octane, 2,4,6-trimethyl-	2,4,6-trimethyloctane	62016-37-9	975	hydrocarbon	0	1	0	0	0	0	0
C200	Octane, 2,5-dimethyl-	2,5-dimethyloctane	15869-89-3	922	hydrocarbon	0	0	0	0	0	0	0
C201	Octane, 3,3-dimethyl-	3,3-dimethyloctane	4110-44-5	1017	hydrocarbon	0	1	0	0	0	0	0
C202	Octane, 3-methyl-	3-methyloctane	2216-33-3	873	hydrocarbon	0	0	0	0	0	0	0
C203	Octane, 4-ethyl-	4-ethyloctane	15869-86-0	958	hydrocarbon	0	1	0	0	0	0	0
C204	Octane, 4-methyl-	4-methyloctane	2216-34-4	863	hydrocarbon	0	1	0	0	0	0	0
C205	o-Terphenyl	1,2-diphenylbenzene	84-15-1	1968	terpene	1	1	0	0	0	0	0
C206	p-Dicyclohexylbenzene	1,4-dicyclohexylbenzene	1087-02-1	2043	hydrocarbon	1	1	0	0	0	0	0
C207	Pentadecane, 2,6,10,14-tetramethyl-	2,6,10,14-tetramethylpentadecane	1921-70-6	1709	hydrocarbon	0	1	0	0	0	0	0
C208	Pentadecane, 2-methyl-	2-methylpentadecane	1560-93-6	1565	hydrocarbon	1	1	0	0	0	0	0
C209	Pentadecane, 4-methyl-	4-methylpentadecane	2801-87-8	1559	hydrocarbon	0	1	0	0	0	0	0
C210	Pentanal	pentanal	110-62-3	713	aldehyde	0	1	0	0	0	0	0
C211	Pentane	pentane	109-66-0	619	hydrocarbon	1	1	0	0	0	0	0
C212	Phenol	phenol	108-95-2	1019	phenol	0	1	0	0	0	0	0
C213	Phenol, 2-methoxy-	2-methoxyphenol	90-05-1	1118	phenol	0	1	0	0	0	0	0
C214	Propane, 1-(methylthio)-	3-(prop-2-enyldisulfanyl)prop-1-ene	2179-57-9	1092	organosulphur	0	1	0	0	0	0	0
C215	Propanoic acid	propanoic acid	79-09-4	719	carboxylic acid	0	1	0	0	0	0	0
C216	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	(3-hydroxy-2,4,4-trimethylpentyl) 2-methylpropanoate	74367-34-3	1396	carboxylic acid	1	1	0	0	0	0	0
C217	Propanoic acid, 2-oxo-, methyl ester	methyl 2-oxopropanoate	600-22-6	744	ester	0	1	0	0	0	0	0
C218	Propanoic acid, butyl ester	butyl propanoate	590-01-2	908	ester	0	0	0	0	0	0	0
C219	Propanoic acid, ethyl ester	ethyl propanoate	105-37-3	724	ester	0	1	0	0	0	0	0
C220	Propylene Glycol	propane-1,2-diol	57-55-6	760	alcohol	0	1	0	0	0	0	0
C221	p-Xylene	1,4-xylene	106-42-3	878	terpene	1	1	0	0	0	0	0
C222	Pyrazine, methyl-	2-methylpyrazine	109-08-0	837	nitrogenous	0	1	0	0	0	0	0
C223	S-Methyl 2-propenethioate	(1Z)-1-(allyldisulfanyl)-1-propene	122156-03-0	1109	organosulphur	0	1	0	0	0	0	0
C224	Styrene	styrene	100-42-5	905	hydrocarbon	1	1	0	0	0	0	0
C225	Sulfide, allyl methyl	3-[(E)-prop-1-enyl]disulfanylprop-1-ene	122156-02-9	1114	organosulphur	1	1	0	1	1	1	1
C226	Supraene	(6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene	111-02-4	2709	terpene	0	0	0	0	0	0	0
C227	Tetradecane, 4-ethyl-	4-ethyltetradecane	55045-14-2	1520	hydrocarbon	0	1	0	0	0	0	0

