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Investigation of flavour-related sulphur metabolism in *Actinidia chinensis* genotypes

Catrin Sonja Günther

*The University of Auckland, November 2010*

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry
Für Rasmus
Innovation in kiwifruit flavour is desired. This can be achieved by affecting secondary metabolites. Here the biosynthesis of flavour-related methylsulfanyl (MeS)-volatiles in *Actinidia chinensis*, their genetic background and regulation by ethylene were investigated.

Initially, headspace solid-phase microextraction (HS-SPME) and dynamic headspace sampling (DHS) parameters were systematically optimised to ensure highest extraction yields of MeS-volatiles from kiwifruit tissue using GC-MS. However, the qualitative and quantitative MeS-volatile profiles were improved using DHS compared with HS-SPME, making this the more sensitive and preferred method.

MeS-volatile levels were then quantified during ripening and storage (1.5ºC) of *A. chinensis* ‘Hort16A’. The majority of MeS-compounds were specific for eating-ripe fruit and their concentrations increased in parallel with the climacteric rise in ethylene. No ethylene production was observed after long-term storage (4–6 months) and the levels of all MeS-volatiles, except methional, declined by 98–100% during that period. Transcript accumulation (RT-PCR) of six putative alcohol acyltransferase (*AAT*) was ripening-specific and these levels decreased after prolonged cold storage concomitantly with (MeS)alkanoate ester production of fruit and cell-free extracts. However, ester production and gene expression was recovered by ethylene treatment after five months at 1.5ºC, indicating that the biosynthesis of (MeS)alkanoate esters was likely to be ethylene-regulated in ‘Hort16A’.

The limiting steps of ethylene-dependent (MeS)alkanoate ester biosynthesis were then further investigated using closely related *A. chinensis* genotypes. Quantification of MeS-compounds revealed little variation in their volatile composition but remarkable differences in the magnitude of their fruit volatile levels. Enzyme kinetic studies using cell-free extracts were performed to test whether this was caused by the catalytic properties of genotype-specific AATs. The substrate preferences were similar for most genotypes, thus suggesting substrate availability as the limiting factor for (MeS)alkanoate ester biosynthesis in *A. chinensis*. A link between precursor formation and ethylene biosynthesis in kiwifruit is proposed.
Finally, ripening-specific AATs from *Actinidia* were characterised after recombinant expression. Striking differences in substrate preferences were found for two phylogenetically distinct AATs either exhibiting benzoyl-CoA: alcohol O-acyltransferase or acetyl-CoA: alcohol O-acyltransferase activity. An increased preference for benzoyl-CoA was associated with increased specificity for (MeS)alkanoyl-CoAs, thus suggesting a role of three likely orthologues in their production.
ACKNOWLEDGEMENTS

I was often asked why I had chosen to do my PhD in New Zealand and there was actually a list of reasons and answers. Now, however, I seem to be facing another question:

*Was this a good decision?*

It truly was. The successful completion of my interdisciplinary research project, my progress with the kiwenglish language and the beauty of this country are important reasons. But my main appreciation goes to people and there are many faces and gestures I wish to acknowledge. First, I would like to thank my supervisor Ken Marsh who was a great support and available whenever needed. From him I learned taking things the kiwi way and truly everything was working out 😊 Next I respectfully acknowledge my academic supervisor Laura Nicolau for her input. It was great having a synthetic chemist on ones’ side; my work wouldn’t have been what it is without Adam Matich’s help and advice. I am especially grateful to my other advisor William Laing who has been a true mentor and great inspiration for this work.

I very much appreciate the expert advice of Ross Atkinson and Richard Newcomb and their interest in my project. Their constructive feedback clearly helped me to develop my writing skills. Great thanks to Mindy Wang who introduced me to the GC-MS and headspace sampling equipment. I think you never realised how much I enjoyed your help and in turn giving you a hand, which made me feel really professional. Edwige Souleyre, it was always great fun working with you! There are no words for the ups and downs we have shared on our 3-year journey that eventually had a happy ending. Our combined stubbornness and persistence finally succeeded in taming the moods of recombinant AATs! You have become a dear friend on this adventure that has taken us to France. Here I would like to dearly acknowledge Prof. Christian Chervin and Jean-Claude Pech from ENSAT for giving us the opportunity to learn recombinant expression in yeast in their lab and make us feel very welcome. Special thanks to Salma and Bridgett!

I am also grateful to Anne White, Ringo Feng and Allan Wolf for their expert advice in postharvest research, a field that was completely new to me at the start of my project. I further wish to thank Paul Datson and Mark McNeilage for kindly and reliably bringing along weekly kiwifruit samples from Te Puke during the 2008 season. I also wish to emphasise the great effort my graphic-designer friend Fiona Ward has put into mating
 zigzags with a square and a circle, presented in Fig. 4.4. It has always been helpful talking to Niels Niewenhuizen, Sol Green and Robert Winz. Thank you so much for your interest and time.

I am very grateful for the full scholarship and funding I have received from the New Zealand Institute for Plant & Food Research Limited. Moreover, the great enjoyment of my PhD-project here was very much influenced by its friendly work environment. I deeply appreciate Sarah Johnsten, Marcela Martinez-Sanchez, Simona Nardozza, Christelle Andre, Miriam Farrell, Rebecca Henry and Yael Salzman for their friendship and scientific support.

I wish to highlight how happy I feel about the many good friends I have made in New Zealand. They have become an important and cheerful part of my life. I also thank my and Rasmus’ family with all my heart for supporting our adventure to the other side of the world despite missing us. I especially appreciate my mother for her unconditional love and encouragement to do what I enjoy doing.

Rasmus, I deeply appreciate your commitment following me to Auckland. We have filled many chapters here together, parts of our life story. In these years, your company has been most important to me and I thank you for your loving patience.
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<tr>
<td>1-MCP</td>
<td>1- Methylcyclopropene</td>
</tr>
<tr>
<td>AAT</td>
<td>Alcohol acyltransferase</td>
</tr>
<tr>
<td>ACE</td>
<td>Apparent catalytic efficiency</td>
</tr>
<tr>
<td>ACO</td>
<td>1-aminocyclopropane-1-carboxylate oxidase</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcohol dehydrogenase</td>
</tr>
<tr>
<td>AFS</td>
<td>Acoustic firmness sensor</td>
</tr>
<tr>
<td>APCI-MS</td>
<td>Atmospheric pressure chemical ionisation-mass spectrometry</td>
</tr>
<tr>
<td>AT</td>
<td>Acyltransferase</td>
</tr>
<tr>
<td>BAHD</td>
<td>Benzyl alcohol-acetyl-, anthocyanin-O-hydroxy-cinnamoyl-, anthranilate-N-hydroxy-cinnamoyl/benzoyl-, deacetyl-vindoline</td>
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<tr>
<td>CA</td>
<td>Controlled atmosphere</td>
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<td>DHS</td>
<td>Dynamic headspace sampling</td>
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<td>DMS</td>
<td>Dimethyl sulfide</td>
</tr>
<tr>
<td>DSIR</td>
<td>Department of Scientific and Industrial Research</td>
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<tr>
<td>DTD</td>
<td>Direct thermal desorption</td>
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<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
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<tr>
<td>FI</td>
<td>Firmness index</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
</tr>
<tr>
<td>fob</td>
<td>Free on board</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
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<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GC-O</td>
<td>Gas chromatography-olfactometry</td>
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<td>HS-SPME</td>
<td>Head space solid-phase microextraction</td>
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<tr>
<td>KMBA</td>
<td>2-keto-4-methylsulfanyl-butyric acid</td>
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<tr>
<td>LLE</td>
<td>Liquid-liquid-extraction</td>
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LOD  Limit of detection
LOQ  Limit of quantification
LOX  Lipid peroxidation
LTB  Low temperature break down
MeS  Methylsulfanyl
Mp   Mapping population
NC   Non-comparable
ND   Not detected
NQ   Not quantified
OAV  Odour activity value
PA   Peak area
PG   Polygalacturonase
PL   Pectate lyase
PME  Pectin methylesterase
RSD  Relative standard deviation
RT   Reverse transcriptase
SAM  S-adenosyl-methionine
SSC  Soluble solid concentration
TOF  Time-of-flight
VOSC Volatile organic sulphur compound
DECLARATION

This is to certify, that this thesis comprises only my original work towards the PhD in Chemistry. The experimental design, generation of data, interpretation of results and preparation of the manuscript, including all Figures and Tables, was my own work except where indicated below.

Chapter 2:
The synthesis of internal standards and reference compounds (described in 2.2.2) was performed by Adam J. Matich.

Chapter 4:
The enzyme activity measurements (described in 4.2.5) were carried out by Karolin Heinemann under my supervision as part of her student internship.

Chapter 5:
This study was undertaken in collaboration with Edwige Souleyre. Equal contributions were made to this work, except for the writing of this chapter, which was my own work.

Here I wish to thank my supervisors Laura Nicolau and Ken Marsh and my advisors William Laing and Adam Matich for their intellectual contribution towards the success of this research and the completion of this manuscript.
CHAPTER 1

INTRODUCTION

This interdisciplinary study began as a part of the “taste the future”-programme (objective 2, C06X0403) which has the aim of sustaining research to underpin the breeding of new kiwifruit varieties with novel flavours. In order to realise such breeding targets, the chemical, biochemical and genetic basis for flavour-related volatile metabolism needs to be established. The contribution of this thesis was to identify sulphur-containing compounds in kiwifruit and to study their regulation and genetic background. *Actinidia chinensis* ‘Hort16A’ (Zespri™ GOLD) was chosen as the model cultivar because of its distinct, tropical flavour and relatively high diversity of sulphur-containing compounds that are commonly found in tropical fruit such as melon, pineapple and passion fruit.

1.1 Kiwifruit—a crop plant with great impact

The berries which are commercially available as “kiwifruit” on every continent of the world belong to the genus *Actinidia*, which includes more than 60 species (Ferguson, 1999). However, only two cultivars, the green flesched *Actinidia deliciosa* ‘Hayward’ and the yellow flesched *Actinidia chinensis* ‘Hort16A’ have achieved importance on the global market to date, with the strong brandings ZESPRI™GREEN and ZESPRI™GOLD, respectively. These commercial cultivars were developed in New Zealand, and in 2009 kiwifruit were, at 31.5%, the single most important crop of New Zealand’s horticultural export market (Plant and Food Research 2009). Moreover, kiwifruit export values increased more than 300% within the last 14 years from 320.8 million fob (free on board) in 1995 to 1071.7 million fob in 2009 even though the actual kiwifruit production increased less than 200%. This reflects how one of the most recently domesticated crop plants can convince consumers with novel flavour, appearance and outstanding good nutritional value to pay an extra premium. What is more, kiwifruit that were named after the icon of New Zealand, the flightless, kiwi bird, have become an icon themselves. Although New Zealand is in competition for the leading position in global kiwifruit supply, it is the hub of kiwifruit innovation.
Even though the moist, temperate mountain forests of southwest China have been described as the centre of evolution of the genus *Actinidia* (Ferguson, 1984) the first specimen were collected 1821 in Nepal by Wallich who named them after the Greek word for *ray* (*actis*) to describe the radiating arrangement of the styles in female flowers. Since then mainly botanical characteristics have been considered in the early attempts of classification but more subdivisions took place in later times. For instance, the hairy, green-fleshed and the smooth-skinned yellow-fleshed kiwifruit were treated as different varieties of the same species (*A. chinensis*) until 1984 (Ferguson, 1984) when *A. deliciosa* was classified as a separate species. All *Actinidia* species are perennial and mainly deciduous with vigorous growing, twining stems to climb up or even overgrow trees in their natural habitat (Ferguson, 1984). Fruit of this genus appear singly or in bunches on the vine and display a striking diversity of external and internal attributes like size, shape, skin hairiness, colour and texture of skin and flesh (Fig. 1.1). Around 1903 E.H. Wilson was the first person to report about the genetic diversity of *Actinidia* and their potential as temperate crops. He sourced plants from China and introduced these to North America, Great Britain and Europe but their cultivation was not successful (Ferguson, 2004).

![Fruit diversity in Actinidia, with the commercial cultivars A. deliciosa ‘Hayward’ and A. chinensis ‘Hort16A’ for comparison. Taken from Ferguson (1999).](image-url)

It was Isabel Fraser who, in 1904, brought with her to New Zealand some “mao-erh-tao” seeds from Ichang on the northern banks of the Yangzi River. These same seeds were passed on to Alexander Allison who reported in 1910 about a new vine bearing “Chinese gooseberries” (Ferguson and Bollard, 1990). Nurserymen took interest in their cultivation and were offering plants and fruit for sale by 1917. Finally, Hayward Wright, a horticulturist from Auckland, developed the variety that bears his name.

In the 1930s, the first commercial kiwifruit orchards were established, first in Wanganui and later in the Bay of Plenty, which is now the main kiwifruit-growing area in New Zealand. ‘Hayward’ proved to be the best cultivar suitable for export because of its long storage life. Thirteen tonnes were shipped to England in 1953. In 1959, the fruit packing company Turners & Growers Ltd from Auckland proposed the name “kiwifruit” to improve marketing, particularly in North America (Ferguson and Bollard, 1990). With the successful development of new export markets, The New Zealand Kiwifruit Licensing Authority was formed and in 1978 a research committee was established following discussions between the kiwifruit industry and the Department of Scientific and Industrial Research (DSIR), part of which is the Crown research institute ‘The New Zealand Institute for Plant & Food Research Ltd’ (formerly HortResearch) today. The New Zealand Kiwifruit Marketing Board (formerly New Zealand Kiwifruit Licensing Authority) was formed in 1988 and corporatised 12 years later as ZESPRI Group Limited, a grower-owned and -controlled company, representing New Zealand’s kiwifruit industry in the 21st century (Martin, 2003).

Most commercial plantings of green kiwifruit throughout the world (except for Chinese ones) can be traced back to the seeds that have been introduced to and developed in New Zealand (Seager, 1999). However, this implies that only a small part of the gene pool has been exploited and there is great potential for the breeding of new varieties. With the establishment of kiwifruit breeding programmes in New Zealand, Actinidia chinensis seeds were imported from China in 1977 in order to breed a novel cultivar. In 1992, one offspring was selected from the Plant & Food Research (formerly HortResearch) orchard in Te Puke from block 37, row 1, bay 16, Position A and therefore named ‘Hort16A’ (Martin and Luxton, 2005). Besides good characteristics for horticultural success and postharvest handling, the novel flavour and flesh colour of this kiwifruit has favoured its fast and successful commercialisation. The flavour of ‘Hort16A’ fruit is distinct from ‘Hayward’ and changes from a sweet lime at the firmer end of the eating-firmness range to an intense tropical aroma at the softer end (Patterson et al., 2003). Most consumers who participated
in a sensory study (Wismer et al., 2005) liked this cultivar. Its success was predicted due to its special flavour profile, which created a new product niche and met consumers’ desire for novelty.

1.2. Ripening-specific changes in kiwifruit

Anatomically, fruit are engorged ovaries that are enlarged through cell division and increased in volume. Their development is concomitant with seed maturation after fertilisation. Reaching maturity is a dynamic metabolic process and is characterised by changes in fruit flavour, colour and texture. Many forms have evolved to protect and disperse seeds and Actinidia species bear fleshy fruit that are not dehiscent and therefore rely on distribution after consumption by frugivores, mainly monkeys in their natural habitat. The laxative effect kiwifruit can have especially in elderly people (Rush et al., 2002) can be seen as a natural tactic to increase seed dispersion. However, an attractive combination of sensorial and visual aspects must be achieved during maturation to attract frugivores at the right time—when seeds are fully developed. Nowadays, consumers from western societies generally purchase fresh fruits from stores rather than picking them off the plant once fully ripe. Therefore, the commercial use of fruit is strongly influenced by their harvest criteria, storage abilities and shelf life.

The commercial kiwifruit cultivars can be stored at low temperatures for several months. However, chilling for more than five months is considered as long-term storage and disorders like low temperature breakdown (LTB) can occur (Bauchot et al., 1999). Kiwifruit stored below 0ºC were especially affected by LTB, causing extreme softening and grainy appearance of fruit, whereas only 2% of green kiwifruit stored at 2.5ºC developed these symptoms. Therefore, Bauchot and coworkers (Bauchot et al. 1999) concluded that storage temperatures between 0 and 2.5ºC are more suitable for longer storage periods. Generally, kiwifruit are ripened at room temperature (20ºC) until they reach optimum palatability. This ripening process is accelerated by the ripening hormone ethylene. Even though kiwifruit do not share all aspects of a classic climacteric fruit (Pratt and Reid, 1974), they show a burst of ethylene production and increased respiration during ripening. They are therefore considered as climacteric fruit (Whittaker et al., 1997). In contrast to non-climacteric fruit (e.g. strawberries), ripening can be induced by exogenous ethylene, and postharvest treatments aiming to control the production of this ripening hormone are commonly applied in order to extend storage and shelf life of kiwifruit. Although a
minimum threshold of kiwifruit sensitivity to ethylene-induced ripening has not been defined, a concentration of 0.03 ppm at 0ºC was shown to induce fruit softening. Therefore, special care must be taken during kiwifruit storage and transport to avoid sources of ethylene, such as prematurely ripe or damaged fruit and even fuel burning motors (McDonald, 1990). On the other hand, ethylene treatment (e.g. 100–500 ppm for 12 h at 20ºC) is commonly used to initiate ripening of commercial kiwifruit. Treated fruit will simultaneously reach eating ripeness in 1 week (McDonald, 1990). It becomes clear that storage conditions can influence the timing of kiwifruit reaching their eating-ripe stage, which is comparatively short and creates a fine line between consumers’ delight and disappointment. But which are the most important indicators for eating-ripe kiwifruit?

1.2.1 Changes in fruit texture

Probably the most important marker for kiwifruit ripeness and predictor for shelf life is fruit firmness, which declines with ongoing ripening. Fruit softening is accompanied by changes in fruit texture and juiciness. These changes are primarily caused by dissolution of primary cell walls together with regulated swelling but the molecular processes are not yet fully understood. However, they involve modifications in the network of pectic polysaccharides, which are constituents of plant primary cell walls. Pectic polysaccharides form calcium cross-links between adjacent chains of homogalacturanon, which enhances cell adhesion of the middle lamella. During ripening, polygalacturonase (PG) and pectin methylesterase (PME) influence the integrity of those cross-links and therefore the incorporation of calcium ions. However, suppression of PG only slightly affects fruit softening rate and no effect was observed for PME suppression (Brummell and Harpster, 2001). Marin-Rodriguez and others (Marin-Rodriguez et al. 2002) reviewed the role of pectate lyases (PL) in catalysing the calcium-dependent cleavage of de-esterified pectin. Until 1989, PLs were reported only to be secreted by plant pathogens (e.g. Erwinia carotovora) but in the following years, the accumulation of ripening-specific PL-like sequences was reported in various higher plants, including fruits like bananas, strawberries and grapes. Finally, increased fruit firmness in transgenic strawberries with incorporated antisense PL-sequence (Jimenez-Bermudez et al., 2002) provided evidence for a central role of PLs in fruit softening. However, cell wall metabolism during fruit ripening is a complex multi-enzyme trait that is not controlled by the manipulation of individual genes.
Kiwifruit are harvested mature (seeds fully developed) but unripe at a fruit firmness of 60–90 N for ‘Hayward’ (Beever and Hopkirik, 1990) and 40–50 N for ‘Hort16A’ (Patterson et al., 2003) and are considered eating ripe at 8–5 N and 10–5 N, respectively. The softening pattern (decrease of firmness per time) of both cultivars at 20ºC follows a biphasic, sigmoid shape curve with a slow initial phase, rapid decline and a slower phase towards a lower asymptote (Beever and Hopkirik, 1990; White et al., 2005). There are several destructive and non-destructive methods to measure kiwifruit firmness. Using conventional, intrusive methods, the penetrating force is given as kgf (1 kgf = 9.88 N) whereas non-invasive devices for example utilise an acoustic signal. The resonance frequency (f) of the first elliptical mode (Hz) and the fruit mass (kg) is then expressed as firmness index (FI in 10^6 Hz^2 g^2/3) by an acoustic firmness sensor (AFS):

\[ FI = f^2 m^{2/3} \]

Kinetic models following texture changes of Actinidia chinensis ‘Hort16A’ fruit using AFS in comparison with intrinsic methods have been developed (Schotsmans et al., 2008). Thus, it was shown that the AFS is a reliable and convenient tool for monitoring kiwifruit softening. It was also concluded in an annual, unpublished report (Ringo Feng) that the AFS (Aweta™ Nootdorp, Holland) was well suited for evaluating kiwifruit firmness of all tissue zones. Furthermore, it correlated well with physiological attributes like soluble solids content (SSC), respiration and ethylene production rate in Actinidia chinensis ‘Hort16A’. Unfortunately, there is no equation available to express FI (10^6 Hz^2 g^2/3) in N. However, ‘Hort16A’ fruit can be considered as eating ripe between 10 and 4 x 10^6 Hz^2 g^2/3 using the standard settings, established in our laboratory (microphone gain 80, tick power 16).

### 1.2.2 Changes in kiwifruit flesh colour

Ripe Actinidia delicosa ‘Hayward’ and Actinidia chinensis ‘Hort16A’ fruit are very distinctive in flesh colour (Fig.1.2). The flesh of the unripe cultivars is green and ‘Hayward’ does not convert chloroplasts into chromoplasts during ripening whereas this may be the case in ‘Hort16A’ (McGhie and Ainge, 2002). Analysis of fruit flesh pigments revealed, moreover, that the yellow flesh colour of ‘Hort16A’ was caused by a depletion of chlorophyll rather than an accumulation of carotenoids. Both cultivars contained similar levels and compositions of carotenoids and xanthophylls but ‘Hort16A’ produced more esterified derivatives, typically found in chromoplasts, thus indicating the presence of these
organelles (McGhie and Ainge, 2002). Therefore, the change in flesh colour can be used as an indicator for ripeness of *A. chinensis* but not for *A. deliciosa*.