C228	Tetradecane, 4-methyl-	4-methyltetradecane	25117-24-2	1461	hydrocarbon	1	1	0	0	0	0	0
C229	Tetradecane, 5-methyl-	5-methyltetradecane	25117-32-2	1454	hydrocarbon	0	1	0	0	0	0	0
C230	Tetrasulfide, dimethyl	3-ethenyl-3,6-dihydrodithiine	62488-52-2	1215	organosulphur	0	1	0	1	0	0	0
C231	Thiophene, 2,4-dimethyl-	3-ethenyl-3,4-dihydrodithiine	62488-53-3	1244	organosulphur	0	1	0	1	0	0	0
C232	Tridecane	tridecane	629-50-5	1301	hydrocarbon	1	1	0	0	0	0	0
C233	Tridecane, 5-methyl-	5-methyltridecane	25117-31-1	1355	hydrocarbon	0	1	0	0	0	0	0
C234	Trisulfide, di-2-propenyl	3-(prop-2-enyltrisulfanyl)prop-1-ene	2050-87-5	1327	organosulphur	1	1	0	1	1	1	1
C235	Undecane	undecane	1120-21-4	1099	hydrocarbon	1	1	0	0	0	0	0
C236	Undecane, 2,6-dimethyl-	2,6-dimethylundecane	17301-23-4	1234	hydrocarbon	0	1	0	0	0	0	0
C237	Undecane, 2,9-dimethyl-	2,9-dimethylundecane	17301-26-7	1271	hydrocarbon	0	1	0	0	0	0	0
C238	Undecane, 2-methyl-	2-methylundecane	7045-71-8	1167	hydrocarbon	1	1	0	0	0	0	0
C239	Undecane, 3-methyl-	3-methylundecane	1002-43-3	1174	hydrocarbon	1	1	0	0	0	0	0
C240	Undecane, 4,8-dimethyl-	4,8-dimethylundecane	17301-33-6	1229	hydrocarbon	0	1	0	0	0	0	0
C241	Undecane, 4-methyl-	4-methyldecane	2847-72-5	1164	hydrocarbon	0	1	0	0	0	0	0
C242	Undecane, 6,6-dimethyl-	6,6-dimethylundecane	17312-76-4	936	hydrocarbon	1	1	0	0	0	0	0
C243	Unknown C15-16 methyl ester	NA	NA	1942	ester	0	1	0	0	0	0	0
C244	Unknown Dodecene	NA	NA	1189	hydrocarbon	0	1	0	0	0	0	0
C245	Z-1-Allyl-2-(prop-1en-1-yl) disulfane	(methyltetrasulfanyl)methane	5756-24-1	1583	organosulphur	1	1	1	0	0	0	0

8.22. Comparison of the number of VOCs detected here to the literature

Table S3.4: The total number of VOCs detected in five studies on garlic and the number of which were also detected here.

	Total No. Compounds Identified	No. Compounds Similar
Vernin et al., 1985	7	6
Calvo-Gómez et al., 2004	47	15
Yu et al., 1989	35	11
Lee et al., 2003	21	9
Molina-Calle et at., 2016	45	19

8.23. VOCs identified in warehouse trials

Table S5.1: Compounds identified by TD-GC-ToFMS in warehouse sampled VOCs. Nist Name = name originally assigned to the compound after identification by comparison of spectra and RI to NIST 2011 mass spectral database. IUPAC name = International Union of Pure and Applied Chemistry systematic name. CAS No. = Chemical Abstracts Service registry number. RI = mean retention index as measured across all samples for a compound, calculated with reference to a linear alkane standard (C8-C20).