Fig. 1.2: Eating-ripe kiwifruit. Left: *Actinidia chinensis* 'Hort16A' right: *Actinidia deliciosa* 'Hayward'.
Taken from: Plant & Food Research image database

1.2.3 Kiwifruit taste

The key components of kiwifruit taste are the perceived sweetness and acidity, which are primarily influenced by the SSC. The SSC accumulates during maturation and ripening because of the breakdown of starch into sugars. It can therefore be used as markers for harvest maturity and eating ripeness. Rossiter and coworkers (Rossiter et al., 2000) provided evidence that consumer liking for 'Hayward' kiwifruit increased with SSC levels above 11% by adding sugar solutions to unripe kiwifruit pulp. This preference was caused by changes in panellists’ perception of "acid intensity", which decreased with increasing sweetness. This was confirmed by Marsh et al. (2003) who concluded that sugar levels in kiwifruit pulp are the key feature for the taster’s response. In a later study, the effects of the addition of endogenous acids (citric, malic, quinic or ascorbic acid) and sugars on taste and flavour perception of 'Hayward and 'Hort16A' fruit were investigated (Marsh et al., 2006). Interestingly, consumers’ acidity rating did not change after sugar addition but the perception of banana flavours increased in pulp of both kiwifruit cultivars. On the other hand, it was found that the addition of any endogenous acid reduced sweetness and increased lemon flavour attributes in 'Hayward' fruit. In contrast, only quinic and ascorbic acid reduced the perception of sweetness in ‘Hort16A’ and panellists’ perception of banana flavours. However, no changes in headspace volatiles known for
banana or lemon flavour attributes were identified using gas chromatography-mass spectrometry (GC-MS) analysis after sugar and acid addition. It was shown that consumers are sensitive to even small changes in sugar and acid composition of kiwifruit pulp. Thus, it can be deduced that the degree of kiwifruit ripeness, which correlates with the sugar content of the fruit, attracts consumers with different taste preferences.

1.3 Kiwifruit characteristic flavour compounds

Besides visual characteristics typical flavours were found to be critical differentiators in defining consumers’ preferences for ‘Hayward’ and ‘Hort16A’ kiwifruit (Jaeger et al., 2003). Flavour attributes like fresh, sweet-tart with grassy, green notes have been ascribed to the commercial green kiwifruit (Actinidia deliciosa ‘Hayward’) whereas the yellow fleshed cultivar (Actinidia chinensis ‘Hort16A’) is perceived as being sweeter with strong blackcurrant and candy floss flavours and a tropical aroma, reminiscent of melon and banana (Jaeger et al., 2003). However, both cultivars are recognised by their kiwifruit specific flavour mainly owing to increased levels of the predominant esters methyl and ethyl butyrate and also to elevated amounts of hexenal and hexanal. Those latter compounds were shown to enhance this characteristic flavour intensity using a model system (Gilbert et al., 1996). Within the last 25 years, various publications elucidated the flavour chemistry of different Actinidia cultivars using solvent or headspace extraction techniques mainly combined with GC-MS as a powerful tool.

Young and Paterson (1985) and Takeoka et al. (1986) were the first scientists to research fruit volatiles in A. deliciosa ‘Hayward’ using GC-MS after dynamic headspace sampling (DHS) and liquid-liquid extraction, respectively (LLE). They found that lipid oxidation (LOX) products, such as C₆-alcohols and C₆-aldehydes, contribute with over 90% to the typical kiwifruit volatile profile. The impact of harvest maturity (5.7%SSC -10.8%SSC), storage time (0–6 months) and firmness (11–3 N) on the volatile composition of green ‘Hayward’ fruit was investigated by Young and Paterson (1985). In this study, evidence was given for the negative impact of early harvest maturity and long term storage at 0°C on total volatile levels. Furthermore, it was shown that overripe fruit contained greater amounts of headspace volatiles. Especially the composition and concentrations of fruit esters dramatically increased with fruit softness (except ethyl 2-methylpropionate which decreased with softness) but decreased with storage time. Compared with ripe fruit, unripe ‘Hayward’ fruit contained considerably higher levels of C₆-aldehydes, thus resulting
in a high aldehyde/ester ratio that declined with fruit firmness. How these changes in chemical composition affect sensorial properties of ‘Hayward’ kiwifruit was demonstrated in a later study (Paterson et al., 1991). Here tangy/acid attributes were correlated with fruit containing low ester levels (e.g. firm fruit, long term storage) and sweetness scores complied with soft fruit and high ester levels. More recently aroma active components were studied in green kiwifruit puree and essence using GC-MS and gas chromatography-olfactometry (GC-O) analysis (Jordan et al., 2002). It was confirmed that various C₆-alcohols and aldehydes (LOX-products) were major volatiles and the high aroma impact of methyl- and ethyl butyrate was pointed out. Interestingly, hexyl hexanoate and the mint-flavoured terpene eucalyptol could neither be detected in kiwifruit essence nor in fruit purée using GC-MS but were found to be of remarkable organoleptic importance after GC-O analysis. Hence, these latter compounds were described as aroma active compounds by these authors.

Ripening related changes in fruit volatile profiles of A. chinensis ‘Hort16A’ were recently investigated and similar trends to those observed in ‘Hayward’ reported (Wang et al., 2011). For example, the levels of fruity, pineapple flavoured ethyl butyrate (Burdock, 2004) exceeded its odour threshold in kiwifruit when softened to less than 6 N and increased in intensity with further ripening. In comparison to ‘Hayward’, aldehyde levels were 100-fold lower in ‘Hort16A’ fruit. In particular, hex-E2-nal with fresh, green and grassy notes contributed only 47%, (opposed to 85% in ‘Hayward’) to total aldehydes measured in the yellow-fleshed cultivar. As previously pointed out by Friel et al. (2007), ethanol was by far the major alcohol, resulting in a great variety of ethyl esters as dominating volatile esters from the headspace of ‘Hort16A’ fruit (Wang et al., 2011). In general, ester levels were strongly influenced by fruit firmness resulting in markedly higher amounts in soft compared to firm eating-ripe kiwifruit. The aroma impact of ‘Hort16A’ volatiles was studied using GC-O and by testing the real-time release of compounds after maceration by the means of atmospheric pressure chemical ionisation-mass spectrometry (APCI-MS) (Friel et al., 2007). In this study, panellists were asked to breathe through their nose into an APCI-MS port in certain intervals while chewing yellow-fleshed kiwifruit. This revealed that the most prominent compounds, ethanol and acetaldehyde, were followed by hex-E2-enal and hexanal in firm (6–8 N) ‘Hort16A’ and by ethyl-esters in soft (4–6 N) fruit. What is more, the persistence of dimethyl sulphide (DMS) throughout this in vivo and additional GC-O analysis indicated its impact on the typical ‘Hort16A’ aroma. Odour descriptors of this compound after GC resolution included “sulphury” and “‘Hort16A’-
aroma”. DMS levels were similar in firm and soft fruit and maximal levels were consistently exhaled with the first breath indicating the foremost perception of this compound. DMS is probably best known as a potent effector of global climate because its oxidation yields sulphate that precipitates to cloud condensation nuclei (Charlson et al., 1987). As a flavour compound, however, DMS is mainly appreciated in wine (Segurel et al., 2004) and this was the first time DMS was described as aroma active in fresh fruit (Friel et al., 2007). However, DMS levels and intensity were unaltered in firm and soft kiwifruit (Friel et al., 2007) whereas the perception of tropical flavours were most strongly perceived in soft eating-ripe fruit (Patterson et al., 2003). This points towards a potential flavour impact of sulphur-containing esters, potentially (methylsulfanyl)alkanoate (MeS) esters, which have been identified in various tropical fruits.

1.4 The potential impact of methylsulfanyl-volatiles on tropical fruit flavours

1.4.1 Sulphur-containing compounds in tropical fruits

Yellow passion fruit (Passiflora edulis spp.) was described as “having a floral, estery aroma with an exotic tropical sulphury note” and 47 different sulphur volatiles, including thiols, sulphides and methylsulfanyl-compounds (MeS-compounds) were identified by Werkhoff et al. (1998). The sulphur-volatile profile of yellow passion fruit was dominated by 26 different MeS-compounds, mainly esters and alcohols. Their odour descriptors from aqueous solutions containing reference compound (1–10 mg L\(^{-1}\)) contained fruity, sweet and sulphury organoleptic characters, reminiscent of general or specific tropical fruits (guava, pineapple, passion fruit, melon, mango, durian) but also tinted with vegetable notes (cauliflower, radish, mushroom, kohlrabi, asparagus, potato, leek). However, a significant impact on the typical aroma of passion fruit was questioned by the authors (Werkhoff et al., 1998) because of their comparatively high odour thresholds in comparison to thiols. Nevertheless, (MeS)alkanoate esters belong to the best characterised sulphur volatiles in tropical fruits, especially 2-MeS acetates and 3-MeS propionates. Haagen-Smit et al. (1945) was the first chemist to prove the presence and structure of a “sulphur-containing ester” (methyl 3-MeS propionate) in pineapple and estimated its approximate quantity to 1 mg kg\(^{-1}\) fruit pulp. In later, GC-MS based studies, five additional (MeS)alkanoate esters were identified in pineapple fruit pulp but only the main esters methyl and ethyl 3-MeS propionate exceeded their odour thresholds by an odour activity value (OAV) of 3.3 and 3.9, respectively (Takeoka et al., 1989; Takeoka et
The major sulphur compounds found amongst various melon (*Cucumis melo*) cultivars were 2-MeSethyl acetate and 3-MeSpropyl acetate and the presence of the corresponding MeS-alcohols in fruit pulp was also presented (Aubert and Bourger, 2004; Wyllie and Leach, 1992). In addition, methyl and ethyl 3-MeSpropionate and 2-MeSAcetate were commonly found in the volatile profiles of melon (Aubert and Pitrat, 2006; Beaulieu and Grimm, 2001) but only methyl 2-MeSacetate was shown to be aroma active in musk melon fruit purée (Jordan et al., 2001). Interestingly, methyl 2-MeSacetate was also recognised as an important contributor to the authentic aroma of green kiwifruit purée (Jordan et al., 2002) using GC-O analysis. A great diversity of sulphur-volatiles was also identified from durian fruit (Weenen et al., 1996). In this study, 3-MeSpropionate esters were not detected although methyl and ethyl 2-MeSacetate were present in most durian varieties. Asian pear on the other hand exclusively formed ethyl 3-MeSpropionate, ethyl 3-MeSprop-2-enoate and 3-MeSpropyl acetate esters (Takeoka et al., 1992). MeS-volatiles, commonly found in tropical fruit and their sensorial properties are summarised in Table 1.1.

### 1.4.2 Sulphur-containing compounds in *Actinidia chinensis* genotypes

The initial *Actinidia chinensis* mapping population (Mp) was planted in 1997 in the experimental Plant & Food Research orchard in Te Puke. It consists of 134 female fruit-bearing and 138 male vines. 'Hort16A' is a sibling of the male parent of these plants. From 2002 to 2004, 470 further female and 507 male vines were planted and are available as an extension of this initial Mp for research purposes.

There was an extensive, preliminary phenotyping study (unpublished) of the initial *Actinidia chinensis* mapping population undertaken in 2002. In this survey, fruit volatiles, sensorial properties, firmness, SSC, dry matter and pH were analysed. As a result, 19 sulphur-containing compounds were identified among 133 genotypes using LLE and DHS. The majority of these compounds were identified as MeS-volatiles previously found in tropical fruits. The most prominent sulphur-ester, found in 82.7% of the studied *A. chinensis* genotypes was ethyl 3-MeSpropionate followed by methyl 3-MeSpropionate (47.4%) and ethyl 2-MeSacetate (21%, Table 1.2). These three (MeS)alkanoate esters exhibit sweet and fruity odours with pineapple, melon and vegetable notes (Table 1.1). Interestingly, 3-(methylsulfanyl)propanol (methionol) occurred in 27.8% and 2-MeSethanol
<table>
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<th>Odour descriptor</th>
<th>Asian Pear&lt;sup&gt;1&lt;/sup&gt; (Pyros serotina)</th>
<th>Durian fruit&lt;sup&gt;2&lt;/sup&gt; (Durio zibethinus)</th>
<th>Guave&lt;sup&gt;3&lt;/sup&gt; (Psidium guajava)</th>
<th>Melon&lt;sup&gt;4&lt;/sup&gt; (Cucumis melo)</th>
<th>Passion fruit&lt;sup&gt;5&lt;/sup&gt; (Passiflora edulis)</th>
<th>Pineapple&lt;sup&gt;6&lt;/sup&gt; (Ananas comosus)</th>
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<td>Methyl 3-(methylsulfanyl)propionate</td>
<td>Sulphurous&lt;sup&gt;5&lt;/sup&gt;, tropical&lt;sup&gt;5&lt;/sup&gt;, sweet&lt;sup&gt;1&lt;/sup&gt;, cabbage&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Ethyl 3-(methylsulfanyl)propionate</td>
<td>Melon&lt;sup&gt;1&lt;/sup&gt;, fresh&lt;sup&gt;1&lt;/sup&gt;, fruity&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Methyl 3-(methylsulfanyl)prop-E2/Z2-enoate</td>
<td>E: yoghurt&lt;sup&gt;5&lt;/sup&gt;, pineapple&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Ethyl 3-(methylsulfanyl)prop-E2/Z2-enoate</td>
<td>E: sweet&lt;sup&gt;1&lt;/sup&gt;, metallic&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Methyl 2-(methylsulfanyl)acetate</td>
<td>Sulphurous&lt;sup&gt;1&lt;/sup&gt;, roasted oil&lt;sup&gt;5&lt;/sup&gt;, pungent&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>Ethyl 2-(methylsulfanyl)acetate</td>
<td>Fruity&lt;sup&gt;5&lt;/sup&gt;, sweet&lt;sup&gt;1&lt;/sup&gt;, juicy&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>Methyl 2-(methylsulfanyl)ethyl acetate</td>
<td>Sulphur&lt;sup&gt;5&lt;/sup&gt;, rotten&lt;sup&gt;4&lt;/sup&gt;, creamy&lt;sup&gt;5&lt;/sup&gt;, cauliflower&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Ethyl 2-(methylsulfanyl)ethyl acetate</td>
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<td>Methionol</td>
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<sup>1</sup>(Takeoka et al., 1992), <sup>2</sup>(Weenen et al., 1996), <sup>3</sup>(Steinhaus et al., 2008), <sup>4</sup>(Wyllie and Leach, 1992), <sup>5</sup>(Werkhoff et al., 1998), <sup>6</sup>(Takeoka et al., 1989; Takeoka et al., 1991), <sup>7</sup>(Burdock, 2004), <sup>8</sup>(Jordan et al., 2001), <sup>9</sup>(Jordan et al., 2002).
in 3% of all the genotypes (Table 1.2) but 3-MeSpropyl acetate and 2-MeSethyl acetate were not detected in A. chinensis kiwifruit. The tropically-flavoured bis-1-(methylene)disulphide (diisopropyl disulphide) was previously identified in 29.8% of all A. chinensis lines and has recently been reported to occur in A. arguta (Matich et al., 2003) and yellow passion fruit (Werkhoff et al., 1998). However, bis-1-(methylene)disulphide was found only in samples prepared by LLE. In general, LLE yielded a higher number of sulphur volatiles but relative standard deviations between technical replicates were often >30%. Methyl 3-MeSprop-2-enoate for instance was only found in one of three replicates of one single cultivar after LLE and ethyl 2-mercaptopropionate was never detected in more than one of three replicates per line although it appeared to be present in six different lines using both extraction methods. Of those sulphur compounds identified in members of the Actinidia chinensis mapping population, (MeS)alkanoate esters indicated the highest potential as aroma compounds owing to their pleasant organoleptic properties at low concentrations and their presence in various tropical fruits.

**Table 1.2: Sulphur volatiles detected in 133 different genotypes of the Actinidia chinensis mapping population (Mp) in a preliminary study.**

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Mp genotypes in %</th>
<th>Compound name</th>
<th>Mp genotypes in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-(methylsulfanyl)ethanol</td>
<td>3</td>
<td>Ethyl 2-mercaptopropionate</td>
<td>4.5</td>
</tr>
<tr>
<td>Methionol</td>
<td>27.8</td>
<td>3-hydroxy-2-mercapto-pyridine</td>
<td>0.75</td>
</tr>
<tr>
<td>Methional</td>
<td>0.75</td>
<td>Benzothiazole</td>
<td>21.8</td>
</tr>
<tr>
<td>Methyl 2-(methylsulfanyl)acetate</td>
<td>1.5</td>
<td>2-pentyliothiope</td>
<td>0.75</td>
</tr>
<tr>
<td>Ethyl 2-(methylsulfanyl)acetate</td>
<td>21</td>
<td>Bis-1-(methylene)disulphide</td>
<td>29.3</td>
</tr>
<tr>
<td>Methyl 3-(methylsulfanyl)propionate</td>
<td>47.4</td>
<td>Carbon disulphide</td>
<td>0.75</td>
</tr>
<tr>
<td>Ethyl 3-(methylsulfanyl)propionate</td>
<td>82.7</td>
<td>Dimethyl sulphide</td>
<td>0.75</td>
</tr>
<tr>
<td>Propyl 3-(methylsulfanyl)propionate</td>
<td>0.75</td>
<td>Dimethyl sulphoxide</td>
<td>9.8</td>
</tr>
<tr>
<td>Butyl 3-(methylsulfanyl)propionate</td>
<td>13.5</td>
<td>Dimethyl sulphone</td>
<td>3</td>
</tr>
<tr>
<td>Methyl 3-(methylsulfanyl)prop-2-enoate</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The frequency of occurrence of each compound in 133 genotypes is given in %.

Data were taken from the HortResearch Compound database (unpublished, IP protected).

Six experienced panellists assessed fruit of these same A. chinensis genotypes for their key flavours, one of which was described as “tropical” in odour and taste. In this sensory trial, 39 out of 133 genotypes were described as having a tropical odour and taste by at
least 50% (3/6) of all panellists. Those kiwifruit lines with either high scores for tropical fruit flavour, high diversity and/or high relative concentrations (semi-quantitative study) of sulphur-esters are presented in Table 1.3. It is obvious that a direct correlation between tropical flavour and sulphur-ester composition or concentration cannot be drawn. In the case of ‘Mp145’ total (MeS)alkanoate ester concentrations reached high levels (<1000) compared with most other lines of the A. chinensis mapping population and five and three out of six panellists perceived tropical odour and taste attributes, respectively. However, ‘Mp258’ was also characterised as tropically flavoured by the same number of panellists but sulphur-containing esters have not been detected at all (Table 1.3). The highest diversity in sulphur-esters, including the thiol ethyl 2-mercaptопropionate with sulphurous, fruity odour properties (Burdock, 2004), was reported for ‘Mp201’. This line also produced high amounts of sulphur-esters in general (<1000) but only one third (2/6) of the panellists scored for tropical odour and taste. In contrast, only one (MeS)alkanoate ester was identified from ‘Mp124’, ‘Mp161’, ‘Mp256’ and ‘Mp263’ at <10-fold lower levels compared with ‘Mp201’ but the perception of tropical flavour attributes was above average. Because these preliminary results appear controversial at first, in-depth research is needed to reveal the true potential of MeS-volatiles for the characteristic, tropical aroma of some A. chinensis lines and ‘Hort16A’ in particular. For example, a direct quantification approach will give a first indication whether the actual odour thresholds of individual MeS-compounds are exceeded in selected genotypes. Furthermore, in this pilot study, individual fruit were consumed by sensory panellists and fruit-to-fruit variation of sulphur volatiles is a possibility. In this respect, it will be of great value to investigate the regulation of MeS-volatile biosynthesis in kiwifruit. This knowledge, based on reliable analytical methods will be the prerequisite for future studies targeting the aroma impact of MeS-volatiles in Actinidia genotypes.

1.4.3 Proposed biosynthesis of MeS-volatiles

Haagen-Smit suggested in 1945 that methyl 3-MeSpropionate may be derived from cysteine because of its similarity to the chemical structure of this amino acid. Almost 50 years later, Wyllie and Leach (1992) studied the abundance of six (MeS)alkanoate esters in approximately 30 melon cultivars and concluded that the formation of MeS-volatiles was under genetic control. Furthermore, these authors hypothesised that methionine may be the common precursor of (MeS)alkanoate esters. They also mentioned the possibility that precursors may be shared with the ethylene biosynthetic pathway and concluded
Table 1.3: **Sensorial scores and relative sulphur-ester concentrations in selected genotypes of the Actinidia chinensis mapping population assessed from a preliminary study.**

<table>
<thead>
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</thead>
<tbody>
<tr>
<td>Tropical odour</td>
<td>5/6</td>
<td>5/6</td>
<td>4/6</td>
<td>5/6</td>
<td>1/6</td>
<td>5/6</td>
<td>4/6</td>
<td>4/6</td>
<td>2/6</td>
<td>3/6</td>
<td>4/6</td>
<td>5/6</td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Tropical taste</td>
<td>3/6</td>
<td>3/6</td>
<td>4/6</td>
<td>3/6</td>
<td>0/6</td>
<td>3/6</td>
<td>4/6</td>
<td>4/6</td>
<td>2/6</td>
<td>4/6</td>
<td>5/6</td>
<td>3/6</td>
<td>5/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Relative concentration</td>
<td>19</td>
<td>8</td>
<td>129</td>
<td>1013</td>
<td>5</td>
<td>99</td>
<td>5</td>
<td>1031</td>
<td>6</td>
<td>8</td>
<td>0</td>
<td>12</td>
<td>530</td>
<td></td>
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<tr>
<td>(total esters)</td>
<td></td>
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<tr>
<td>Methyl 2-[(methylsulfanyl)acetate]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.3%</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Ethyl 2-[(methylsulfanyl)acetate]</td>
<td>-</td>
<td>-</td>
<td>2.5%</td>
<td>2.4%</td>
<td>-</td>
<td>100%</td>
<td>-</td>
<td>-</td>
<td>2.7%</td>
<td>1.7%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl 3-[(methylsulfanyl)propionate]</td>
<td>65.5%</td>
<td>-</td>
<td>-</td>
<td>4.6%</td>
<td>1.7%</td>
<td>-</td>
<td>1%</td>
<td>-</td>
<td>0.25%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30.7%</td>
<td></td>
</tr>
<tr>
<td>Ethyl 3-[(methylsulfanyl)propionate]</td>
<td>34.5%</td>
<td>100%</td>
<td>81.5%</td>
<td>93%</td>
<td>98%</td>
<td>-</td>
<td>99%</td>
<td>100%</td>
<td>97%</td>
<td>90%</td>
<td>100%</td>
<td>-</td>
<td>100%</td>
<td>64.5%</td>
</tr>
<tr>
<td>Propyl 3-[(methylsulfanyl)propionate]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.3%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Butyl 3-[(methylsulfanyl)propionate]</td>
<td>-</td>
<td>-</td>
<td>16%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.8%</td>
</tr>
<tr>
<td>Ethyl 3-mercaptopropionate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.05%</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

For sensory evaluation the scores per total scores are given. The contributions of individual sulphur-esters are displayed in % of total sulphur-ester concentration per genotype as determined in a semi-quantitative study.

Data were taken from the HortResearch Compound database (unpublished, IP protected)
“(...) if this is the case, then their generation and concentration may very well be
dependent on fruit maturity and harvest time”. Indeed, (MeS)alkanoate ester levels
markedly increased in ethylene-producing C. melo fruit (Aubert and Bourger, 2004), which
was also mentioned to affect the musky note of melon aroma. Starting point for this study
was the finding that short shelf-life ‘charentais cantaloupe’ (C. melo ‘cantalupensis’)
cultivars with a sharp climacteric phase were superior in taste to mid or long shelf-life
cultivars with low levels of ethylene production. MeS-volatiles were found to be 4–17 fold
reduced in long shelf-life cultivars, thus suggesting a link between their biosynthesis and
the ethylene pathway.

The formation of volatile organic sulphur compounds (VOSC) from methionine was
primarily studied in microorganisms in the context of food fermentation (Landaud et al.,
2008). The authors indicated that the central precursor 2-keto-4-MeS-butyric acid (KMBA)
was formed by aminotransferase from methionine. In lactic acid bacteria KMBA was
decarboxylated to 3-(methylsulfanyl)propanal (methional) that was either reduced to
methionol by catalysis of alcohol dehydrogenase (ADH) or oxidised to 3-MeSpropionic
acid (Vallet et al., 2008). These same authors pointed out that KMBA may possibly be
converted into 3-MeSpropionyl-CoA via oxidative decarboxylation. (MeS)alkanoyl-CoAs
are likely to be utilised by alcohol acyltransferases (AAT) for (MeS)alkanoate ester
formation with alcohols.

In plants, KMBA is an intermediate of the methionine salvage pathway or Yang cycle
(Yang and Hoffman, 1984), delivering substrates for ethylene biosynthesis.

1.5 Alcohol acyltransferases are key-enzymes for fruit ester formation

Alcohol acyltransferases (AAT) catalyse the final step of ester formation by
acylation of an alcohol acceptor using acyl-CoAs as donor molecules (Fig. 1.3). All AATs
are members of the BAH (benzyl alcohol-acetyl-, anthocyanin-O-hydroxy-cinnamoyl-, anthranilate-N-hydroxy-cinnamoyl/benzoyl-, deacetyl-vindoline) acyltransferase (AT)
superfamily. Because many functionally important structures are formed by BAH ATs,
such as lignin, alkaloids, phenolics, phytoalexins, anthocyanins and volatiles esters, this
protein family is of great importance for plant secondary metabolism (D'Auria, 2006).
The most common structural features of BAHD proteins are two conserved motifs in their primary structure. The first one (HXXXD) was shown to be located in the catalytic site of crystallised vinorine synthase and the second motif (DFGWG) was suggested to be structure-related since it was found on the C-terminus of the protein (Ma et al., 2005). A key role was particularly proposed for the HXXXD region that is located in the centre of the reaction channel, allowing independent access of substrate and co-substrate from both sides. A reaction mechanism was also suggested (Ma et al., 2005) with Histidine acting as a general base catalyst, favouring the initial deprotonation of the alcohol acceptor molecule and consequently the electrophilic attack of the carbonyl-carbon atom of the CoA-thioester. In this reaction a ternary complex is likely to be formed before the release of the alkyl ester and CoA-SH (Ma et al., 2005).

In general, a broad range of substrates can be utilised by BAHD ATs. However, individual members were classified in different phylogenetic clades (D’Auria, 2006) that appeared to have similar trends in substrate preference for the acyl-CoA donor. For example, representatives of clade III appeared to primarily use acetyl-CoA whereas a subgroup of clade V preferred benzoyl-CoA as substrate. Nevertheless, the authors emphasise that predictions about the substrate specificity and physiological function of the protein cannot be made on the basis of the protein sequence alone (D’Auria, 2006).

Flavour-related AAT-genes have been characterised from several fruits including banana (Beekwilder et al., 2004), strawberry (Beekwilder et al., 2004), apple (Souleyre et al., 2005), melon (El-Sharkawy et al., 2005) and papaya (Balbontín et al., 2010) after recombinant expression. These recombinant AAT proteins were generally reported to be active as monomers and approximately 50 kDa in size. Souleyre et al. (2005) observed in
MpAAT1 from apple that enzyme preference for the alcohol substrate was influenced by its concentration and therefore concluded that the binding of the alcohol acceptor was rate limiting compared with the binding of the acyl-CoA substrate. In melon (*Cucumis melo*) a multi gene family consisting of four AATs has been identified and kinetic studies of the recombinant isoymes revealed preferences for the acylation of individual alcohol substrates (El-Sharkawy et al., 2005). One member of this gene family (Cm-AAT4) was even exclusively devoted to using acetyl-CoA as acyl-donor (Aggelis et al., 1997). It was demonstrated in a later study (Lucchetta et al., 2007) that most recombinant melon AATs were able to acetylate MeS-alcohols. However, ester production using (MeS)alkanoyl-CoAs has not been tested. The gene transcription of potentially flavour-related *CmAATs* in melon correlated well with the climacteric rise in fruit ethylene production and a concomitant increase in AAT activity was also reported (El-Sharkawy et al., 2005). This confirmed the findings of a previous study (Shalit et al., 2001) stating a drastic increase in volatile acetates arising in Arava melon in parallel with the climacteric peak in ethylene production. In comparison, transcript levels of ripening-related AATs were significantly reduced or completely inhibited in 1-aminocyclopropane-1-carboxylate oxidase (ACO) repressed melons and in fruit treated with the ethylene antagonist 1-Methylcyclopropene (1-MCP). Volatile ester levels were clearly reduced in those fruit. Ethylene-treated fruit recovered *CmAAT* transcripts and fruit ester biosynthesis, thus providing evidence for the impact of climacteric ethylene on aroma-related ester formation. The regulation of volatile production has also been researched in antisense ACO-apples (Schaffer et al., 2007) and it was concluded that ethylene primarily controls the first and final step of ester biosynthesis and therefore AAT expression. Ethylene-regulated fruit ester production was also shown in Mountain papaya fruit and transcript levels of a ripening-specific AAT (*VpAAT1*) appeared to be modulated by this ripening hormone (Balbontín et al., 2010). It is discussed in this study that the highest catalytic activities of AATs from papaya and melon were observed for the production of esters that have not been found in fruit. Therefore, the production of fruit-characteristic esters was suggested to be primarily dependent on substrate availability. This conclusion was made by several authors studying ethylene-dependent ester formation in conjunction with AAT-activity in climacteric fruits (Ferenczi et al., 2006; Holland et al., 2005; Lara et al., 2007; Matich and Rowan, 2007; Wyllie and Fellman, 2000). Nevertheless, cultivar specific acetylation of aliphatic alcohols was demonstrated after partial purification of AATs from five different strawberry varieties (Olias et al., 2002). On the basis of enzyme kinetic studies, these authors concluded that substrate affinity increased with carbon chain length of the alcohol-
acceptor molecule. Furthermore, straight-chained alcohols with even-numbered carbon atoms (LOX-products) were preferred to odd-numbered ones as substrates for ester formation. These findings point to a complex regulation of fruit ester biosynthesis, orchestrated by genotype-specific substrate preferences of AATs and precursor availability.

As a result of an *Actinidia* expressed sequence tag (EST) sequencing program, 26 contig sequences (25 full-length clones) with putative AT-function were isolated recently from five different kiwifruit species (Crowhurst et al., 2008). On the basis of phylogenetic studies with characterised, plant-derived ATs, these *Actinidia* AT contigs were grouped in six different clades, demonstrating the diversity of their potential biosynthetic products (Fig.1.4). Interestingly, two separate flavour-related clades were defined with nine proposed *Actinidia* AATs being classified as clade 1 and three sequences belonging to clade 2. These contig-sequences offer a great resource for studying the regulation of (MeS)alkanoate ester biosynthesis on the transcriptional level. Moreover, substrate preferences can be studied after recombinant expression. This may lead to conclusions about the genotype-specific (MeS)alkanoate ester profiles of *A. chinensis* fruit and aid targeted breeding approaches.

![Phylogenetic relationship and proposed function of putative Actinidia-derived acyltransferases.](image)

*Fig. 1.4:* Phylogenetic relationship and proposed function of putative Actinidia-derived acyltransferases. Modified from Crowhurst et al. (2008).
1.6 Principal aims of this study

This is the first co-ordinated approach investigating (MeS)alkanoate ester biosynthesis in *A. chinensis* ‘Hort16A’ and related genotypes. The stages of this interdisciplinary study are structured in four chapters. The first chapter is focussed on method development, using static and dynamic headspace sampling techniques and GC-MS as analytical tools. These extraction methods were used in the second approach to monitor MeS-volatile levels in ‘Hort16A’ during fruit ripening and after cold storage. In this part (MeS)alkanoate ester formation by AATs, changes in enzyme activity and steady-state transcript levels are researched in order to obtain first indications of their regulation. Enzyme kinetic studies were employed in the third report aiming to elucidate the biochemical regulation of MeS-volatile production in selected *A. chinensis* genotypes. Here a linkage between the MeS-volatile and the ethylene biosynthetic pathway is suggested. Finally, substrate preferences of recombinant AATs are presented using molecular and functional characterisation, targeted to evaluate the impact of specific AAT-genes on (MeS)alkanoate ester biosynthesis in fruit. The higher aim of this thesis is to provide a substantial foundation for ongoing research in this field.

The specific goals of this research were:

- To establish quantitative methods for the analysis of MeS-volatiles from the headspace of kiwifruit pulp. These techniques are likely to be suitable for the analysis of MeS-volatiles from other (kiwi)fruit species.
- To qualitatively and quantitatively identify MeS-volatiles in *A. chinensis* ‘Hort16A’ and in related members of the mapping population. This will allow first indications of their aroma impact and is likely to aid the selection of genotypes with desired levels of particular MeS-volatiles.
- To investigate the regulation of (MeS)alkanoate ester biosynthesis and the impact of postharvest cooling. Increased knowledge about these factors will provide approaches to synchronise and standardise fruit quality.
- To distinguish the limiting steps of MeS-volatile production and to propose a possible biosynthetic route of their formation in *A. chinensis*. This may lead to potential areas of future research.
- To identify genes encoding for flavour-related AATs. This information may be useful for the targeted breeding of new cultivars with novel flavours.
CHAPTER 2

DEVELOPMENT OF A QUANTITATIVE METHOD FOR HEADSPACE ANALYSIS OF METHYLSULFANYL-VOLATILES FROM KIWIFRUIT TISSUE

2.1. Introduction

Kiwifruit are widely appreciated for their high levels of vitamin C (Rassam and Laing, 2005) and health-promoting effects (Collins et al., 2001; Hunter et al., 2008). Nevertheless, flavour is one of the most important attributes of fruit quality and a substantial factor influencing consumers' liking and product preference (Harker et al., 2009). Long-term storage and postharvest techniques aiming to extend shelf life of climacteric fruit such as apple (Marin et al., 2009), melon (Beaulieu, 2005) and kiwifruit (Günther et al., 2010; Young and Paterson, 1985) were shown to reduce aroma-related volatile formation, especially fruit ester biosynthesis. Volatile esters are often key aroma compounds of edible fruit and therefore indispensable for plant-food recognition and eating pleasure. Ethyl butyrate, for example, is perceived by consumers to be a kiwifruit-specific aroma compound, increasing the acceptance of kiwifruit (Gilbert et al., 1996). Two kiwifruit cultivars are of commercial importance on the global market, the well established green kiwifruit (Actinidia deliciosa ‘Hayward’) and the more recent, yellow-fleshed cultivar (Actinidia chinensis ‘Hort16A’). The latter cultivar is distinguished from the green variety by its sweeter taste, with strong blackcurrant and candy floss flavours and a tropical aroma reminiscent of melon and banana (Jaeger et al., 2003). Flavour-related ‘Hort16A’ volatiles have been investigated (Friel et al., 2007; Wang et al., 2011), and it was shown that ethyl esters dominate the volatile profile at the softer end of the eating-firmness range. However, minor compounds such as MeS-volatiles have not been identified in these studies using the extraction methods applied, thus disclosing the need of more targeted sampling techniques for their investigation. MeS-compounds from tropical fruits such as pineapple (Takeoka et al., 1989; Takeoka et al., 1991), passion fruit (Werkhoff et al., 1998), Asian pear (Takeoka et al., 1992) and melon (Aubert and Bourger, 2004; Wyllie and Leach, 1992) include (MeS)alkanoate esters some of which exhibit tropical flavour notes (Burdock, 2004). Methional is a likely precursor to these compounds and has been

\[1\] This chapter is based on the manuscript Günther et al., Food Research International 2011, 44, 1331-1338
identified as a key aroma compound in pink guava (Steinhaus et al., 2009). Traditionally, solvent extraction techniques were employed before GC-MS analyses to isolate sulphur-containing volatiles from fruits but these approaches are time-consuming, require large amounts of fruit material and produce toxic waste. This chapter describes the development of fast and reliable headspace-sampling techniques for MeS-volatile analysis from kiwifruit tissue that can also be applied to other fruit matrices. Two methods were systematically optimised for this purpose: Firstly, the static headspace extraction method HS-SPME that is known as a simple and fast technique (Kataoka et al., 2000) and secondly, DHS, which requires more handling and specific, often purpose-built equipment. In addition, “salting-out” and “salting-in” effects on MeS-volatiles were investigated.

2.2 Materials and Methods

2.2.1 Kiwifruit material

*Actinidia chinensis* Planch. var. *chinensis* ‘Hort16A’ kiwifruit were harvested at commercial maturity from three different orchards in 2008 and then colour-conditioned for five days at 5°C (Satara Kiwifruit Supply Ltd, Bay of Plenty, New Zealand). After cold storage at 1.5°C for one month, a sample of ten kiwifruit from each orchard was ripened at 20°C to eating firmness (4 × 10^6 Hz^2 g^2/3, AWETA® acoustic firmness sensor, microphone gain 80, tick power 16). Fruit were then pooled by spooning the pulp into liquid nitrogen, homogenised with a stone crus her (Rocklabs, New Zealand) and stored at -80°C before headspace volatile extraction.

2.2.2 Reference compounds

Stock solutions (10 mM) of all standards were prepared in isopropanol and stored at -20°C prior to use. The following reference compounds were purchased from Sigma-Aldrich New Zealand Ltd:
2-MeSethanol, 3-MeSpropanol, 3-MeSpropanal, methyl and ethyl 2-MeSacetate, methyl and ethyl 3-MeSpropionate.

Propyl and butyl 3-MeSpropionate were synthesised by reaction of propanol or butanol with 1 equivalent of 3-MeSpropionic anhydride, which was synthesised by stirring 2.3 mmol of dicyclohexylcarbodiimide with 4.2 mmol 3-MeSpropionic acid in 10 mL of dry Et₂O under N₂ for 24 h, and was used without further purification. Propanol or butanol (1
mmol), 3-MeSpropionic anhydride, and p-toluene sulphonic acid (10 mg) were stirred for five days in 5 mL of dry Et₂O. 40 mL of Et₂O was added, and the reaction quenched with 2 x 10 mL saturated NaHCO₃ (aq.). The organic phase was dried (MgSO₄), the solvent removed and the product purified by flash chromatography on silica gel. Propyl 3-MeSpropionate (99% pure²); EI-MS, m/z (rel. int.): 74 (100), 61 (88), 41 (60), 75 (48), 43 (47), 162 (40, M⁺), 47 (32), 103 (27), 55 (20), 73 (18), 120 (15), 77 (14), 119 (10), 102 (7), 89 (7), 147 (1). Butyl 3-MeSpropionate (99% pure); EI-MS, m/z (rel. int.): 74 (100), 61 (88), 41 (60), 75 (48), 43 (47), 176 (40, M⁺), 47 (32), 103 (27), 55 (20), 73 (18), 120 (15), 77 (14), 119 (10), 102 (7), 89 (7), 120 (1).

Propyl and butyl 2-MeSacetate were synthesised by reaction of propanol or butanol with 1 equivalent of 2-MeSacetic anhydride, which was synthesised by stirring 6.3 mmol of dicyclohexylcarbodiimide with 3.33 mmol 2-MeSacetic acid in 10 mL of dry Et₂O under N₂ for 24 h, and was used without further purification. Propanol or butanol (1.6 mmol), 2-MeSacetic anhydride (1.67 mmol), and p-toluene sulphonic acid (10 mg) were stirred for five days in 10 mL of dry Et₂O. 20 mL of Et₂O was added, and the reaction quenched with 2 x 5 mL saturated NaHCO₃ (aq.). The organic phase was dried (MgSO₄), the solvent removed, and the product purified by flash chromatography on silica gel. Propyl 2-MeSacetate (>99% pure); EI-MS, m/z (rel. int.): 61 (100), 43 (36), 148 (30, M⁺), 41 (19), 42 (11), 45 (11), 60 (11), 35 (9), 47 (7), 63 (4), 62 (4), 149 (4), 102 (4), 77 (4), 106 (4), 46 (3), 89 (3). Butyl 2-MeSacetate (99% pure); EI-MS, m/z (rel. int.): 61 (100), 41 (51), 56 (37), 57 (30), 162 (28, M⁺), 106 (21), 42 (15), 60 (12), 35 (12), 62 (11), 45 (10), 46 (10), 39 (9), 47 (7), 43 (7).

The internal standard, ethyl 3-[D₃]MeSpropionate (Fig. 2.1), was synthesised by reaction of CD₃I with ethyl 3-mercaptopropionate according to Sen and Grosch (1991), and was purified (97.5%) by distillation at 65°C under reduced pressure. EI-MS, m/z (rel. int.): 77 (100), 64 (71), 151 (58, M⁺), 78 (48), 106 (20), 43 (19), 55 (16), 49 (13), 46 (12), 80 (11), 59 (11), 50 (10), 45 (10).

For the synthesis of [D₅]ethyl 2-MeSacetate (Fig. 2.1), 1.58 mmol of 2-MeSacetic anhydride (5.74 mmol 2-MeSacetic acid and 3.15 mmol dicyclohexylcarbodiimide reacted as described above) were stirred with 1.75 mmol d₆-ethanol and p-toluene sulphonic acid (5 mg) for 3 h under N₂. Pentane (10 mL) was added to the reaction mixture, which was

² Purity determined from chromatogram according to peak size of compound.
then quenched with 2 x 10 mL saturated NaHCO₃ (aq.). The aqueous layer was extracted twice with 5 mL pentane before combining and drying (MgSO₄) the organic fractions. After the solvent was removed, the product was purified (>99%) by distillation at 50ºC under reduced pressure. El-MS, m/z (rel. int.): 61 (100), 139 (82, M⁺), 93 (56), 63 (9), 45 (6), 74 (5), 140 (5), 46 (4), 141 (4), 73 (4).

2.2.3 Sampling conditions

2.2.3.1 HS-SPME optimisation

Unless stated otherwise, three replicates per sample were analysed, each containing 30 g of pulverised fruit tissue in a 50-mL Erlenmeyer flask with a Quick Fit® (Sigma-Aldrich) cap, and pierced polytetrafluoroethylene (PTFE)-lined silicone septa to allow fibre extraction in close proximity to the sample surface. Either no salt or saturating amounts of CaCl₂ (24.4 g), NaCl (10.8 g), MgSO₄ (10.7 g) or (NH₄)₂SO₄ (25.3 g), purchased from Sigma-Aldrich, New Zealand Ltd, were added to the fruit pulp before introducing the internal standard mixture. After 5-min equilibration time at the corresponding sampling temperature (20, 40, 60 or 80 °C), the headspace volatiles were extracted, with gentle agitation, using a 65-μm polydimethylsiloxane-divinylbenzene (1 cm, PDMS-DVB) HS-SPME fibre (Supelco, Bellefonte, PA, USA). The standard sampling time was 20 min but times ranging from 5 to 40 min were also tried at the optimised extraction parameters. Finally, the performance of a 65-μm carbowax-divinylbenzene (CW-DVB) fibre coating was compared with the PDMS-DVB coating. All fibres were conditioned before sampling according to the manufacturer’s recommendations.