Compound Number	NIST Name	IUPAC Name	CAS No.	RI
C1	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	(1R,5R)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	7785-70-8	944
C2	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	84-69-5	1879
C3	1-Allyl-2-isopropyl disulfane	1-allyl-2-isopropyl disulfane	67421-85-6	1109
C4	1-Butanol, 2-methyl-	1-butanol, 2-methyl-	137-32-6	742
C5	1-Butanol, 3-methyl-	1-butanol, 3-methyl-	123-51-3	738
C6	1-Heptene	hept-1-ene	592-76-7	701
C7	1-Hexanol	1-hexanol	111-27-3	871
C8	1-Hexanol, 2-ethyl-	1-hexanol, 2-ethyl-	104-76-7	1032
C9	1-Nonene	1-nonene	124-11-8	892
C10	1-Octene	1-octene	111-66-0	792
C11	1-Pentanol	1-pentanol	71-41-0	772
C12	1-Penten-3-ol	pent-1-en-3-ol	616-25-1	693
C13	1-Phenyl-1-butene	but-1-enylbenzene	1005-64-7	1104
C14	1-Propanol	propan-1-ol	71-23-8	612
C15	1-Propene, 1-(methylthio)-, (Z)-	1-propene, 1-(methylthio)-, (Z)-	52195-40-1	730
C16	1-Propene, 3,3'-oxybis-	3-prop-2-enoxyprop-1-ene	557-40-4	700
C17	2(3H)-Furanone, 5-ethyldihydro-	5-ethyldihydro-2(3H)-thiophenone	570-03-6	1068

C18	2,3-Butanediol	2,3-butanediol	513-85-9	785
C19	2,6-Diisopropylnaphthalene	2,6-di(propan-2-yl)naphthalene	24157-81-1	1758
C20	2,6-Dimethyldecane	2,6-dimethyldecane	13150-81-7	1099
C21	2-Butenal, 2-methyl-, (E)-	(E)-2-methylbut-2-enal	497-03-0	750
C22	2-Heptanone	heptan-2-one	110-43-0	894
C23	2-n-Butyl furan	2-butylfuran	4466-24-4	896
C24	2-Octene	(E)-oct-2-ene	13389-42-9	814
C25	2-Pentanone	pentan-2-one	107-87-9	695
C26	2-Propanol, 1-methoxy-	1-methoxypropan-2-ol	107-98-2	683
C27	2-Propen-1-ol	prop-2-en-1-ol	107-18-6	608
C28	2-Propenal	prop-2-enal	107-02-8	589
C29	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	2-ethylhexyl (Z)-3-(4-methoxyphenyl)prop-2-enoate	177352-99-7	2313
C30	2-Undecanone	undecan-2-one	112-12-9	1298
C31	3-Carene	3,7,7-trimethylbicyclo[4.1.0]hept-3-ene	13466-78-9	1020
C32	3-Heptanone	heptan-3-one	106-35-4	890
C33	3-Octanone	octan-3-one	106-68-3	990
C34	3-Octene, (E)-	(E)-oct-3-ene	14919-01-8	793
C35	3-Vinyl-1,2-dithiacyclohex-5-ene	3-Vinyl-3,4-dihydro-1,2-dithiine	62488-53-3	1248
C36	4-Heptenal	(E)-hept-4-enal	929-22-6	854
C37	4-Penten-1-ol, 2-methyl-	2-methylpent-4-en-1-ol	5673-98-3	825
C38	4-Pentenal, 2-methyl-	2-methylpent-4-enal	5187-71-3	759
C39	4-Phenyldibenzofuran	4-phenyldibenzofuran	74104-10-2	2332
C40	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	6,10-dimethylundeca-5,9-dien-2-one	689-67-8	1457
C41	5-Hexen-2-one	hex-5-en-2-one	109-49-9	779
C42	5-Hexenal	hex-5-enal	764-59-0	794
C43	Acetaldehyde	acetaldehyde	75-07-0	576
C44	Acetic acid	acetic acid	64-19-7	625