2.2.3.2 DHS optimisation

Four replicates each of 10 g pulverised ‘Hort16A’ kiwifruit pulp were prepared in 250-mL Erlenmeyer flasks to provide a large sample surface area. Deuterated internal standards and either the carboxy esterase inhibitor Paraoxon (0.1 mM; Sigma-Aldrich, New Zealand Ltd) or 8 g (NH₄)₂SO₄ were added to the pulp. After hermetically sealing the equipment, purified air (BOC) was purged over the sample matrix at room temperature and concentrated in direct thermal desorption (DTD) traps (ATAS GL International), each containing 100 mg Tenax®-TA resin (60–80 mesh, Chrompack, Netherlands). The flow rate (15, 25, 35 mL min⁻¹) was optimised at an extraction time of 20 h. Before sampling, the Tenax®-filled DTD traps were conditioned for 2 h at 235 °C with a nitrogen flow of 20 mL min⁻¹.
2.2.4 GC-MS settings

GC separations were on a 30 m × 0.25 mm × 0.25 μm film thickness DB-Wax (J & W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent Technologies) with helium as the carrier gas. The GC was coupled to a time-of-flight mass spectrometer (TOF-MS, Leco Pegasus III, St. Joseph, MI, USA). The transfer line temperature was set to 220°C and a detector voltage of 1700 V was applied. The ion source temperature was kept at 200°C and ionisation energy of 70 eV was used for electron impact ionisation. Ion spectra from 26 to 250 amu were collected with a data acquisition rate of 20 Hz. The total ion chromatograms were processed using the LECO ChromaTOF software.

2.2.4.1 HS-SPME settings

The fibres were manually injected into the GC injection port at 150°C, followed by a ramp of 10°C s\(^{-1}\) to 250°C. Injections were splitless for 2 min with a column flow of 2 mL min\(^{-1}\), followed by a column flow of 1 mL min\(^{-1}\). The GC oven ramp was 30°C for 2 min, 5°C min\(^{-1}\) to 220°C, and then held for 1 min.

2.2.4.2 DTD settings

The Focus (ATAS GL) autosampler was operated via the PAL Cycle Composer software 1.5.4. Volatiles were desorbed from the DTD traps in the injection port with a temperature ramp of 35°C to 220°C at 16°C sec\(^{-1}\) and cryofocused on the front of the capillary column by a liquid nitrogen-cooled cryogenic trap at -120°C before increasing the trap temperature to 175°C at 50°C min\(^{-1}\) (Optic 3 thermal desorption system, ATAS GL). A 15:1 split was employed while the volatiles were transferred onto the capillary column at a column flow of 1 mL min\(^{-1}\). The GC oven ramp was 30°C for 1 min, 3°C min\(^{-1}\) to 220°C, and then held for 2.3 min.

2.2.5 Qualitative and quantitative analysis

Retention times and mass spectra of authentic reference compounds were used to identify MeS-volatiles, except for ethyl 3-MeSprop-2-enoate whose spectrum was matched to the National Institute of Standards and Technology (NIST) library. All peaks were selected and integrated manually using the molecular ion. The 3-MeSpropionate esters, methional and methionol were quantified with respect to the internal standard ethyl 3-[D3]MeSpropionate. The 2-MeSacetate esters and 2-MeSethanol were quantified with respect to [D5]ethyl 2-MeSacetate. Ethyl 3-MeSprop-2-enoate was quantified in
equivalents of ethyl 3-MeSpropionate. Changes of absolute molecular ion peak areas in response to the sample treatment were compared for method optimisation. For quantification of MeS-volatiles after DHS, standard curves were acquired by standard dilution series in a ‘Hort16A’ model solution (5.5 g L⁻¹ malic acid, 11.2 g L⁻¹ citric acid, 20.6 g L⁻¹ quinic acid, 1.3 g L⁻¹ myo-inositol, 15 g L⁻¹ sucrose, 39.3 g L⁻¹ glucose, 49.6 g L⁻¹ fructose, pH 4.5; (Marsh et al., 2006)) with and without (NH₄)₂SO₄ (8 g).

2.2.6 Repeatability

The repeatability was determined in % relative standard deviation (RSD) of six (HS-SPME) and eight (DHS) replicates containing the same mixture of all available reference compounds in synthetic ‘Hort16A’ model solution.

2.2.7 Statistical analysis

Student’s t-tests, analysis of variance (ANOVA) and post-hoc Tukey’s honestly significant difference test (Tukey’s HSD₀.₀₅) were performed using Microsoft® Excel 2003 or Origin 7.5 SR4 (Originlab corporation, USA) programs.

2.3. Results and Discussion

2.3.1 HS-SPME optimisation

2.3.1.1 The effect of salt saturation and sampling temperature

The so called “salting-out” effect is commonly used to improve the release of organic volatiles from an aqueous sample matrix to its headspace. This is caused by changes of phase boundary properties between sample and matrix and a general reduction in their solubility (Yang and Peppard, 1994). Also important is the effect of sampling temperature on the extraction yield of headspace volatiles, since the vapour pressure generally increases with temperature, resulting in decreased partition coefficients for most compounds (Snow and Bullock, 2010). In order to define the most sensitive conditions, the effect of four inorganic salts (NaCl, MgSO₄, CaCl₂, (NH₄)₂SO₄) on MeS-volatiles from ‘Hort16A’ fruit pulp was investigated at different extraction temperatures (20, 40, 60, 80°C) using HS-SPME. Two main classes of (MeS)alkanoate esters, namely alkyl 2-MeSacettes and alkyl 3-MeSpropionates, the MeS-alcohols 2-
MeSethanol and methionol and the aldehyde methional (Fig. 2.1) were identified from ‘Hort16A’ fruit pulp.

Fig. 2.1: Structures of MeS-volatiles quantified in Actinidia chinensis ‘Hort16A’ kiwifruit and the corresponding deuterium-labelled internal standard.

However, salt treatment and sampling temperature appeared to have a major effect on the composition of MeS-volatiles that were detected from kiwifruit pulp as presented in Table 2.1. For example, only three MeS-compounds, namely ethyl 2-MeSacetate, methyl and ethyl 3-MeSpropionate, were extracted from the headspace of untreated kiwifruit pulp at 20°C whereas ethyl 3-MeSprop-2-enoate and methional were also detected at 60°C and 80°C, respectively.
**Table 2.1:** The impact of sampling temperature and salinity on methylsulfanyl-volatiles extracted from *Actinidia chinensis* ‘Hort16A’ pulp using HS-SPME (PDMS-DVB fibre; 20 min).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>NaCl</th>
<th>CaCl₂</th>
<th>MgSO₄</th>
<th>(NH₄)₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methyl</em> 2-(methylsulfanyl)acetate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>40°C</td>
</tr>
<tr>
<td><em>Ethyl</em> 2-(methylsulfanyl)acetate</td>
<td>20-80°C</td>
<td>20-80°C</td>
<td>20-60°C</td>
<td>20-80°C</td>
<td>20-80°C</td>
</tr>
<tr>
<td>Methional</td>
<td>80°C</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>40-80°C</td>
</tr>
<tr>
<td><em>Methyl</em> 3-(methylsulfanyl)propionate</td>
<td>20-60°C</td>
<td>20-60°C</td>
<td>60°C</td>
<td>20-40°C</td>
<td>20-40°C</td>
</tr>
<tr>
<td>2-(methylsulfanyl)ethanol</td>
<td>ND</td>
<td>20°C</td>
<td>ND</td>
<td>ND</td>
<td>40°C</td>
</tr>
<tr>
<td><em>Ethyl</em> 3-(methylsulfanyl)propionate</td>
<td>20-80°C</td>
<td>20-80°C</td>
<td>20-80°C</td>
<td>20-80°C</td>
<td>20-40°C</td>
</tr>
<tr>
<td>Methionol</td>
<td>ND</td>
<td>20-60°C</td>
<td>ND</td>
<td>ND</td>
<td>20-80°C</td>
</tr>
<tr>
<td><em>Ethyl</em> 3-(methylsulfanyl)prop-2-enoate</td>
<td>60°C</td>
<td>40-80°C</td>
<td>ND</td>
<td>ND</td>
<td>40°C</td>
</tr>
</tbody>
</table>

ND: not detected

In contrast, methionol and 2-MeSethanol were detectable at 20°C after saturating the fruit pulp with NaCl but methional was not found at any temperature studied. The beneficial use of NaCl is well described in literature and a remarkable effect on the extraction efficiency of sulphur mustard (bis(2-chloroethyl) sulphide) has been reported (Røen et al., 2010). Interestingly, the commonly used salt CaCl₂ decreased the diversity of MeS-volatiles that were extracted from the headspace and the sampling temperature for methyl 3-MeSpropionate detection was raised from 20°C to 60°C compared with the untreated sample. This points towards a "salting-in" effect that was also observed after MgSO₄ saturation. Each volatile can respond differently to varying salts or salt concentrations (Falabella and Teja, 2008; Yang and Peppard, 1994) and the use of MgSO₄ and CaCl₂ did not appear beneficial for the MeS-volatile release from the kiwifruit matrix. However, when (NH₄)₂SO₄ was used, the best extraction yield for MeS-volatiles was achieved. The advantages of salting out with (NH₄)₂SO₄ include detection of methyl 2-MeSacetaate in addition to the above-mentioned MeS-compounds. Furthermore, the minimum sampling temperature for methional was lowered from 80°C to 40°C compared with unsalted
samples. This allowed sampling of all MeS-volatiles at this lower temperature. Finally, the absolute peak areas obtained for the main esters, ethyl 2-MeSacetate and ethyl 3-MeSpropionate and their corresponding, deuterated internal standards, were significantly higher at 40ºC compared with the pure sample or any other salt tested (Fig. 2.2). It was recently reported that the beneficial effects of (NH₄)₂SO₄ addition on the extraction efficiency of fruit volatiles, including (MeS)alkanoate esters and MeS-alcohols, were superior to the use of NaCl and CaCl₂ using liquid-liquid microextraction (Aubert et al., 2005) and we can confirm this finding for headspace sampling on the basis of our results.

![Fig. 2.2: Salt and temperature effects on HS-SPME of the main (methylsulfanyl)alkanoate esters and their corresponding deuterated internal standards from Actinidia chinensis 'Hort16A' pulp. A: ethyl 2-(methylsulfanyl)acetate; B: ethyl 3-(methylsulfanyl)propionate; C: [D₅]ethyl 2-(methylsulfanyl)acetate; D: ethyl 3-[(D₃)methylsulfanyl]propionate. The error bars represent the standard error of the mean of three replicates. The treatment indicated with an asterisk is significantly different from all other salt treatments at any temperature (Tukey's HSD₀.₀₅). Note: Other observed significant differences are not marked in this.]

2.3.1.2 Sampling time for HS-SPME

Consistent extraction times have been described as the most critical parameter necessary for a high degree of reproducibility between samples using HS-SPME (Holt, 2001). This is especially important if equilibrium has not been reached among the three phases (sample, headspace, fibre) because a linear relationship exists between the
amount of analyte adsorbed on the HS-SPME fibre and the sampling time (Kataoka et al., 2000). The impact of sampling time (5, 10, 15, 20, 40 min) on absolute peak areas of MeS-volatiles at the optimised sampling conditions (40°C, 25.3 g (NH₄)₂SO₄) is shown in Fig. 2.3. Different types of response curves were observed for individual compounds. Nevertheless, peak areas after 15–20 min sampling time were generally significantly higher compared to 5 and 10 min (Fig. 2.3A–D). In some cases a unique extraction optimum was observed after 15 min (Fig. 2.3D) or 20 min (Fig. 2.3B), respectively. However, 40-min extraction generally resulted in significantly lower peak areas except for methyl 2-MeSacetate, for which a trend similar to ethyl 2-MeSacetate was observed. This implies that a stable equilibrium was not reached for any MeS-compound within this experiment, which may be explained by decreasing concentrations of the

**Fig. 2.3:** The impact of sampling time on absolute peak areas of methylsulfanyl-volatiles from Actinidia chinensis ‘Hort16A’ pulp using PDMS-DVB coated HS-SPME fibres (40°C, (NH₄)₂SO₄ saturation). A: ethyl 2-(methylsulfanyl)acetate; ethyl 3-(methylsulfanyl)propionate. B: methyl 3-(methylsulfanyl)propionate; 2-(methylsulfanyl)ethanol. C: methyl 2-(methylsulfanyl)acetate; ethyl 3-(methylsulfanyl)prop-2-enoate. D: methional; methionol. The error bars represent the standard error of the mean of four replicates. Letters are used to indicate the significance of treatment (Tukey’s HSD₀.₀₅).
particular volatile in the biological, acidic sample due to chemical rearrangement at warm temperatures (ester hydrolysis, oxidation), or by competitive binding on the SPME fibre. The latter would explain why the extraction yield of ethyl 3-MeSprop-2-enoate increased with extraction time (Fig. 2.3C) whereas the reverse effect was observed for saturated (MeS)alkanoate esters. Since there was no single optimal extraction time for all MeS-volatiles, 20 min was chosen as a suitable time and to prevent “over-sampling”.

2.3.1.3 Repeatability of manual HS-SPME

In general, the % RSD of absolute peak areas was high, (up to 33.7% methionol, Table 2.2) and the use of internal standards was crucial for achieving a reasonable repeatability. Deuterated (MeS)alkanoate esters worked best as internal standards for this family of compounds (3.1–7.9% RSD) but additional standards with chemical properties similar to methional, methionol and 2-MeSethanol would be necessary to improve the % RSD (16.9–22.5 %) of their corrected peak areas.

Table 2.2: Repeatability of manual HS-SPME using reference compounds in synthetic ‘Hort16A’ solution at optimised conditions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>%RSD absolute peak area n=6</th>
<th>%RSD corrected peak area n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 2-(methylsulfanyl)acetate</td>
<td>11.8</td>
<td>7.6</td>
</tr>
<tr>
<td>[D5]Ethyl 2-(methylsulfanyl)acetate</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>Ethyl 2-(methylsulfanyl)acetate</td>
<td>20.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Methional</td>
<td>26.1</td>
<td>22.2</td>
</tr>
<tr>
<td>Methyl 3-(methylsulfanyl)propionate</td>
<td>19.3</td>
<td>7.9</td>
</tr>
<tr>
<td>2-(methylsulfanyl)ethanol</td>
<td>31.5</td>
<td>16.9</td>
</tr>
<tr>
<td>Ethyl 3-(methylsulfanyl)propionate</td>
<td>25.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Ethyl 3-([D3]methylsulfanyl)propionate</td>
<td>26.6</td>
<td></td>
</tr>
<tr>
<td>Methionol</td>
<td>33.7</td>
<td>22.5</td>
</tr>
</tbody>
</table>

RSD = relative standard deviation
2.3.1.4 The performance of different fibre coatings

The absolute peak areas of MeS-volatiles obtained by using polar CW-DVB-coated fibres and optimised sampling parameters (40°C, 25.3 g (NH₄)₂SO₄, 20 min) were similar compared with those using PDMS-DVB fibres. However, ANOVA and Student’s *t*-test analysis revealed a significant 30% decrease in peak size (data not shown) for ethyl 3-MeSpropionate using the polar CW-DVB fibre but no significant differences for the remaining MeS-volatiles were observed. Therefore, both fibres were considered suitable for MeS-compound analysis from ‘Hort16A’ pulp, but the PDMS-DVB coating was preferred because of its higher sensitivity for the main (MeS)alkanoate ester.

2.3.2 Optimisation of dynamic headspace sampling

2.3.2.1 Impact of flow rate

The effect of different gas flow rates between 15–35 mL min⁻¹ on the extraction yield of the main MeS-compounds was marginal. For example, there was no significant (ANOVA 0.05) difference observed for (MeS)alkanoate ester levels and methional with changing flow rate (Fig. 2.4). However, the peak areas of MeS-alcohols were significantly (Tukey’s HSD 0.05) increased by about two-fold at the higher flow rates (25 and 35 mL min⁻¹), in comparison with 15 mL min⁻¹. This implies that the sampling flow rate had a greater impact on the less volatile MeS-compounds, possibly because quantitatively more molecules were transferred from the headspace into the adsorption trap. A flow rate of 30 mL min⁻¹ was chosen for further analysis to provide an optimal gas flow for the extraction of all MeS-volatiles.
Fig. 2.4: The effect of different flow rates on extraction yields of the main methylsulfanyl-volatiles from Actinidia chinensis 'Hort16A' pulp using dynamic headspace sampling (Tenax®, 20 h). Methionol; methional; ethyl 2-(methylsulfanyl)acetate; 2-(methylsulfanyl)ethanol; ethyl 3-(methylsulfanyl)propionate. The error bars represent the standard error of the mean of four replicates. a, b indicate the significant differences (Tukey’s HSD0.05) in response to changes in flow rate for each individual compound.

2.3.2.2 Advantage of (NH₄)₂SO₄ addition for MeS-volatile extraction with DHS

Linear regression models of reference compounds in a ‘Hort16A’ model solution with and without (NH₄)₂SO₄ saturation were acquired for direct quantification of fruit samples (Table 2.3). The coefficients of determination were generally ≥ 0.994 for both treatments, confirming a good linearity within the concentration range tested. The % RSD of most of the MeS-volatiles was also independent of the treatment and less than 10% in most cases, indicating good repeatability of this method. The limit of detection (LOD) was estimated using a simplified approach by interpolation of the slope, gathered from plotting the signal-to-noise ratio (S/N) over the corresponding concentration of a single standard dilution series, to a S/N value of 2. Therefore, the LOD values given in Table 2.3 need to be seen as approximate measures. However, the LOD of all the MeS-volatiles was generally lower than the concentrations reported as odour thresholds for the corresponding MeS-compounds in the literature, where available (Aubert and Bourger, 2004; Ferreira et al., 2005; Steinhaus et al., 2009; Takeoka et al., 1989). Furthermore, the sensitivity of the method was markedly increased by addition of (NH₄)₂SO₄ because the LOD of all the MeS-volatiles was concomitantly decreased (from 1.5-fold for ethyl 2-MeSacetate to 29.2-fold for 3-MeSpropanal Table 2.3).
**Table 2.3:** Evaluation of the linearity, sensitivity and repeatability of the dynamic headspace sampling method applied to unsalted and salted synthetic ‘Hort16A’ solution containing reference compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Untreated</th>
<th>(NH₄)₂SO₄</th>
<th>Fold decrease LOD (treated/untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;std&lt;/sub&gt; (ug L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>LOD (ng L&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Methyl 2-(methylsulfanyl)acetate</td>
<td>0.011-5.5</td>
<td>0.999</td>
<td>2.28</td>
</tr>
<tr>
<td>Ethyl 2-(methylsulfanyl)acetate</td>
<td>0.003-5.9</td>
<td>0.996</td>
<td>4.99</td>
</tr>
<tr>
<td>Methional</td>
<td>0.8-26</td>
<td>0.999</td>
<td>720.7</td>
</tr>
<tr>
<td>Methyl 3-(methylsulfanyl)propionate</td>
<td>0.0013-8.8</td>
<td>0.999</td>
<td>4.51</td>
</tr>
<tr>
<td>2-(methylsulfanyl)ethanol</td>
<td>0.54-138</td>
<td>0.997</td>
<td>174.5</td>
</tr>
<tr>
<td>Ethyl 3-(methylsulfanyl)propionate</td>
<td>0.14-141</td>
<td>0.999</td>
<td>55.5</td>
</tr>
<tr>
<td>Propyl 3-(methylsulfanyl)propionate</td>
<td>0.025-3.2</td>
<td>0.999</td>
<td>16.11</td>
</tr>
<tr>
<td>Methionol</td>
<td>2-1060</td>
<td>0.999</td>
<td>1863.5</td>
</tr>
<tr>
<td>Butyl 3-(methylsulfanyl)propionate</td>
<td>0.11-1.8</td>
<td>0.999</td>
<td>78.0</td>
</tr>
</tbody>
</table>

C<sub>std</sub>: concentration range of each reference compound used for a standard dilution series in ‘Hort16A’ model solution without (untreated) and with (NH₄)₂SO₄ treatment. R<sup>2</sup>: coefficient of determination; LOD: Limit of detection as estimated concentration resulting in a signal-noise ratio of 2 by interpolation. %RSD: repeatability expressed as relative standard deviation of eight replicates. NC: not comparable (only detected in salt-treated sample).
Table 2.4 provides an overview of the MeS-volatiles detected in A. chinensis ‘Hort16A’ pulp with and without the addition of (NH₄)₂SO₄ using the optimised DHS approach before GC-MS analysis. Two additional esters, propyl- and butyl 3-MeSpropionate, were detected using DHS, although the latter compound required “salting out”. The salt addition affected the absolute peak areas of all MeS-compounds: (MeS)alkanoate esters increased approximately two-fold, MeS-alcohols 30-fold and methional 16-fold. The estimated concentrations of most MeS-compounds from ‘Hort16A’ fruit were in agreement to within 20% for salted and unsalted pulp, except for methional and ethyl 3-MeSprop-2-enoate, for which the concentration values differed by 41% and 31%, respectively. The latter compound was quantified in equivalents of ethyl 3-MeSpropionate because an authentic standard was not available and the difference in concentration points towards a different “salting-out” effect on both esters. The %RSD of methional appeared to be relatively high (13.5–14.9%) between replicates of the reference compound in the synthetic “Hort16A” matrix (Table 2.3) and between fruit samples (13.9–16.4%, Table 2.4).

Since methional is a rather unstable compound that can be formed by oxidation of methionol and is further oxidised to 3-MeSpropionic acid, its concentrations are likely to fluctuate between samples, and an additional internal standard with the same chemical properties (e.g. deuterated methional) could help reducing this variability. We therefore conclude that DHS appears to be a suitable approach for MeS-volatile analysis at trace amounts and its sensitivity can be significantly increased by the saturation with (NH₄)₂SO₄.
Table 2.4: Direct quantification of methylsulfanyl-volatiles from untreated and (NH₄)₂SO₄-saturated kiwifruit tissue and comparison of extraction yield (absolute peak areas).

<table>
<thead>
<tr>
<th>Volatile</th>
<th>C&lt;sub&gt;Untreated&lt;/sub&gt; (ug kg⁻¹ FW)</th>
<th>%RSD (n=4)</th>
<th>C&lt;sub&gt;(NH₄)₂SO₄&lt;/sub&gt; (ug kg⁻¹ FW)</th>
<th>%RSD (n=4)</th>
<th>Fold increase PA&lt;sub&gt;abs&lt;/sub&gt; (treated/untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 2-(methylsulfanyl)acetate</td>
<td>0.08</td>
<td>6.74</td>
<td>0.10</td>
<td>6.89</td>
<td>2.3</td>
</tr>
<tr>
<td>Ethyl 2-(methylsulfanyl)acetate</td>
<td>0.27</td>
<td>1.31</td>
<td>0.32</td>
<td>4.54</td>
<td>2.2</td>
</tr>
<tr>
<td>Methional</td>
<td>17.37</td>
<td>13.93</td>
<td>10.24</td>
<td>16.43</td>
<td>16.2</td>
</tr>
<tr>
<td>Methyl 3-(methylsulfanyl)propionate</td>
<td>1.01</td>
<td>5.98</td>
<td>0.98</td>
<td>9.67</td>
<td>2.2</td>
</tr>
<tr>
<td>2-(methylsulfanyl)ethanol</td>
<td>12.95</td>
<td>7.73</td>
<td>15.45</td>
<td>11.83</td>
<td>31</td>
</tr>
<tr>
<td>Ethyl 3-(methylsulfanyl)propionate</td>
<td>8.73</td>
<td>4.75</td>
<td>9.32</td>
<td>4.19</td>
<td>2.5</td>
</tr>
<tr>
<td>Propyl 3-(methylsulfanyl)propionate</td>
<td>0.04</td>
<td>9.21</td>
<td>0.04</td>
<td>4.36</td>
<td>2.6</td>
</tr>
<tr>
<td>Methionol</td>
<td>63.81</td>
<td>4.35</td>
<td>50.60</td>
<td>10.36</td>
<td>33.1</td>
</tr>
<tr>
<td>Ethyl 3-(methylsulfanyl)prop-2-enoate</td>
<td>0.13</td>
<td>8.49</td>
<td>0.18</td>
<td>7.93</td>
<td>7.1</td>
</tr>
<tr>
<td>Butyl 3-(methylsulfanyl)propionate</td>
<td>ND</td>
<td>ND</td>
<td>0.12</td>
<td>4.22</td>
<td>NC</td>
</tr>
</tbody>
</table>

PA<sub>abs</sub>: absolute peak area; FW: fresh weight; %RSD: repeatability expressed as relative standard deviation of eight replicates; NC: not comparable (only detected in salt treated sample); ND: not detectable

2.3.2.3 Comparison of DHS with HS-SPME

DHS qualitatively and quantitatively improved MeS-volatile extraction compared with HS-SPME under optimised conditions. For example, propyl 3-MeSpropionate and butyl 3-MeSpropionate were exclusively detected using DHS (Table 2.4), but the latter compound required “salting out” with (NH₄)₂SO₄ to enable its detection at room temperature. Fig. 2.5 demonstrates that not only were the extraction yields of all MeS-volatiles remarkably increased using DHS but also the peak separation was much improved, resulting in well arranged chromatograms with sharper peaks. A similar finding was reported by (Povolo and Contarini, 2003). In part, this can be explained by a higher
extraction yield of the porous resin (usually Tenax®) because of a larger surface area (Bicchi et al., 2008) and lower compound selectivity compared with that from HS-SPME fibre coatings. In addition, exposing the sample to a continuous flow of gas can be used to drive more volatiles into the headspace and cryogenic focusing usually leads to improved chromatography due to compound enrichment and band concentration (Kolb, 1999). Thus, three times the amount of fruit tissue (30 g) was necessary to reliably sample most MeS-volatiles using HS-SPME compared with DHS. Therefore, DHS was a more sensitive method for sampling the volatiles of interest and in general required less compound-specific optimisation. Nevertheless, HS-SPME under optimised conditions was proven to be suitable for the identification of the main MeS-volatiles from kiwifruit pulp with much shorter sampling times (20 min versus 20 h) and less complex equipment (i.e. flow rate control, nitrogen-cooled cryogenic trap). This makes HS-SPME the higher throughput method, especially for qualitative analysis and if coupled to an autosampler.

In addition, HS-SPME appeared to be the quicker and therefore preferred option for optimisation purposes, for example testing the “salting-out” effects of different salts on

Fig. 2.5: Chromatograms of reference compounds in (NH₄)₂SO₄-saturated ‘Hort16A’ model solution using (A, C) dynamic headspace sampling and (B, D) headspace solid-phase microextraction. The m/z = 61 (A, B) peaks and molecular ion peaks (C, D) are displayed. Concentrations of standard compounds are given in [μg L⁻¹]. The first value refers to HS-SPME and the second to DHS sampling: 1. methyl 2-(methylsulfanyl)acetate [30, 5]; 2. [D5]ethyl 2-(methylsulfanyl)acetate; 3. ethyl 2-(methylsulfanyl)acetate [67, 2]; 4. methional [520, 22]; 5. methyl 3-(methylsulfanyl)propionate [201, 6]; 6. 2-(methylsulfanyl)ethanol [2300, 60]; 7. ethyl 3-([D3]methylsulfanyl)propionate; 8. ethyl 3-(methylsulfanyl)propionate [37, 55]; 9. methionol [1325, 265]
Chapter 2

MeS-volatiles. Whether the peak separation can be improved by coupling HS-SPME with a cold trap would be interesting to investigate.

2.4. Concluding remarks

It was shown that static (HS-SPME) and dynamic (DHS) headspace sampling techniques are suitable means for trace MeS-volatile analysis from frozen kiwifruit pulp using GC-MS analysis. Furthermore, a considerable “salting-out” effect, after saturation with (NH₄)₂SO₄, was observed for this family of compounds. In general, DHS was more sensitive and required less fruit tissue for reliable MeS-compound analysis, but HS-SPME was much more time-efficient and easier to use once the best sampling conditions were established. We suggest the use of HS-SPME for preliminary, qualitative analysis of MeS-compounds from fruit tissue and to test the effect of specific treatments on the volatiles of interest. This information can be further used to improve the performance of DHS as a quantitative approach to determine MeS-volatile concentrations in different kiwifruit cultivars or other fruit matrices.
CHAPTER 3

(METHYLSULFANYL)ALKANOATE ESTER BIOSYNTHESIS IN ACTINIDIA CHINENSIS AND CHANGES DURING COLD STORAGE

3.1 Introduction

Since 1998, the novel kiwifruit cultivar Actinidia chinensis ‘Hort16A’ has been commercially available under the trade name ZESPRI® GOLD. This cultivar is distinguished from the green variety not only by its yellow flesh colour but also by a sweeter taste with a fruity aroma reminiscent of tropical fruits (Jaeger et al., 2003). (MeS)alkanoate esters, in particular methyl and ethyl 3-MeSpropionate as well as ethyl 2-MeSacetate, display fruity, sweet and tropical odour characteristics (Burdock, 2004) and were reported to occur in several tropical fruits (Takeoka et al., 1989; Takeoka et al., 1992; Werkhoff et al., 1998; Weenen et al., 1996). Wyllie and Leach (1992) assigned these and other (MeS)alkanoate esters a significant impact on the characteristic aroma of certain melon cultivars due to their OAV. Moreover, methyl and ethyl 2-MeSacetate have previously been quantified in Actinidia deliciosa ‘Hayward’ kiwifruit puree by Jordan et al. (2002) and an aroma impact was described for the cooked nut or roasted oil odour of the first ester using GC-O.

General fruit ester production by AATs has been studied in apple (Matich and Rowan, 2007; Souleyre et al., 2005), banana (Wyllie and Fellman, 2000), strawberry (Olias et al., 2002) and melon (El-Sharkawy et al., 2005). It was shown that recombinant Cucumis melo AATs were capable of using MeS-alcohols as substrates for ester synthesis with acetyl-CoA (Lucchetta et al., 2007) but (MeS)alkanoyl-CoAs have not been tested as substrates for ester synthesis. In apple and melon, the gene expression of ripening-specific AATs appeared to be regulated by ethylene (Schaffer et al., 2007; Yahyaoui et al., 2002) and the production of volatile esters was also correlated with ethylene synthesis in these climacteric fruit (Aubert and Bourger, 2004; Johnston et al., 2009).

Kiwifruit in general is known to produce climacteric ethylene during ripening but only very low levels of ethylene can be detected in Actinidia chinensis ‘Hort16A’ during cold storage.

3 This chapter is based on the manuscript Günther et al., Phytochemistry 2010, 71 (7), 742-750
(Patterson et al., 2003) which is the common postharvest practice to maintain flesh firmness. Kiwifruit firmness is widely used as the defining character for ripeness and the non destructive AFS was suggested as a suitable alternative to destructive devices for monitoring textural changes of ‘Hort16A’ kiwifruit during storage (Schotsmans et al., 2008). The AFS measures fruit stiffness in $10^6 \text{ Hz}^2 \text{ g}^{2/3}$ and to date there is no equation available to express kiwifruit stiffness in N. However, ‘Hort16A’ fruit are considered as eating ripe between 10 and $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$ using the settings applied in this study.

The levels of MeS-volatiles during ripening and after cold storage of ‘Hort16A’ fruit are outlined in this chapter. Further it was tested whether (MeS)alkanoate esters can be synthesised by ripening specific AATs and if transcript levels of proposed putative AATs are influenced by chilling and ethylene. Thirdly, it was of interest to use the quantitative MeS-volatile data to obtain a first indication of their potential impact on the tropical flavour of eating-ripe fruit.

### 3.2 Materials and Methods

#### 3.2.1 Fruit material

*Actinidia chinensis* ‘Hort16A’ kiwifruit from three different growers were harvested in 2008 at commercial maturity (Satara Kiwifruit Supply Ltd, Bay of Plenty, New Zealand), colour-conditioned for five days at 5°C and randomised. A random sample of the fruit was ripened at 20°C and sampled according to their firmness using AWETA® AFS (microphone gain 80, tick power 16). The other fruit were cold stored at 1.5°C for six months and monthly samples were taken without ripening and after ripening to an average firmness of 8 and $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$, respectively. An additional sample was treated with 100 ppm ethylene for 24 hours after five months of cold storage and ripened to $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$. At each time-point, kiwifruit pulp from 25–30 fruit was snap frozen with liquid nitrogen, pulverised with a stone crusher (Rocklabs, New Zealand) and stored at -80°C prior to sampling.

#### 3.2.2 Ethylene production

Six fruit of each time point were individually placed in airtight plastic containers with rubber seal apertures. In addition, one empty container was prepared as a control. After one hour, 1 mL was taken with a syringe from the headspace and directly injected
into a Phillips PU 4500 GC with 1.5 m x 6 mm x 4 mm 80/100 mesh activated alumina F1 column (Pye Unicam) and FID-detector (210°C) that was calibrated with 1 ppm ethylene standard. The injector and column temperatures were set at 130°C.

3.2.3  Headspace volatile analysis

3.2.3.1 Standard compounds

Authentic reference compounds and internal standards were purchased and synthesised according to Section 2.2.2

3.2.3.2 Dynamic headspace sampling

Four replicates, each containing 10 g of pulverised 'Hort16A' fruit pulp, 1 µL of each deuterated internal standard (10 mM), and 0.1 mM of Paraaxon (Sigma-Aldrich) were prepared in 250 mL Erlenmeyer flasks (Quick Fit™). The addition of the carboxy esterase inhibitor Paraaxon significantly increased peak areas of (MeS)alkanoate esters and internal standards and was therefore crucial for quantification (Matich et al., 2008). After sealing the equipment hermetically, dry air (BOC) was purged through the system with a flow rate of 30 mL min⁻¹ and headspace volatiles were trapped with 100 mg Tenax®-TA resin for 20 h at room temperature. Prior to sampling, the Tenax®-filled DTD vials (ATAS GL International) were conditioned for two hours at 235°C with a nitrogen flow of 20 mL min⁻¹.

3.2.3.3 GC-MS-TOF analysis

Automated DTD injection (Focus, ATAS GL) and cryofocussing (-120°C) of headspace volatiles was utilised as described in Section 2.2.4.2. The volatiles were then transferred onto a 30 m x 0.25 mm x 0.25 μm film thickness DB-Wax (J & W Scientific, Folsom, CA, USA) capillary column in a HP6890 GC (Agilent Technologies). A linear GC-programme of 3°C min⁻¹ from 30°C for 1 min to 220°C for 2.3 min was applied with a column flow (Helium) of 1 mL min⁻¹. Peaks were identified by TOF-MS (Leco Pegasus III, St. Joseph, MI, USA) according to Section 2.2.4.

3.2.3.4 Qualitative and quantitative analysis

Headspace volatiles were analysed as described in Section 2.2.5.
3.2.4 Quantitative real-time RT-PCR analysis

Three RNA extractions were made from each sample according to Lopez-Gomez and Gomez-Lim (1992) and quantified with NanoDrop™ (Thermo scientific). 1 μg RNA of each replicate was treated with DNase I amplification grade (Invitrogen) prior to cDNA synthesis. First-strand cDNA was synthesised using the SuperScript™ VILO cDNA synthesis-kit (Invitrogen) according to the manufacturer’s instructions and diluted 100-fold before use. The same procedure without addition of reverse transcriptase (RT) was applied for each replicate to test for gDNA contamination. Real-time gene transcription analysis of Actinidia ESTs with proposed AAT-function (Crowhurst et al., 2008) were performed relative to the reference gene Ubiquitin conjugating protein 9 (UBC9) on a LightCycler® 480 platform using LightCycler® 480 SYBR Green master mix in a reaction volume of 5 μl. Two technical replicates of each biological replicate per sample were used and cDNA was replaced by water to test for false amplification. A standard dilution series with plasmid DNA was generated to evaluate the primer efficiencies of each primer, which were ≥ 1.9. The results were analysed using the LightCycler 480 software (Roche) and quantified according to Pfaffl (2001). Programme: 1 min at 95°C; 40 cycles of 10 s at 95°C, 10 s at 60°C, and 12 s at 72 °C; followed by melting curve analysis (95°C 5 s, 65°C 60 s then ramping at 0.18°C s⁻¹ to 95°C) to confirm that only one single PCR-product was formed. The following primer pairs (Table 3.1) were used for real-time RT-PCR:

Table 2.1: Primer sequences used for quantitative real-time RT-PCR analysis and their predicted product sizes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward (5’-3’)</th>
<th>Primer Reverse (3’-5’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBC9</td>
<td>CCATTTCAGGTGTTGCTT</td>
<td>TACTTGTTCCCGGTCCGTCTT</td>
<td>109 bp</td>
</tr>
<tr>
<td>AT1</td>
<td>TCCCTTATCCCATTCCAG</td>
<td>CGGCATCTCCATAAACACG</td>
<td>198 bp</td>
</tr>
<tr>
<td>AT2</td>
<td>CTCCCTCATACATCCGCCA</td>
<td>CGGCATCTCCATAAACACG</td>
<td>199 bp</td>
</tr>
<tr>
<td>AT15</td>
<td>ACAACAGCAACAGCATGACC</td>
<td>AGTGCAGCATCTCATAAAC</td>
<td>142 bp</td>
</tr>
<tr>
<td>AT16</td>
<td>CCATCACATTCCAGCACTAC</td>
<td>GTGTGGGTGAAAAATGTCCTTC</td>
<td>133 bp</td>
</tr>
<tr>
<td>AT17</td>
<td>TGGCAGAGGGAGTTTCTAGC</td>
<td>TGGGTTCAGATGCCATTTCT</td>
<td>160 bp</td>
</tr>
<tr>
<td>AT18</td>
<td>ACCCCGTCAAGTCACTCAGA</td>
<td>GCAATCCACCACAAGTTTCT</td>
<td>107 bp</td>
</tr>
</tbody>
</table>
3.2.5 (MeS)alkanoate ester production of cell free extract

All chemicals were purchased by Sigma-Aldrich New Zealand LTD unless stated otherwise.

3.2.5.1 (MeS)alkanoyl-CoA synthesis

2-MeSacetyl-CoA (99%) and 3-MeSpropionyl-CoA (>99.5%) were synthesised by reaction of the corresponding (MeS)alkanoyl anhydride (see 3.2.3.1) and Coenzyme A Lithium salt according to Goldman and Vagelos (1961). The crude product was purified by RP-HPLC after Pourfarzam and Bartlett (1991), and quantified using the hydroxamate assay, modified from Lipmann and Tuttle (1945).

3.2.5.2 Protein crude extraction

0.5 g of pulverised tissue was extracted with 1 mL 0.25 M potassium phosphate buffer (pH 7) containing Complete™ protease inhibitors (Roche Applied Science), 1% Triton and Polyvinylpolypyrrolidone (PVPP). The supernatant was desalted using a NAP-5 column (Illustra™) and assay buffer (0.25 M potassium phosphate buffer, pH 8, 10% glycerol, Complete™ protease inhibitors) following manufacturer’s instructions. Total protein concentrations were quantified according to Bradford (1976) using the Quick start™ Bradford protein assay (Bio-Rad) and a standard curve with bovine serum albumine (BSA, 99%). Two replicates were extracted per time-point.

3.2.5.3 AAT assay

10-mL glass vials with pierced Teflon liners were used to mix 600 μg of crude protein, assay buffer, 3 mM methanol, ethanol, propanol and butanol, 1 μM of each deuterated internal standard and 300 μM of each (MeS)alkanoyl-CoA in a reaction volume of 2 mL. After one hour at 33°C, a 50µm PDMS-DVB HS-SPME fibre (Supelco) was injected into the headspace of the sample and volatiles were adsorbed onto the fibre for 15 min. The same GC-MS-TOF equipment was utilised as described above but HS-SPME fibres were injected manually with an injector temperature of 220°C in splitless mode. A GC-oven programme of 90°C for 1 min, 5°C min⁻¹ to 200°C, and hold for 1 min was applied. The spectra were compared with those obtained from boiled and substrate-free protein extracts and only additional peak areas were considered for integration.
(MeS)alkanoate esters were identified according to the retention times and mass spectra of the reference compounds and quantified in equivalents of the corresponding deuterated internal standard.

3.3 Results and Discussion

3.3.1 MeS-volatiles accumulate during softening of eating-ripe ‘Hort16A’ kiwifruit

Methyl, ethyl, propyl, and butyl esters of 3-MeSpropionic acid, methyl and ethyl 2-MeSacetae, ethyl 3-MeSprop-2-enote, 2-MeS-ethanol, methionol and methional were quantified in A. chinensis ‘Hort16A’ using GC-MS-TOF after dynamic headspace sampling. None of these compounds was detected in unripe fruit after harvest at 38 $\times$ 10$^6$ Hz$^2$ g$^{2/3}$ and only methyl and ethyl 2-MeSacetae and methional (Fig. 3.1B and 3.1D) were present, at low concentrations (less than 0.5 $\mu$g kg$^{-1}$), before ‘Hort16A’ kiwifruit had softened to eating firmness. As shown in Fig. 3.1 A-D most MeS-volatiles just appeared to be detectable in eating-ripe fruit after the climacteric rise in ethylene production. In fact, all MeS-compounds increased with fruit softness, except for methyl 2-MeSacetae, which displayed highest levels (0.42 ± 0.01 $\mu$g kg$^{-1}$) in firm (8 $\times$ 10$^6$ Hz$^2$ g$^{2/3}$) eating-ripe fruit. Its levels dropped to under the detection limit during further ripening (Fig. 3.1B). Peak levels of MeS-volatiles were quantified in soft fruit (4 $\times$ 10$^6$ Hz$^2$ g$^{2/3}$) with ethyl 3-MeSpropionate being the main (MeS)alkanaeate ester (168 ± 6.7 $\mu$g kg$^{-1}$) and methional 429 ± 25.8 $\mu$g kg$^{-1}$) being the major MeS-compound in ‘Hort16A’ fruit pulp. Friel et al. (2007) reported that the levels of most fruit esters, especially ethyl esters, increased during ripening with high levels in soft ‘Hort16A’ fruit and this was also found to be the case for (MeS)alkanoate-esters in this study, indicating that these compounds are specific for eating-ripe A. chinensis ‘Hort16A’.
Fig. 3.1: Ethylene production and direct quantification of MeS-volatiles in non-stored ‘Hort16A’ with respect to fruit firmness during ripening. Error bars: Standard deviations of average values; MeS-volatiles: four replicates; ethylene: six biological replicates; me/et/pr/bu: methyl/ethyl/propyl/butyl; MeS: methylsulfanyl; ac: acetate; prop: propionate; propEN: prop-2-enoate; 2MeSOH: 2-MeS-ethanol; FW: fresh weight.