C45	Acetic acid, butyl ester	butyl acetate	123-86-4	816
C46	Acetoin	3-hydroxybutan-2-one	513-86-0	716
C47	Acetone	propan-2-one	67-64-1	592
C48	Acetophenone	1-phenylethanone	98-86-2	1084
C49	Allyl n-propyl sulphide	1-prop-2-enylsulfanylpropane	27817-67-0	880
C50	á-Pinene	2,6,6-trimethylbicyclo[3.1.1]hept-2-ene	80-56-8	991
C51	Azulene	azulene	275-51-4	1215
C52	Benzaldehyde	benzaldehyde	100-52-7	979
C53	Benzene	benzene	71-43-2	677
C54	Benzene, (1-methylethyl)-	cumene	98-82-8	933
C55	Benzene, 1,2,3-trimethyl-	1,2,3-trimethylbenzene	526-73-8	1018
C56	Benzene, 1,2-dimethoxy-	1,2-dimethoxybenzene	91-16-7	1154
C57	Benzene, 1-ethyl-2,4-dimethyl-	1-ethyl-2,4-dimethylbenzene	874-41-9	1036
C58	Benzene, 1-ethyl-4-methyl-	1-ethyl-4-methylbenzene	622-96-8	972
C59	Benzene, propyl-	propylbenzene	103-65-1	964
C60	Benzeneacetaldehyde	2-phenylacetaldehyde	122-78-1	1061
C61	Benzenesulfonamide, N-butyl-	N-butylbenzenesulfonamide	3622-84-2	1819
C62	Benzoic acid	benzoic acid	65-85-0	1165
C63	Benzophenone	diphenylmethanone	119-61-9	1671
C64	Benzothiazole	1,3-benzothiazole	95-16-9	1259
C65	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)-	2,2-dimethyl-3-methylidenebicyclo[2.2.1]heptane	5794-03-6	964
C66	Bicyclo[3.1.0]hex-3-en-2-one, 6,6-diphenyl-	6,6-diphenylbicyclo[3.1.0]hex-3-en-2-one	13304-07-9	991
C67	Biphenyl	1,1'-biphenyl	92-52-4	1412
C68	Butanal, 2-ethyl-3-methyl-	2-ethyl-3-methylbutanal	26254-92-2	844
C69	Butanal, 2-methyl-	2-Methylbutanal	202-485-6	682
C70	Butanal, 3-methyl-	3-methylbutanal	590-86-3	673
C71	Butanenitrile, 2-methyl-	2-methylbutanenitrile	18936-17-9	732
C72	Carbon disulfide	methanedithione	75-15-0	608
C73	Caryophyllene	(1R,4E,9S)-4,11,11-trimethyl-8-	87-44-5	1450

		methylidenebicyclo[7.2.0]undec-4-ene		
C74	Cyclobutanone, 2,2,3-trimethyl-	2,2,3-trimethylcyclobutan-1-one	1449-49-6	789
C75	Cyclohexane	cyclohexane	110-82-7	675
C76	Cyclohexane, ethyl-	ethylcyclohexane	1678-91-7	843
C77	Cyclohexane, methyl-	methylcyclohexane	108-87-2	733
C78	Decanal	decanal	112-31-2	1214
C79	Decane	decane	124-18-5	1000
C80	Decane, 4-methyl-	4-methyldecane	2847-72-5	1060
C81	Decane, 5-methyl-	5-methyldecane	13151-35-4	1057
C82	Diallyl disulphide	3-(prop-2-enyldisulfanyl)prop-1-ene	2179-57-9	1096
C83	Diallyl sulfide	3-prop-2-enylsulfanylprop-1-ene	592-88-1	867
C84	Dimethyl sulfide	methylsulfanylmethane	75-18-3	593
C85	Dimethyl trisulfide	(methyltrisulfanyl)methane	3658-80-8	989
C86	Diphenyl sulfide	phenylsulfanylbenzene	139-66-2	1630
C87	Diphenyl sulfone	benzenesulfonylbenzene	127-63-9	1980
C88	Disulfide, dimethyl	(methyldisulfanyl)methane	624-92-0	753
C89	Docosane	docosane	629-97-0	2187
C90	Dodecane, 2,6,11-trimethyl-	2,6,11-trimethyldodecane	31295-56-4	1320
C91	Dodecane, 4,6-dimethyl-	4,6-dimethyldodecane	61141-72-8	1274
C92	E 1- Allyl -2 -prop-1-en-1-yl disulfane	(methyltrisulfanyl)methane	3658-80-8	1116
C93	Eicosane	icosane	112-95-8	2000
C94	Ethanol	ethanol	64-17-5	581
C95	Ethyl Acetate	ethyl acetate	141-78-6	644
C96	Ethylbenzene	ethylbenzene	100-41-4	870
C97	Furan, 2-pentyl-	2-pentylfuran	3777-69-3	995
C98	Heptadecane	heptadecane	629-78-7	1700
C99	Heptanal	heptanal	111-71-7	908
C100	Heptane	heptane	142-82-5	702