3.3.2 The effect of cold storage on MeS-volatile production

After one month at 1.5°C, the composition and concentration of all MeS-volatiles in eating-ripe ‘Hort16A’ fruit decreased dramatically (Table 3.2) and only methyl esters of 2-MeSacetic acid and 3-MeSpropionic acid could be detected in firm, eating-ripe fruit ($8 \times 10^6$ Hz$^2$ g$^{2/3}$), with levels corresponding to 10% and 20% of those present in non-stored fruit respectively. After two months of cold storage, methional was the only MeS-volatile detectable at this firmness and its levels remained constant until six months at 1.5°C. After this period two-fold increased methional levels ($12.8 \pm 2.2 \mu g \ kg^{-1}$) were observed compared with non stored fruits ($6.6 \pm 1 \mu g \ kg^{-1}$) at $8 \times 10^6$ Hz$^2$ g$^{2/3}$. Although, ethyl 3-MeSpropionate and ethyl 2-MeSacetate were not detected in firm eating-ripe ‘Hort16A’ fruit stored for 1–3 months (1.5°C) small amounts (0.19± 0.01 μg kg$^{-1}$ and 0.11± 0.002 μg kg$^{-1}$, Table 3.2) of these esters were identified after long-term cold storage.
Even though the composition of MeS-volatiles in very soft kiwifruit ($4 \times 10^6$ Hz$^2$ g$^{2/3}$) did not change (Table 3.2) after one month at 1.5°C their levels were significantly reduced for up to 95% (ethyl 3-MeSpropionate; 8.3 μg kg$^{-1}$), followed by a further gradual decline with storage time at 1.5°C. After four months of cold storage, only trace amounts of ethyl 2-MeSacetate and ethyl 3-MeSpropionate esters remained detectable, at levels less than 3% of those before storage. These results are in good agreement with those of Young and Patterson (1985), who observed that fruit volatiles—especially total esters—increased in *Actinidia deliciosa* ‘Hayward’ with fruit softness, but decreased with storage time at 0°C prior to ripening. Methional, however, was less affected by chilling (3–5 fold decrease in concentration), and remained present during the whole storage trial, even after methionol concentrations were below detection limit. In lactic acid bacteria methional can act as an intermediate for 3-MeSpropionic acid formation from methionine (Landaud et al., 2008; Pripis-Nicolau et al., 2004; Vallet et al., 2008) and a similar role may be possible in plants. In this situation, methional would act as a biosynthetic precursor for AAT substrates, namely 3-MeSpropionyl- and 2-MeSacetyl-CoA, after oxidation to 3-MeSpropionic acid.
Table 3.2: Methylsulfanyl-volatile concentrations (μg kg\(^{-1}\)) in ‘Hort16A’ kiwifruit at firmness 8 and 4 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \) before and after cold storage (1.5ºC).

<table>
<thead>
<tr>
<th></th>
<th>Methyl 2- (methyl sulfanyl) acetate</th>
<th>Ethyl 2- (methyl sulfanyl) acetate</th>
<th>Methional</th>
<th>Methyl 3- (methyl sulfanyl) propionate</th>
<th>2-(methyl sulfanyl) ethanol</th>
<th>Ethyl 3- (methyl sulfanyl) propionate</th>
<th>Propyl 3- (methyl sulfanyl) propionate</th>
<th>Methionol</th>
<th>Ethyl 3- (methyl sulfanyl) prop-2-enoate</th>
<th>Butyl 3- (methyl sulfanyl) propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C(μg kg(^{-1}))</td>
<td>SD</td>
<td>%</td>
<td>C(μg kg(^{-1}))</td>
<td>SD</td>
<td>%</td>
<td>C(μg kg(^{-1}))</td>
<td>SD</td>
<td>%</td>
<td>C(μg kg(^{-1}))</td>
</tr>
<tr>
<td>0m8</td>
<td>0.35</td>
<td>10</td>
<td>0.18</td>
<td>5</td>
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<td>15</td>
<td>0.23</td>
<td>12</td>
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<td>-</td>
</tr>
<tr>
<td>1m8</td>
<td>0.03</td>
<td>10</td>
<td>nd</td>
<td>-</td>
<td>5.4</td>
<td>9</td>
<td>0.04</td>
<td>5</td>
<td>nd</td>
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<td>nd</td>
<td>-</td>
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<td>nd</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>nd</td>
<td>-</td>
<td>2.7</td>
<td>14</td>
<td>nd</td>
<td>-</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>4m8</td>
<td>nd</td>
<td>-</td>
<td>0.07</td>
<td>2</td>
<td>4.2</td>
<td>7</td>
<td>nd</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>4c8</td>
<td>nd</td>
<td>-</td>
<td>nd</td>
<td>-</td>
<td>3.3</td>
<td>27</td>
<td>nd</td>
<td>-</td>
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**Odour threshold**
- 25 μg kg\(^{-1}\)
- 0.43 μg kg\(^{-1}\)
- 180 μg kg\(^{-1}\)
- 7 μg kg\(^{-1}\)
- 2500 μg kg\(^{-1}\)
- 246 μg kg\(^{-1}\)

SD%: Standard deviation of the average of four technical replicates given in %; 0m8…6m8: 0…6 months at 1.5ºC, ripened to 8 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \); 0m4…6m4: 0…6 months at 1.5ºC, ripened to 4 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \); 4c8: 4 months of softening to 8 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \) at 1.5ºC; 6c4: 6 months of softening to 4 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \) at 1.5ºC; 5et4: ethylene treatment after 5 months at 1.5ºC, ripened to 4 \( \times 10^6 \) Hz\(^2 \) g\(^{2/3} \); nd: not detectable

3.3.3 Ethylene production of ‘Hort16A’ kiwifruit is altered by cold storage

Interestingly, the pattern of ethylene production was also altered after cold storage (Fig. 3.2). For instance there was a 25-fold decrease of ethylene levels measured in firm, eating-ripe fruit ($8 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$) from the first to the second month of cold storage and no ethylene was detected in eating-ripe ‘Hort16A’ after four months at 1.5ºC. In non-stored fruit, a climacteric peak of ethylene production was observed within the eating-firmness range that appeared in parallel to MeS-volatile formation (3.1A), suggesting a link between this hormone and fruit ester biosynthesis. In non-stored fruit low levels ($0.023 \pm 0.008 \text{ ppm g}^{-1} \text{ h}^{-1}$) of ethylene were detectable before fruit had reached eating firmness ($12 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$) and ethylene levels further increased during softening to peak concentrations ($0.33 \pm 0.07 \text{ ppm g}^{-1} \text{ h}^{-1}$) at $5 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$, with a further decline towards the very soft end of the eating-firmness range ($4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$, $0.14 \pm 0.02 \text{ ppm g}^{-1} \text{ h}^{-1}$).

The depletion of ethylene production by ‘Hort16A’ after cold storage is unlikely to depend on the actual length of ripening time at 20ºC (data not shown); for example, after two months of chilling, fruit at $8$ and $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$ were both ripened for 13 days but soft, eating-ripe fruit produced 200-fold more ethylene than firm, eating-ripe fruit. After an additional two months at 1.5ºC, fruit were ripened for 11 days to $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$ but ethylene was not detectable at all. During the six-months storage trial, ethylene production was never observed for fruit that had been taken directly from cold storage without ripening, suggesting that the biosynthesis of this ripening hormone in ‘Hort16A’ is inhibited by chilling at 1.5ºC. Ritenour et al. (1999) discovered that ethylene preconditioning of A. deliciosa at 0ºC within the first two weeks of cold storage induced fruit softening but at a 2-fold slower rate than fruit which were ethylene treated at 20ºC prior to cold storage. Moreover, the softening pattern of green kiwifruit that were kept for at least three weeks at 0ºC prior to preconditioning was not altered in comparison with non-treated fruit. On the other hand, Antunes and Sfakiotakis (2002) stated that short-term chilling (0–10ºC) for 12 days of ‘Hayward’ kiwifruit actually enhanced the onset of ethylene production in parallel with its precursor and biosynthetic enzymes after re-warming. However, since the effects of mid and long-term storage were not investigated by these authors, the impact of prolonged chilling on ethylene biosynthesis in kiwifruit remains to be elucidated.

It would be interesting to investigate whether the lack of ethylene production in cold stored ‘Hort16A’ fruit may be associated with enhanced competition for S-adenosyl-methionine (SAM), a common precursor of the ethylene, polyamine and S-methyl-methionine biosynthetic pathway. These latter metabolites were shown to increase resistance to cold
stress in certain crops (Szego and Horvath, 2007; Cuevas et al., 2008; Groppa and Benavides, 2008).

3.3.4 Gene expression of putative AATs is regulated by ethylene in A. chinensis ‘Hort16A’

Gene transcription of MpAAT1, an AAT that has been previously reported to be of importance for ‘Royal Gala’ apple (Malus domestica) fruit flavour (Souleyre et al., 2005), was shown to be regulated by ethylene (Schaffer et al., 2007). Yahyaoui et al. (2002) reported that gene expression of CmAAT1 from melon (Cucumis melo) increased during ripening. Furthermore, transcript levels of this gene were reduced in fruit treated with 1-MCP and also in antisense ACO fruit. These findings indicate a regulatory role of ethylene for AAT gene transcription in climacteric fruit. Six Actinidia ESTs with putative flavour-related AAT function (AT15, AT18, AT2, AT17 AT1 and AT16) were shown to be phylogenetically related to those genes mentioned above and closely to each other (Crowhurst et al., 2008). Steady-state transcript levels of these contig sequences were measured in A. chinensis ‘Hort16A’ using quantitative real time RT-PCR. In unripe fruit (38 x 10^6 Hz^2 g^2/3), transcripts were not observed apart from low levels of AT16 (Fig. 3.3A). Transcript accumulation of further three sequences (AT2, AT17, AT1) were detectable before fruit had softened to eating firmness (15 x 10^6 Hz^2 g^2/3) for the first time and transcripts of AT15 and AT18 occurred only in eating-ripe fruit (8–4 x 10^6 Hz^2 g^2/3).
Therefore, AT2, AT17, AT1, AT15 and AT18 are suggested to be ripening-related and a role in fruit ester formation, including (MeS)alkanoate esters is implicated. After short-term cold storage for one month, expression of AT2, AT17 AT1 and AT16 was significantly reduced in fruit at $8 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$ (Fig. 3.3B), but transcripts of these contigs in soft, eating-ripe fruits ($4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$) displayed similar levels to those before storage, except for AT2, which appeared to be two-fold reduced. After five months at 1.5ºC, only transcripts of AT2 and AT16 with levels less than 1% and 50% compared to non stored fruit were detected in eating-ripe ‘Hort16A’ (8 and $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$). However, ethylene treatment of long-term stored fruit before ripening was led to increased transcripts of these six contigs to levels before storage or after one month at 1.5ºC. This suggests that transcription of these putative Actinidia AATs is regulated by ethylene.

![Fig. 3.3: Relative transcript levels of six putative Actinidia AATs relative to UBC9 in non-stored and stored ‘Hort16A’ kiwifruit. Error bars: Standard errors of the means of three biological with two technical replicates each. 0m38…4: no storage, 38…4 $\times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$; 1m4…5m4: 1 month and 5 months, ripened to $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$; 1m8…5m8: 1 month and 5 months, ripened to $8 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$; 5et4: 5 months at 1.5ºC, ethylene-treated and ripened to $4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$]

### 3.3.5 Ripening specific AATs produce (MeS)alkanoate esters in ‘Hort16A’ kiwifruit

The production of most MeS-volatiles present in non-stored ‘Hort16A’ fruit was restored after ethylene-treatment of long-term stored fruit (five months, 1.5ºC) but much lower levels were detected (Table 3.2). For example, in soft eating-ripe fruit ($4 \times 10^6 \text{ Hz}^2 \text{ g}^{2/3}$), volatile levels of ethyl 2-MeSacetate were recovered to 8%, methyl 3-MeSpropionate
to 3% and ethyl 3-MeSpropionate to 2% compared with no storage, or 100%, 10% and 40% after one month at 1.5ºC respectively. Therefore, it was of interest to investigate whether this was caused by reduced enzyme expression or substrate depletion. We hypothesised that 2-MeSacetate and 3-MeSpropionate esters were formed by AATs using straight chain alcohols and the corresponding (MeS)alkanoyl-CoA. To test this hypothesis, these substrates were added to cell-free protein extracts. Ethyl, propyl, butyl 2-MeSacetates and 3-MeSpropionates were produced by crude extracts from non stored eating-ripe fruit and after one month at 1.5ºC (Fig. 3.4), indicating that (MeS)alkanoate esters in ‘Hort16A’ are produced by AATs. In addition, there was no (MeS)alkanoate ester production in cell-free extracts from unripe fruit (38 x 10^6 Hz^2 g^2/3) and after five months of cold storage (4 x 10^6 Hz^2 g^2/3), pointing towards AAT depletion. However, protein extracts from ethylene-treated eating-ripe fruit (4 x 10^6 Hz^2 g^2/3) produced (MeS)alkanoate esters after five months at 1.5ºC at similar levels to cell-free extracts of ‘Hort16A’ fruit after one month of cold storage. This finding correlated well with changes in steady-state transcript levels but not with volatile levels of fresh fruit, thus confirming recovery of AAT-enzyme levels and suggesting reduced availability of (MeS)alkanoyl-CoAs or alcohols in ethylene-treated kiwifruit after five months of chilling.

Fig. 3.4: (Methylsulfanyl)alkanoate ester production of cell-free protein extracts from ‘Hort16A’ kiwifruit. Error bars: Standard deviations of the average of two biological replicates. 0m38: no storage, 38 x 10^6 Hz^2 g^2/3; 0m4: no storage, 4 x 10^6 Hz^2 g^2/3; 1m4…5m4: 1 and 5 months at 1.5ºC, 4 x 10^6 Hz^2 g^2/3, 5et4: 5 months at 1.5ºC, ethylene-treated and ripened to 4 x 10^6 Hz^2 g^2/3.
3.3.6 The potential impact of MeS-volatiles on ‘Hort16A’ flavour

In this study, MeS-volatiles occurred in trace amounts in ‘Hort16A’ fruit pulp. Literature values of odour thresholds for most MeS-volatiles in water or synthetic wine (methionol) are listed in Table 3.2 and it is obvious that methional is the only MeS-compound that exceeds its odour threshold in firm and soft eating-ripe fruit after cold storage for at least six months. This mashed potato or soup-like odorant was reported to be aroma-active in non-processed pink guava (Steinhaus et al., 2008), lychee (Mahattanatawee et al., 2007) and one blackberry cultivar (Klesk and Qian, 2003). However, since methional levels are also above odour threshold in unripe fruit ($33 \times 10^6$ Hz$^2$ g$^{2/3}$), its impact on the characteristic flavour of ripe fruit is questionable. In wine, high OAVs of methional are associated with off-flavours and mainly caused by chemical oxidation of methionol (Escudero et al., 2000). In this study, headspace volatiles were trapped with air for 20 hours and oxidation products as artefacts can therefore not be excluded.

Ethyl 3-MeSpropionate also exceeded its odour thresholds but only in non-stored, soft, ($4 \times 10^6$ Hz$^2$ g$^{2/3}$) kiwifruit (OAV=24) and after one month at 1.5°C (OAV=1). The concentrations of this tropical, sweet odorant stayed well below its odour threshold after further storage at 1.5°C. However, OAVs that are generally estimated from odour thresholds in a simplified matrix are an indicative means for the aroma impact of an individual compound. For a better understanding of their impact on the overall aroma, matrix influence (Marsh et al., 2006) and the interaction between volatile compounds (Escudero et al., 2007) need to be considered. Furthermore, Escudero et al. (2004) demonstrated a key role of low OAV compounds on wine aroma and more recent research showed that volatiles of the same chemical family, which occurred below odour threshold, produced a quite specific odour impression probably due to additive effects (Pineau et al., 2009). These findings make the use of OAVs as critical parameter even more controversial. Whether MeS-volatiles contribute to the tropical flavour of A. chinensis ‘Hort16A’ kiwifruit will therefore need to be investigated using a systematic GC-O approach and aroma reconstitution studies. However, since commercial ‘Hort16A’ kiwifruit is often stored for up to nine months, a contribution of (MeS)alkanoate esters on the aroma of long-term stored fruits seems very unlikely, since the levels of these compounds markedly decreased below the detection limit with storage time. Therefore, it would be of interest to investigate the impact of cold storage on the general flavour perception of
‘Hort16A’. After all, total ester production is likely to be influenced by decreased AAT expression and enzyme activity.

3.4.  Concluding remarks

It is concluded that most MeS-volatiles specifically occur in eating-ripe A. chinensis ‘Hort16A’ and increase during softening. Cold storage in general leads to a marked decrease of (MeS)alkanoate esters and transcript levels of putative AATs, which can be partially restored by ethylene treatment. This finding was confirmed by (MeS)alkanoate ester production of cell-free extracts with added (MeS)alkanoyl-CoAs and alcohols. Therefore, we suggest that (MeS)alkanoate ester production in ‘Hort16A’ fruit is likely to depend primarily on gene expression of ripening specific AATs, which are likely to be regulated by ethylene and inhibited by prolonged cold storage. On the basis of estimated OAVs ethyl 3-MeSpropionate appears to be odour active in soft, non-stored fruit. If cumulative or synergistic effects of (MeS)alkanoate esters contribute to characteristic aroma notes of non or short-term stored ‘Hort16A’ will have to be investigated. Finally, the changes in MeS-volatile levels with ripening stage and storage time also demonstrate the impact of postharvest treatment on fruit volatiles and potential flavour abundance.
CHAPTER 4

ETHYLENE-REGULATED (METHYLSULFANYL)ALKANOATE ESTER BIOSYNTHESIS IN ACTINIDIA CHINENSIS GENOTYPES

4

4.1 Introduction

Kiwifruit are one of the most recently domesticated temperate fruit crops developed by traditional breeding methods. An example is the yellow-fleshed cultivar Actinidia chinensis Planch. var. chinensis ‘Hort16A’ (marketed as ZESPRI®GOLD Kiwifruit) which is well liked by consumers for its sweet taste and tropical fruit flavour (Ferguson, 1999). The volatile profile of eating-ripe ‘Hort16A’ is dominated by ethyl esters (Friel et al., 2007) and ethyl 3-MeSpropionate has been suggested to contribute to the tropical aroma of non-stored kiwifruit (Günther et al., 2010). Little is known about the biosynthetic pathway leading to (MeS)alkanoate ester formation in plants but studies on microorganisms suggest their derivation from methionine (Vallet et al., 2008). The final step of general fruit ester biosynthesis is catalysed by AATs (EC 2.3.1.x), which therefore have a key role in fruit flavour formation. In climacteric fruit such as apple and melon, ethylene-controlled regulation of flavour-related AATs was proposed based on studies using antisense ACO transformed plants (Defilippi et al., 2005; Flores et al., 2002). Despite a clear reduction of volatile esters from the headspace of ACO-suppressed fruit, AAT activity remained high. This suggests the action of additional, ethylene-independent AATs and emphasises a potential impact of ethylene on AAT-substrate formation in apple and melon (Flores et al., 2002, Defilippi et al., 2005). Substrate availability as the primary control for ester biosynthesis was supported by studies on ester synthesis in controlled atmosphere (CA) stored apples using deuterium-labelled substrates (Matich and Rowan, 2007) and by biochemical analysis of precursor-forming enzymes (Lara et al., 2007).

Thus, the impact of ethylene on volatile ester production in climacteric fruit is well accepted but the limiting steps of this pathway are yet to be established.

Kiwifruit may not behave in all aspects like a classic climacteric fruit (Pratt and Reid, 1974), but they are very responsive to external ethylene, which accelerates fruit ripening. However, the impact of ethylene on ripening-specific changes in secondary metabolism

4 This chapter is based on the manuscript Günther et al., Journal of Plant Physiology, 2011, 168 (7), 629-638
such as fruit volatile production is not yet well understood. In this chapter, the investigation of (MeS)alkanoate ester profiles and their biosynthesis in response to ethylene using selected genotypes from an *Actinidia chinensis* mapping population (Mp) that is closely related to ‘Hort16A’ is described. Furthermore, we are testing whether differences in (MeS)alkanoate ester production in these fruit are determined by genotype specific AAT enzyme activity and gene expression of putative AATs.

### 4.2 Materials and Methods

#### 4.2.1 Plant materials

##### 4.2.1.1 Ethylene-treated fruit.

Kiwifruit of five different *Actinidia chinensis* genotypes (‘Hort16A’, ‘Mp060’, ‘Mp104’, ‘Mp145’, ‘Mp201’) were harvested in April 2008 from the Plant & Food Research orchard in Te Puke, New Zealand. Those genotypes are members of a mapping population (Fraser et al., 2004) and they were selected because of their diversity in MeS-volatiles as determined in a preliminary study (not published), described in Chapter 1. ‘Hort16A’ is a sibling of the male parent of this mapping population. Ten mature but unripe, firm fruit (approximately 58.8 N average fruit firmness) from each genotype were picked from different levels in the vine canopy and divided into two samples of five fruit. The fruit flesh of one sample was cut into small pieces and was flash frozen with liquid nitrogen while the whole fruit of the other sample were ethylene treated (100 ppm, 20°C) for 24 h and ripened for 5–7 d until fruit ethylene production was detected before freezing as described above.

##### 4.2.1.2 Vine-ripened fruit.

In order to investigate MeS-volatiles produced after natural ripening, ten vine ripened, soft fruit of each *A. chinensis* line (‘Mp135’, ‘Mp248’, ‘Mp270’, ‘Mp282’, ‘Mp288’, ‘Mp098’, ‘Mp228’; selected based on unpublished data from a preliminary study in 2002) were harvested in July 2008 and split into two samples of five fruit. Ethylene production of each fruit was measured before snap freezing the peeled fruit cubes of one sample. Interestingly, ethylene was not detected from the headspace of these fruit directly after harvest and they were therefore ripened at 20°C for one week until fruit ethylene production was detected. All samples were pulverised while frozen to a fine powder with a
liquid N\textsubscript{2}-cooled stone crusher (Rocklabs, New Zealand) and stored at -80\degree C for further analysis.

4.2.2 Fruit ethylene detection

Ethylene production of five individual fruit per time-point and genotype was measured according to 3.2.2.

4.2.3 Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich Ltd. The internal standards (ethyl 3-[D3]MeSpropionate, [D5]ethyl 3-MeSacetate) and reference compounds (propyl and butyl 3-MeSpropionate, propyl and butyl 2-MeSacetate) were synthesised as described earlier (Section 2.2.2) and AAT substrates (MeSacetyl-CoA, MeSpropionyl-CoA) were made according to Section 3.2.5.1.

[D5]ethyl butyrate was synthesised by stirring [D6]ethanol (0.82 mL, 12.5 mmol, 99% D) with butyric anhydride (2.0 mL, 12.2 mmol, Janssen Chimica, 99%) and \( p \)-toluenesulphonic acid (5 mg) for 16 h at room temperature. Et\textsubscript{2}O (20 mL) was added, and the reaction quenched with 5 mL saturated NaHCO\textsubscript{3} (aq.). The organic phase was separated and the aqueous phase extracted with 2 \( \times \) 20 mL Et\textsubscript{2}O. The organic phases were combined and dried using MgSO\textsubscript{4}. The solvent was removed and the product purified (> 99%) by short path distillation (13 mm Hg, Kugelrohr). El–MS, \( m/z \) (relative intensities in %): 71 (100), 43 (100), 34 (79), 93 (65), 41 (36), 61 (28), 74 (28), 42 (24), 39 (15), 106 (10), 44 (10), 63 (9), 121 (4).

4.2.4 Quantification of MeS-volatiles from the headspace of fruit tissue

A dynamic headspace sampling approach was applied to concentrate MeS-volatiles from fruit samples analogous to Section 3.2.3.2 using the same GC-MS settings as described in Section 2.2.4. Direct quantification of headspace volatiles was undertaken according to 2.2.5.
4.2.5 Development of an automated HS-SPME based assay for AAT-enzyme kinetic measurements

4.2.5.1 Optimising HS-SPME sampling parameters

Reference samples containing 1 μM of each ester (ethyl 2-MeSacetate, ethyl 3-MeSpropionate, ethyl butyrate), internal standard ([D5]ethyl butyrate, ethyl 3-[D3]MeSpropionate) and 1 mM ethanol in 0.1 M sodium phosphate buffer (pH 8, total volume 1 mL) were tested with and without the addition of 0.9% SDS (w/v) to evaluate if this ionic detergent affects headspace concentrations of (MeS)alkanoate esters. These solutions were kept at 2ºC prior to HS-SPME using an autosampler and cooling rack (Gerstel MPS, Germany). To optimise HS-SPME, duplicate samples were equilibrated for 5 min at different temperatures (30ºC, 40ºC, 50ºC, 60ºC) then headspace volatiles extracted at the equilibration temperature for 10 min using a PDMS-DVB coated fibre (65 μm, 23-gauge, Supelco, Bellefonte, USA). In a second survey experiment, fibre exposure times (2 min, 5 min, 10 min) were varied to optimise for this parameter and 5-min equilibration and sampling times at 40ºC were considered as best parameters. Absolute peak areas of the main ion were reduced by approximately 50% after SDS-addition. Nevertheless, a strong linear relationship ($R^2 \geq 0.99$) was acquired using a standard dilution series (0.5–10 μM), corrected against deuterated internal standards in SDS-containing sodium phosphate buffer.

4.2.5.2 GC-MS settings for HS-SPME

HS-SPME fibres were automatically (Gerstel MPS, Germany) injected into the injection port (240ºC). A 1:2 split was applied for 4 min. The GC-program for the analysis of ethyl butyrate was 40ºC for 2 min, 5ºC min$^{-1}$ to 100ºC, 20ºC min$^{-1}$ to 200ºC and held for 2 min. The GC-programme for the analysis of ethyl 2-MeSacetate and ethyl 3-MeSpropionate was 85ºC for 2 min, 5ºC min$^{-1}$ to 150ºC, held 1 min, 10ºC min$^{-1}$ to 200ºC, and held for 2 min. The transfer line temperature was kept at 280ºC. The GC was coupled to a TOF-MS (Leco Pegasus III, St. Joseph, MI, USA) with an ion source temperature of 230ºC. Ion spectra from 26 to 350 amu were collected with a data acquisition rate of 20 Hz. The total ion chromatograms were processed using the LECO chromaTOF software.

4.2.5.3 Enzyme assay

Three individual protein extractions per sample were prepared as described earlier (Section 3.2.5.2). AAT-activity of each genotype was tested in a reaction volume of 1 mL,
by mixing 250 μL of total protein extract (~200 μg protein) on ice with assay buffer (0.1 M sodium phosphate buffer, pH 8, Complete™ protease inhibitor tablet, Roche), 1 mM ethanol, 1 μM of each internal standard ([D5]ethyl butyrate, ethyl 3-[D3]MeSpropionate) with 100 μM of 2-MeSacetyl-CoA or 3-MeSpropionyl-CoA to start the reaction. Samples were incubated at 30ºC for 1 h and kept on ice for 5 min before adding 100 of 10% SDS (w/v) solution, which had been confirmed to stop the enzyme reaction. The samples were immediately vortexed for 30 s, frozen in liquid nitrogen and stored at -20ºC prior to GC-MS analysis.

The pH response of enzyme activity was investigated using butyryl-CoA and ethanol in 0.1 M NaH2PO4/NaH(PO4)2 buffer (pH 2, 4, 6, 8, 10, 12). The pH-optimum was maximal from pH 6 to pH 8 and pH 8 was chosen for further analysis. The reaction time was set for 1 h after ensuring a linear increase of the ester production rate from 30 min to 120 min at 30ºC. The effect of different ethanol concentrations (0.5–5 mM) using 200 μM 2-MeSacetyl-CoA was also determined and there was no increase in reaction rate found using concentrations higher than 1 mM, while concentrations higher than 2.5 mM resulted in a decrease in relative peak area. Boiled extracts (15 min, 95ºC) and those containing only one or no co-substrate produced no product.

4.2.5.4 Enzyme kinetic studies

In most cases, we were unable to saturate the enzyme reaction rate with CoA-substrate, owing to limited availability of the (MeS)alkanoyl-CoAs. Thus we calculated the apparent catalytic efficiency (ACE) of AAT enzymes from cell-free extracts as the initial slope of the enzyme reaction rate under limiting 2-MeSacetyl-CoA (20–180 μM) and 3-MeSpropionyl-CoA (30–240 μM) conditions with 1 mM ethanol. An attempt was made to fit the data using the Michaelis-Menten equation (Equation 1; Origin 7.5 SR4, Originlab Corporation) but this often resulted in large errors for the mathematical $K_M$ and apparent $V'_{\text{Max}}$ ($V'_{\text{Max}}$) values, where $V'_{\text{max}}$ is the apparent maximal velocity in a crude extract (nkat mg total protein$^{-1}$) because of the essentially linear substrate response curves. Therefore, the $V'_{\text{Max}}$, $K_M^{-1}$-value was regarded as the ACE and calculated either using a non-linear fit of the standard Michaelis-Menten equation (Equation 1) when significant curvature in the $v$ versus $[S]$ response curve occurred or from the linear regression of the response of enzyme activity to substrate concentration as shown in Equations 1 and 2. This approach is analogous to the concept of $k_{\text{cat}}$ $K_M^{-1}$ ($V'_{\text{max}} = k_{\text{cat}} [E]$; where [E] is known). Thus ACE is a function of both kinetic parameters ($V'_{\text{Max}}, K_M$) and reflects the enzyme reaction rates
of a total protein extract towards a particular substrate at limiting substrate concentration. This approach appears appropriate to us in order to compare the preference of a protein extract towards different substrates in a typical in vivo situation, where different isozymes, that transform the same substrate, can be present at the same time and the substrate supply is limited ([S]« KM)

Equation 1: \( v = \frac{(V'_{\text{Max}}/K_M) \times [S]}{([S]/K_M + 1)} \)

At low substrate concentrations ([S] « KM)

Equation 2: \( v = \frac{V'_{\text{Max}}}{K_M} \times [S] = \text{ACE} \times [S] \)

where [S] is the substrate concentration in μM and V'_{\text{Max}} is the enzyme activity in nkat mg protein\(^{-1}\).

### 4.2.6 Quantitative real-time RT-PCR analysis

Four individual RNA extractions were made (Lopez-Gomez and Gomez-Lim, 1992) using 400 mg of tissue from each genotype and the RNA was quantified using a NanoDrop™ (Thermo scientific) spectrophotometer. After combining the individual extractions, 4 μg RNA per sample was treated with DNase I amplification grade (Invitrogen). RNA (1 μg) was separated on a 1% agarose gel (UltraPure™, Invitrogen) to confirm the integrity of the RNA. The remaining sample was split into three aliquots and first-strand cDNA was synthesised from two RNA replicates (~1 μg each) per genotype using the SuperScript™ VILO cDNA synthesis-kit (Invitrogen) according to the manufacturer’s instructions. The same procedure without addition of RT was applied for the third replicate to test for gDNA contamination. Because the housekeeping gene (UBC9) was expressed stably during ripening cDNA samples were diluted 32-fold to standardise for equal amplification cycles (C\(p=23\pm1\)) of UBC9. qRT-PCR was performed with three technical replicates per template (\(n = 6\) total replicates) according to Section 3.2.4 (see Table 3.1. for primer pairs and product size).

### 4.3 Results

#### 4.3.1 MeS-volatile levels differ strongly between A. chinensis genotypes

genotypes is listed in Table 4.1. The aldehyde methional was the only MeS-compound present in all samples, independent of the treatment or time point taken. However, its concentration increased 1.4 (‘Mp104’) to 15.6-fold (‘Mp201’) in fruit samples that produced detectable amounts of ethylene compared with those that did not. In “ethylene-treated” lines other MeS-volatiles were exclusively found in ethylene-producing samples. A similar response to ethylene was also observed for most “vine-ripened” genotypes but some of these A. chinensis lines produced small amounts of the main (MeS)alkanoate esters ethyl 2-MeSacetate (‘Mp098’, ‘Mp228’, ‘Mp270’), methyl 3-MeSpropionate (‘Mp098’) and/or ethyl 3-MeSpropionate (‘Mp098’, ‘Mp228’, ‘Mp282’) directly after harvest. However, (MeS)alkanoate ester levels increased 5–100 fold in these lines after the ethylene production rate had exceeded 0.1 ppm g⁻¹ h⁻¹.

In general, the spectrum of MeS-compounds produced by the A. chinensis genotypes was similar but their headspace amounts varied remarkably at similar ethylene production rates. For example, ethyl 3-MeSpropionate was the main (MeS)alkanoate ester in all lines, tested in this study, but its levels varied from 2.4 ± 0.15 µg kg⁻¹ (‘Mp104’) to 205 ± 4 µg kg⁻¹ (‘Mp201’) for “ethylene-treated” fruit (not detected in unripe fruit) and 0.9 ± 0.05 µg kg⁻¹ (‘Mp135’) to 184 ± 4 µg kg⁻¹ for “vine-ripened”, ethylene-producing fruit. The second major (MeS)alkanoate ester was ethyl 2-MeSacetate for the “ethylene-treated” lines (except methyl 3-MeSpropionate for ‘Mp060’) and methyl 3-MeSpropionate for the ‘vine-ripened’ genotypes (except butyl 3-MeSpropionate for ‘Mp282’). The highest diversity between genotypes was observed for MeS-alcohol synthesis; ‘Hort16A’ and ‘Mp201’ were the only A. chinensis lines with detectable amounts of 2-MeSethanol whereas methional was found in all ethylene-producing genotypes except for ‘Mp104’ and ‘Mp135’, which appeared to be deficient of MeS-alcohols. In summary, genotypic differences in MeS-volatile profiles primarily affected the quantities of the individual compounds, with minor genotype effects on the range of individual components. We therefore investigated whether the levels of (MeS)alkanoate esters were determined by genotype-specific differences in AAT-enzyme activity and gene expression.
Table 4.1: *Fruit ethylene and methylsulfanyl-volatile production of different Actinidia chinensis genotypes* (for caption see next page)

<table>
<thead>
<tr>
<th></th>
<th>Ethylene ppm g⁻¹ h⁻¹ (%RSD)</th>
<th>Methyl 2-(methylsulfanyl) acetate µg kg⁻¹ (%RSD)</th>
<th>Ethyl 2-(methylsulfanyl) acetate µg kg⁻¹ (%RSD)</th>
<th>Methyl 3-(methylsulfanyl) propionate µg kg⁻¹ (%RSD)</th>
<th>Ethyl 3-(methylsulfanyl) propionate µg kg⁻¹ (%RSD)</th>
<th>Propyl 3-(methylsulfanyl) propionate µg kg⁻¹ (%RSD)</th>
<th>Butyl 3-(methylsulfanyl) prop-2-enoate µg kg⁻¹ (%RSD)</th>
<th>Methyl 3-(methylsulfanyl) prop-2-enoate µg kg⁻¹ (%RSD)</th>
<th>Ethyl 3-(methylsulfanyl) prop-2-enoate µg kg⁻¹ (%RSD)</th>
<th>Methional µg kg⁻¹ (%RSD)</th>
<th>Methionol µg kg⁻¹ (%RSD)</th>
<th>2-(methylsulfanyl) ethanol µg kg⁻¹ (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hort 16A</strong></td>
<td>nd</td>
<td>0.3 (31)</td>
<td>nd</td>
<td>0.11 (7.6)</td>
<td>nd</td>
<td>2.3 (4.2)</td>
<td>nd</td>
<td>0.41 (7.9)</td>
<td>nd</td>
<td>0.07 (6)</td>
<td>0.08 (4.1)</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 060</strong></td>
<td>nd</td>
<td>0.26 (55)</td>
<td>nd</td>
<td>0.23 (0.7)</td>
<td>nd</td>
<td>0.8 (4)</td>
<td>nd</td>
<td>6.6 (1.4)</td>
<td>nd</td>
<td>0.13 (14)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 104</strong></td>
<td>nd</td>
<td>0.17 (59)</td>
<td>nd</td>
<td>0.77 (4)</td>
<td>nd</td>
<td>0.33 (10)</td>
<td>nd</td>
<td>2.5 (12)</td>
<td>nd</td>
<td>1.7 (7.3)</td>
<td>1.4 (13)</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 145</strong></td>
<td>nd</td>
<td>0.43 (29)</td>
<td>nd</td>
<td>0.88 (2.8)</td>
<td>nd</td>
<td>0.7 (11)</td>
<td>nd</td>
<td>15.1 (3)</td>
<td>nd</td>
<td>0.07 (8.4)</td>
<td>0.53 (6.5)</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 201</strong></td>
<td>0.59 (72)</td>
<td>0.46 (8.4)</td>
<td>nd</td>
<td>9 (3.5)</td>
<td>nd</td>
<td>1.3 (6.8)</td>
<td>nd</td>
<td>205 (3.8)</td>
<td>nd</td>
<td>4 (8.4)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Vine-ripened/ overripe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MP 098</strong></td>
<td>nd</td>
<td>0.14 (61)</td>
<td>nd</td>
<td>0.01 (16)</td>
<td>nd</td>
<td>0.16 (1.5)</td>
<td>nd</td>
<td>0.31 (6.3)</td>
<td>nd</td>
<td>3.8 (9.6)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 135</strong></td>
<td>nd</td>
<td>0.32 (85)</td>
<td>nd</td>
<td>0.02 (13)</td>
<td>nd</td>
<td>0.15 (1.3)</td>
<td>nd</td>
<td>0.23 (7.6)</td>
<td>nd</td>
<td>0.9 (10)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 228</strong></td>
<td>0.0001 (230)</td>
<td>0.19 (41)</td>
<td>0.16 (7.4)</td>
<td>0.26 (5.6)</td>
<td>nd</td>
<td>3.5 (20)</td>
<td>nd</td>
<td>30.9 (14)</td>
<td>nd</td>
<td>0.04 (12)</td>
<td>5.8 (7.2)</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 248</strong></td>
<td>nd</td>
<td>0.004 (111)</td>
<td>nd</td>
<td>0.05 (4.1)</td>
<td>nd</td>
<td>0.12 (3.9)</td>
<td>nd</td>
<td>0.54 (4)</td>
<td>nd</td>
<td>1.9 (3.9)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 270</strong></td>
<td>nd</td>
<td>0.15 (45)</td>
<td>nd</td>
<td>0.02 (12)</td>
<td>nd</td>
<td>0.14 (2.4)</td>
<td>nd</td>
<td>2.7 (1.6)</td>
<td>nd</td>
<td>19.9 (2)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 282</strong></td>
<td>0.11 (72)</td>
<td>0.38 (1.7)</td>
<td>nd</td>
<td>1.6 (2.1)</td>
<td>nd</td>
<td>0.15 (28)</td>
<td>nd</td>
<td>142.5 (3.2)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>MP 288</strong></td>
<td>nd</td>
<td>0.23 (52)</td>
<td>nd</td>
<td>0.03 (14)</td>
<td>nd</td>
<td>0.34 (4.3)</td>
<td>nd</td>
<td>6.7 (6.4)</td>
<td>nd</td>
<td>184.1 (4.3)</td>
<td>0.1 (2.4)</td>
<td>nd</td>
</tr>
</tbody>
</table>
4.3.2 AAT-enzyme activity is exclusive to ethylene-producing kiwifruit

In a survey of the accessions from Table 4.1, aiming to select those *A. chinensis* genotypes with the highest biochemical potential for (MeS)alkanoate ester production, AAT activity was measured using cell-free extracts. Surprisingly, ethyl (MeS)alkanoate ester and ethyl butyrate synthesis were only observed using protein extracts prepared from ethylene-producing fruit samples and was not quantifiably detected from fruit extracts that did not produce any ethylene (Fig. S4.1). It cannot be excluded that *A. chinensis* AATs prefer substrates that were not tested in this study but since ethyl butyrate is a major ester of these eating-ripe fruit (peak saturation in current study, results from unpublished study in 2002), high AAT-production rates for this ester would be expected. Therefore, only the AAT activity from ethylene-producing protein extracts was measured for each genotype. It is shown in Fig. 4.1 that the specific activity for the synthesis of ethyl butyrate (Fig. 4.1E, F) was from about 3-fold ('Mp145') to 18-fold ('Mp288') higher than ethyl 2-MeSacetate (Fig. 4.1A, B) and 4-fold ('Mp145') to 26-fold ('Mp288') higher than ethyl 3-MeSpropionate formation (Fig. 4.1B, C), confirming the higher preference for butyryl-CoA with ethanol. In contrast, ethyl 2-MeSacetate and ethyl 3-MeSpropionate were synthesised at similar rates by most genotypes even though fruit volatile concentrations of the latter compound were 10- to 100-fold higher than ethyl 2-MeSacetate levels. The final activity for ethyl ester production was significantly (Tukey’s HSD$_{0.05}$) higher for ‘Hort16A’, ‘Mp145’ (except for ethyl butyrate), ‘Mp201’, ‘Mp228’ and ‘Mp270’ (Fig. 4.1) than for the other genotypes, and so we selected those lines for further biochemical and gene expression studies.
Fig. 4.1: Alcohol acyltransferase activity of cell-free extracts from different ethylene-producing "ethylene-treated" and "vine-ripened" Actinidia chinensis genotypes. A, B Ethyl 2-((methylsulfanyl)acetae, C, D ethyl 3-(methylsulfanyl)propionate; E, F ethyl butyrate. Error bars represent the standard error of the mean of three replicates. Letters (a, b) indicate significant differences in ester production (Tukey’s HSD_0.05).

4.3.3 Enzyme kinetic studies suggest a rate-limiting role of (MeS)alkanoyl-CoA levels on (MeS)alkanoate ester production

Enzyme kinetic studies were performed using cell-free extracts, reflecting the native, physiological situation in regards to the isozyme pattern of total AAT activity. Fig. 4.2 demonstrates the substrate response curves of (MeS)alkanoyl-CoAs at the subsaturating substrate concentrations able to be applied in this study and Table 4.2 provides the corresponding calculated \( V'_{\text{Max}} K_{\text{M}}^{-1} \)-values, representing the initial slope of the substrate response curve regarded as ACE. The ACE is a function of both the apparent maximum velocity \( V'_{\text{Max}} \) that is directly dependent on the amount of actual enzyme present and the \( K_{\text{M}} \), which is an estimate of the total affinity of all isozymes, using the same substrates. Therefore, the ACE \( (V'_{\text{Max}} K_{\text{M}}^{-1}) \) for 2-MeSacetyl-CoA and 3-MeSpropionyl-CoA with ethanol as alcohol substrate was compared for each of the selected A. chinensis genotypes (‘Hort16A’, ‘Mp145’, ‘Mp201’, ‘Mp228’ and ‘Mp270’). As shown in Table 4.2, cell-free extracts of most lines synthesised ethyl 2-MeSacetate and ethyl 3-MeSpropionate, with a similar ACE from the corresponding CoA-substrate except
for ‘Hort16A’ and ‘Mp270’, which appeared almost 3-fold and 10-fold more selective
towards ethyl 2-MeSacetyl-CoA, respectively. This preference, however, was not reflected
in actual fruit volatile concentrations: Ethyl 3-MeSpropionate levels in fruit were 10-fold
(‘Hort16A’) to 142-fold (‘Mp270’) higher than ethyl 2-MeSacetate concentrations. The
Pearson’s correlation was calculated for the levels of volatile esters measured from
kiwifruit with the corresponding ACE of each (MeS)alkanoyl-CoA with ethanol (illustrated
in Fig.S4.2). The correlation was neither significant for ethyl 2-MeSacetate versus the
ACE of 2-MeSacetyl-CoA (R= -0.33, p=0.59) nor for ethyl 3-MeSpropionate versus the
ACE of 3-MePropionyl-CoA (R=0.56, p=0.33), thus suggesting that final (MeS)alkanoate
ester levels in *A. chinensis* are likely to be controlled by the availability of (MeS)alkanoyl-
CoAs.