C101	Heptane, 2,2,4,6,6-pentamethyl-	2,2,4,6,6-pentamethylheptane	13475-82-6	1006
C102	Heptane, 2-methyl-	2-methylheptane	592-27-8	765
C103	Heptane, 3-methyl-	3-methylheptane	589-81-1	772
C104	Hexanal	hexanal	66-25-1	806
C105	Hexane, 2-methyl-	2-methylhexane	591-76-4	670
C106	Hexane, 3-methyl-	3-methylhexane	589-34-4	678
C107	Hexanedioic acid, bis(2-ethylhexyl) ester	1,6-bis(2-ethylhexyl) hexanedioate	103-23-1	2348
C108	Limonene	1-methyl-4-prop-1-en-2-ylcyclohexene	138-86-3	1041
C109	Longifolene	3,3,7-trimethyl-8-methylidenetricyclo[5.4.0.0^{2,9}]undecane	475-20-7	1445
C110	Mesitylene	1,3,5-trimethylbenzene	108-67-8	1006
C111	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1à,4aá,8aà)-	(1R,4aS,8aS)-7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1H-naphthalene	39029-41-9	1541
C112	n-Hexadecanoic acid	hexadecanoic acid	57-10-3	1964
C113	n-Hexane	hexane	110-54-3	630
C114	Nonadecane	nonadecane	629-92-5	1900
C115	Nonanal	nonanal	124-19-6	1111
C116	Nonane	nonane	111-84-2	900
C117	Octadecane	octadecane	593-45-3	1800
C118	Octanal	octanal	124-13-0	1010
C119	Octane	octane	111-65-9	800
C120	Octane, 1,1'-oxybis-	1-octoxyoctane	629-82-3	1666
C121	Octane, 2,3,6,7-tetramethyl-	2,3,6,7-tetramethyloctane	52670-34-5	1054
C122	Octane, 4-methyl-	4-methyloctane	2216-34-4	863
C123	Oxirane, decyl-	2-decyloxirane	2855-19-8	1316
C124	o-Xylene	1,2-xylene	95-47-6	879
C125	p-Cymene	1-methyl-4-propan-2-ylbenzene	99-87-6	1037
C126	Pentane	pentane	109-66-0	633
C127	Pentane, 3,3-dimethyl-	3,3-dimethylpentane	562-49-2	666
C128	Pentane, 3-methyl-	3-methylpentane	96-14-0	628

C129	Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	1-O-(2-methylpropyl) 4-O-propan-2-yl 2,2-dimethyl-3-propan-2-ylbutanedioate	-	1597
C130	Phenol	phenol	108-95-2	982
C131	p-Xylene	1,4-xylene	106-42-3	901
C132	S-Methyl 2-propenethioate	S-methyl prop-2-enethioate	5883-16-9	809
C133	Styrene	styrene	100-42-5	902
C134	Sulfide, allyl methyl	3-methylsulfanylprop-1-ene	10152-76-8	705
C135	Sulfur dioxide	sulfur dioxide	7446-09-5	567
C136	Tetracosane	tetracosane	646-31-1	2352
C137	Tetradecane	tetradecane	629-59-4	1400
C138	Thiophene, 2,4-dimethyl-	2,4-dimethylthiophene	638-00-6	915
C139	Toluene	toluene	108-88-3	774
C140	Tridecane	tridecane	629-50-5	1300
C141	Trisulfide, di-2-propenyl	3-(prop-2-enyltrisulfanyl)prop-1-ene	2050-87-5	1329
C142	Unknown C15-16 methyl ester	NA	NA	1929
C143	Unknown Dodecene	NA	NA	1193