*Fig. 4.2:* Alcohol acyltransferase activity of total protein extracts from *Actinidia chinensis* genotypes in
response to increased (methylsulfanyl)alkanoyl-CoA substrate concentrations. The continuous curve is the fit
to the data using Michaelis-Menten enzyme kinetics (Equation 1, Section 4.2.5.4), with the exceptions of
‘Hort16A’ and ‘Mp201’ for ethyl 2-(methylsulfanyl)acetyl-CoA which were fitted using linear regression. The
top row of graphs shows the formation of ethyl 2-(methylsulfanyl)acetate using 2-(methylsulfanyl)acetyl-CoA
and ethanol; the bottom row of graphs shows ethyl 3-(methylsulfanyl)propionate production using 3-
(methylsulfanyl)propionyl-CoA and ethanol. The error bars represent the standard deviation of the average of
three replicates.
Table 4.2: The apparent catalytic efficiency (V’Max KM⁻¹) of alcohol acyltransferases from Actinidia chinensis total protein extracts.

<table>
<thead>
<tr>
<th></th>
<th>2-(methylsulfonyl)acetyl-CoA</th>
<th>3-(methylsulfonyl)propionyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V’Max KM⁻¹ (nkat mg⁻¹µM⁻¹)</td>
<td>V’Max KM⁻¹ (nkat mg⁻¹µM⁻¹)</td>
</tr>
<tr>
<td>Hort16A</td>
<td>16.8 ± 0.9LR</td>
<td>6.4 ± 0.3LR</td>
</tr>
<tr>
<td>MP145</td>
<td>23.2 ± 0.8LR</td>
<td>26.2 ± 1.6NL</td>
</tr>
<tr>
<td>MP201</td>
<td>26.5 ± 0.3LR</td>
<td>26.4 ± 6.4NL</td>
</tr>
<tr>
<td>MP228</td>
<td>23.6 ± 0.8LR</td>
<td>14.1 ± 0.6LR</td>
</tr>
<tr>
<td>MP270</td>
<td>114 ± 32NL</td>
<td>11.1 ± 0.5LR</td>
</tr>
</tbody>
</table>

The first three genotypes were “ethylene-treated” and the last two genotypes were “vine-ripened”. LR: fitted using linear regression; NL: fitted using non-linear regression analysis (Equation 1, Section 4.2.5.4).

4.3.4 Multiple AAT isoforms are likely to be involved in (MeS)alkanoate ester formation

4.3.4.1 The role of ethylene on AAT-transcript accumulation

Relative transcript levels of six phylogenetically closely related Actinidia-derived ESTs with proposed flavour-related AAT function (Crowhurst et al., 2008) were measured using qRT-PCR-analysis. We observed that the expression of these putative AAT-contigs was restricted to ethylene-producing genotypes (Fig. 4.3) except for minor transcript levels detected from ‘Mp145’ and ‘Mp228’ directly after harvest. Minute levels of ethylene were detected from ‘Mp228’ directly after picking (Table 4.1), which may explain low transcript levels of AT2 and AT16. In summary, gene expression of AT1, AT2, AT15, AT16, AT17 and AT18 appeared to be dependent on ethylene-induced ripening of A. chinensis fruit, thus suggesting the absence of AAT activity may be caused by a lack of ethylene-regulated AAT-gene transcription.
4.3.4.2 Transcription profiles of putative AAT genes

It was of further interest to investigate if a genotype-specific transcription pattern could be observed and correlated to enzyme activity levels. The differences in relative AT transcript levels between the individual genotypes were 2–4 fold in most cases and some genotypic differences have been observed. For example, AT2 appeared to be significantly (Tukey’s HSD0.05) more highly expressed than the other putative AATs in all genotypes except ‘Mp201’ (Fig. 4.3), which most strongly expressed AT16. Furthermore, AT2 gene expression levels did not differ significantly among the individual genotypes, except for ‘Mp145’, which showed 4-fold enhanced transcription. ‘Mp145’ was also the single highest genotype (16-fold) for AT1 gene expression. Moreover, it is apparent from Fig. 4.3 that AT17 and AT18 were significantly more highly expressed in ‘Mp145’ and ‘Mp201’ whereas relative transcript levels of AT15 were about 5-fold increased in all “ethylene-treated” genotypes (‘Hort16A’, ‘Mp145’, ‘Mp201’) compared with “vine-ripened” fruit (‘Mp228, ‘Mp270). In summary, ‘Mp201’ and ‘Mp145’ expressed most proposed AAT-contigs at significantly higher levels than the other lines, with ‘Mp201’ being the only genotype expressing primarily AT16.
Fig. 4.3: Relative gene expression levels of Actinidia-derived ESTs with proposed, flavour-related AAT function from selected “ethylene-treated” and “vine-ripened” Actinidia chinensis genotypes. The error bars represent the standard error of the mean of six replicates. Letters (a, b) indicate significant differences in transcript accumulation (Tukey’s HSD<sub>0.05</sub>).  = Before ethylene production (all genotypes tested),  = after ethylene production.
4.4 Discussion

The individual members of the *A. chinensis* mapping population differ in flavour attributes (unpublished data), particularly tropical odour and taste, which may be in part influenced by (MeS)alkanoate ester levels as discussed in Section 3.3.6 (Günther et al., 2010). We show that while the MeS-volatile composition was similar between the genotypes, (for example ethyl 3-MeSpropionate was the common major (MeS)alkanoate ester), the individual volatile levels differed strongly (up to 200-fold) between the kiwifruit lines. Others reported a similar finding for different *Cucumis melo* (Wyllie and Leach, 1992) cultivars and suggested (MeS)alkanoate ester formation to be under genetic control. Since the final step of ester formation, including (MeS)alkanoate esters, is catalysed by AAT enzymes, we initially hypothesised that genotype-specific (MeS)alkanoate ester composition and concentration was likely to be determined by the catalytic properties of this family of enzymes. Cultivar-specific substrate selectivity has previously been reported for apple AATs from ‘Fuji’ and ‘Granny Smith’ crude protein extracts (Holland et al., 2005). Moreover, enantioselective acetylation of linalool was also proven to differ between members of the *Lamiaceae* family (Larkov et al., 2008), pointing towards substrate preferences of individual AAT isoforms. In our study, the ACE for 2-MeSacetyl-CoA and 3-MeSpropionyl-CoA in crude extracts of *A. chinensis* genotypes displayed similar values except for ‘Mp270’, which appeared strongly to prefer the first substrate. This contrasts remarkably with the 100-fold increased ethyl 3-MeSpropionate levels compared with ethyl 2-MeSacetate measured from all lines, suggesting that (MeS)alkanoate ester formation is rather determined by genotype-specific AAT-substrate supply than genetically determined ACE. The gene expression pattern of putative AATs for ‘Mp201’ and ‘Mp145’ was significantly different from other genotypes in that the transcript accumulation of most genes was clearly increased in these two lines. However, above-average amounts of (MeS)alkanoate esters were only found in ‘Mp201’ fruit pulp but not for ‘Mp145’ and the corresponding ACE-values for (MeS)alkanoyl-CoAs were in a similar range. In the case of ‘Mp270’, its increased ACE towards 2-MeSacetyl-CoA compared with 3-MeSpropionyl-CoA was not reflected in fruit volatile levels or the gene expression pattern of this line. This may point towards the action of an AAT encoded for by an unknown gene or to a mismatch in the time course of transcript level and actual protein content. However, it appears unlikely that ethyl (MeS)alkanoate esters were produced by specific AATs that were derived from a single gene and enhanced expression of several members of this gene family is likely to have the highest impact on
(MeS)alkanoate ester production rates. Nevertheless, recombinant expression studies are necessary to determine whether individual AATs encoded for by different Actinidia AAT genes are more specific for (MeS)alkanoyl-CoA substrates than others.

In Chapter 3, it was suggested for ‘Hort16A’ that AAT activity, gene expression and (MeS)alkanoate ester production were regulated by ethylene in vivo and in vitro and this was confirmed here for related A. chinensis genotypes. Previously, ethylene-controlled AAT gene expression has been reported for papaya (Balbontín et al., 2010), apple (Schaffer et al., 2007) and melon fruit (Yahyaoui et al., 2002). To the best of my knowledge, a depletion of AAT activity has not been reported before in climacteric fruit, other than kiwifruit. This finding was unexpected, especially for soft, “vine-ripened” A. chinensis and points towards coexisting ethylene-dependent and independent ripening processes similar to those known from melon (Pech et al., 2008). Others have observed AAT activity in fruits that were lacking volatile esters from their headspace, suggesting the control of ester biosynthesis by substrate supply. For example, AAT activity was detected in 1-MCP treated apples (Holland et al., 2005) and after CA storage (Lara et al., 2007), in ACO-antisense C. melo fruit (Flores et al., 2002) and in unripe melon (Shalit et al., 2001). Defilippi et al. (2005) studied the biosynthetic pathway leading to volatile ester formation in ethylene-suppressed transgenic apple fruit with a strong reduction of aroma-related esters from the fruit headspace. High levels of AAT activity and merely minor reduction of ADH and LOX enzyme activity, involved in the generation of AAT-substrate precursors, were found in these fruit. Consequently, the importance of upstream mechanisms as limiting factors for substrate supply and final ester levels were suggested. In green kiwifruit (A. deliciosa ‘Bruno’) the transcript accumulation of two members of the LOX-gene family was shown to be ethylene-dependent (Zhang et al., 2009). This implies that the first step in the formation of aliphatic acyl-CoAs was likely to be regulated by ethylene in kiwifruit. On the basis of these findings, we conclude that in A. chinensis (MeS)alkanoate ester production is likely to be controlled by ethylene in two ways: Firstly by ethylene-induced AAT-gene expression, and secondly by stimulating the biosynthetic pathways leading to AAT-substrate formation. We suggest that biosynthesis of ethylene and (MeS)alkanoyl-CoAs share common pathways (Fig. 4.4). In order to maintain high ethylene production rates in fruit, the supply of methionine-derived SAM will need to be elevated. In A. chinensis, for example, Whittaker et al. (1997) provided evidence that transcript accumulation of SAM-synthetase genes was induced in response to ethylene. De novo biosynthesis of SAM (Katz et al., 2006) but also methionine-recycling over the Yang cycle (Yang and Hoffman,
was shown to be of primary importance to support SAM levels, especially in climacteric fruits or plants that produce high amounts of ethylene (Bürstenbinder et al., 2007). The last intermediate of the Yang cycle is KMBA, which is a key intermediate for MeS-volatile production in lactic acid bacteria (Vallet et al., 2008). These authors reported that methional can be formed by decarboxylation of KMBA and is further reduced to methionol by ADH or oxidised to 3-MeSpropionic acid, which may in turn act as precursor for 3-MeSpropionyl-CoA synthesis by a yet unknown ligase. Furthermore, an alternative route converting KMBA directly into 3-MeSpropionyl-CoA via oxidative decarboxylation has been suggested. That KMBA was directly formed from methionine by the action of an aminotransferase has also been reported in lactic acid bacteria (Pripis-Nicolau et al., 2003). In our study, the occurrence of MeS-volatiles was found either only in ethylene-producing fruit or volatile levels remarkably increased after the onset of ethylene production, which was most obvious for methional. This points towards an ethylene-linked biosynthesis of precursors, presumably KMBA and consequently increased methional, methionol and (MeS)alkanoate ester levels. We therefore hypothesise that genotype-specific (MeS)alkanoate ester composition and concentration in ethylene-producing A. chinensis kiwifruit is controlled by enzymes that catalyse intermediates of the biosynthetic pathway leading to AAT-substrate formation and not by the final step, the esterification itself. However, whether fruit esters are produced or not appears to be dependent on ethylene-controlled AAT enzyme levels in A. chinensis fruit.
Fig. 4.4: Proposed biosynthetic pathway for methylsulfanyl-volatile formation and its linkage to methionine metabolism and ethylene synthesis in plants.
4.5 Supplementary

**Fig. S4.1:** GC peaks of ethyl esters produced by alcohol acyltransferases from *Actinidia chinensis* cell-free extracts before and after fruit ethylene production. The corresponding deuterated, internal standards are displayed in grey. Dashed line: before ethylene production, solid line: after ethylene production, A: Mp270 (1) [D5]ethyl butyrate, m/z = 93; (2) ethyl butyrate, m/z = 88; B: Mp201 (3) ethyl 3-([D3]-methylsulfanyl)propionate, m/z = 77; (4) ethyl 3-(methylsulfanyl)propionate m/z: = 148; C: ‘Hort16A’ (5) ethyl 2-(methylsulfanyl)acetate, m/z: 61.

**Fig. S4.2:** Relationship between *A. chinensis* (■) fruit volatile levels and (○) the apparent catalytic efficiencies from cell-free extracts. (A) ethyl 2-(methylsulfanyl)acetate and (B) ethyl 3-(methylsulfanyl)propionate levels and $V'_{\text{max}} K_M^{-1}$-values for (A) 2-(methylsulfanyl)acyetyl-CoA and (B) 3-(methylsulfanyl)propionyl-CoA.
CHAPTER 5

CHARACTERISATION OF TWO ALCOHOL ACYLTRANSFERASES FROM KIWIFRUIT

5.1 Introduction

Until thirty years ago plant secondary metabolites were mainly recognised as “metabolic waste products” (Peach, 1950). However, this perspective has changed remarkably with more than 200,000 different compounds identified to date. These phytochemicals perform a variety of roles in plants from defence against abiotic and biotic stresses through to attraction and stimulation of its biological environment (Hartmann, 2007). A large number of these molecules are produced by acylation of a hydroxyl, amino or thiol-group, catalysed by acyltransferases. Of particular importance are members of the BAHD AT superfamily that produce a wide range of functionally important compounds such as lignin, phenolics, alkaloids, phytoalexins, anthocyanins and volatile esters (D’Auria, 2006; St-Pierre and De Luca, 2000). BAHD ATs are generally recognised by their active site motif (HXXXD) and by a conserved region (DFGWG) with likely structural significance (Ma et al., 2005). Like most ATs, BAHD proteins use Coenzyme A (CoA)-thioesters as acyl donors and one subgroup specifically forms esters by aliphatic or aromatic O-acylation of alcohol acceptor molecules. Volatile esters produced by these AAT often drive plant-food recognition because they contribute to the “fruity” aroma of edible fruits. Some esters are also responsible for specific, key flavours or odours (Morton and Macleod, 1990). In kiwifruit, for example, elevated levels of methyl and ethyl butyrate have been recognised as characteristic fruit aroma compounds (Gilbert et al., 1996). Furthermore, these compounds dominate (Fig. 5.1) the fruit ester profiles of the commercial kiwifruit cultivars Actinidia deliciosa var. deliciosa ‘Hayward’ and Actinidia Planch. var. chinensis ‘Hort16A’ and reach peak levels at the soft end of the eating-firmness range (Wang et al., 2011). Additional alkyl substituted esters, including (MeS)alkanoate and benzoate esters, have been identified from the volatile profiles of ‘Hayward’ and ‘Hort16A’ kiwifruit (Friel et al., 2007; Günther et al., 2011; Günther et al., 2010; Wang et al., 2011; Young and Paterson, 1985). The major alcohol detected in fruit

\[5\] This chapter is based on the manuscript Günther et al., Phytochemistry 2011, 72, 700-710
of these cultivars is ethanol (Fig. 5.1) with at least 50% of the resulting volatile esters being ethyl esters.

![Diagram of volatile compounds in kiwifruit](image)

**Fig. 5.1:** *Kiwifruit volatiles as % of total volatiles, esters as % of total esters, and alcohols as % of total alcohols.* Volatile data for *Actinidia deliciosa* 'Hayward' were taken from "overripe" fruit (Young and Paterson, 1985) and reflect the average of four different harvest maturities without cold storage. *Actinidia chinensis* 'Hort16A' volatile levels correspond to "soft" fruit as reported by (Friel et al., 2007). Alcohol and ester compounds are displayed in the same order as listed in keys to the right of the figure.

That (MeS)alkanoate ester biosynthesis appears to be ethylene-dependent in *A. chinensis* has been reported in Chapter 4 (Günther et al., 2011; Günther et al., 2010). However, the genes involved in ester biosynthesis remain to be validated. As a result of an EST sequencing project from five different *Actinidia* species (*A. deliciosa, A. chinensis, A. arguta, A. eriantha*) 25 full-length clones with putative AT and AAT functions have been identified (Crowhurst et al., 2008). Twelve of these contig-sequences were suggested to be flavour-related due to their phylogenetic relationship to characterised AATs from melon (CmAAT1, El-Sharkawy et al., 2005), apple (MpAAT1,Souleyre et al., 2005), banana (BanAAT, Beekwilder et al., 2004) and strawberry (SAAT and VAAT,Beekwilder et al., 2004). Recombinant AATs from fruit have been shown to produce aroma-related fruit esters when tested with a range of alcohol and acyl-CoA precursors.
In this Chapter, changes in steady-state transcript levels of *Actinidia* ATs were studied to select sequences, potentially encoding for AATs that may be involved in ester biosynthesis, specific for ripe kiwifruit. Substrate preferences of these encoded enzymes were then investigated using recombinant expression in order to evaluate their potential role for flavour-related fruit ester formation. Finally, the results are discussed in the light of their phylogenetic relationship with fruit and flower-derived AATs from other species.

### 5.2 Materials and Methods

#### 5.2.1 Chemicals

Unless stated otherwise, all chemicals, including alcohols (methanol, ethanol, propanol, butanol) authentic standards (methyl acetate, methyl propionate, methyl butyrate, methyl hexanoate, methyl octanoate, methyl benzoate, methyl 2-MeSacetae, methyl 3-MeSpropionate, ethyl acetate, ethyl propionate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl benzoate, ethyl 2-MeSacetae, ethyl 3-MeSpropionate, propyl acetate, propyl propionate, propyl butyrate, propyl hexanoate, propyl benzoate, butyl acetate, butyl propionate, butyl butyrate, butyl hexanoate, butyl octanoate, butyl benzoate) and CoA-thioesters (acetyl-CoA, propionyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, benzoyl-CoA) were purchased from Sigma-Aldrich New Zealand Ltd.

The reference compounds propyl and butyl 3-MeSpropionate and 2-MeSacetae and the internal standards ethyl 3-[D3]MeSpropionate were synthesised as described in Section 2.2.2; [D5]ethyl butyrate was made according to Section 4.2.3 and the substrates 2-MeSacetyl-CoA and 3-MeSpropionyl-CoA were synthesised and purified as outlined in Section 3.2.5.1.

#### 5.2.2 Quantitative real-time RT-PCR (qRT-PCR) analysis

Levels of steady-state transcripts for 26 *Actinidia* AT ESTs (Crowhurst et al., 2008) were determined using cDNA made either from *Actinidia chinensis* ‘Hort16A’ or *Actinidia deliciosa* ‘Hayward’ fruit that was sampled unripe, ripened (20°C) and after ethylene treatment as described recently (Nieuwenhuizen et al., 2007). The cDNA was diluted 100-fold prior to use and negative controls without RT reaction were used to test for gDNA contamination. Four technical replicates were used and cDNA was replaced by water to test for false amplification. Any cycle threshold superior to 35 amplification cycles was
<table>
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<tr>
<th>Gene</th>
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<th>Primer Reverse (3’-5’)</th>
<th>Product size</th>
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<tr>
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<td>138 bp</td>
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considered as very low transcription and not considered for evaluation. A standard dilution series with plasmid DNA was generated to evaluate the primer efficiency of each primer, which was greater than 1.9. Transcription analysis of Actinidia-derived AT ESTs was performed relative to the reference gene UBC9 as described in 3.2.4. Primers were designed within the open reading frame of the full-length contig (Table 5.1; the prefix indicates the species from which the sequence was originally isolated; Aa: A. arguta, Ac: A. chinensis, Ad: A. deliciosa, Ae: A. eriantha, Ap: A. polygama).

5.2.3 Expression of Actinidia ATs in Saccharomyces cerevisiae

5.2.3.1 Cloning and recombinant expression

Following manufacturer’s instructions (Invitrogen, pYES2.1 TOPO®TA Expression Kit), the selected sequences were cloned into the pYES2.1 TOPO® TA vector and verified by enzyme restriction analysis and sequencing. Protein sequence authenticity (AT1 [HO772635]; AT16 [HO772640]; AT9 [HO772637]) was confirmed for all clones although substitutions were noted for AT9 (Ser407 to Ile) and AT16 (Ala404 to Thr) which displayed a mutation in clear distance to the active site motif. A Kozac consensus sequence (TCCACAATG) was linked to the start of each gene to facilitate translation in yeast (Miyasaka, 1999). Auto-ligated construct was used as negative control. The Saccharomyces cerevisiae cell line INVSc1 (Invitrogen) was transformed with the pYES2.1 vector, harbouring the relevant construct. Vector containing the lacZ-gene was used as positive control for recombinant protein expression. Several pre-cultures of the positive transformants were grown in selective SC-U minimal medium without uracil (Yeast Nitrogen Base, Difco) and 2% glucose at 30°C and 250 rpm before gene induction. For recombinant expression, a culture with OD600 0.4 was prepared in SC-U medium with 2% galactose and the conditions described above. Yeast cells were harvested by centrifugation (1800 g, 10 min at 4°C) after 20 h, flash frozen in liquid nitrogen and stored at -80°C until needed.

5.2.3.2 Purification of recombinant Actinidia AATs

Cell pellets from a 400 mL cell culture were resuspended in 20 mL cold extraction buffer (20 mM Na2H2PO4 at pH 7.2, 1 M NaCl, 10 mM Imidazole, Complete™ protease inhibitor tablet-EDTA-free, Roche). The EmulsiFlex-C5 high pressure homogeniser (AVESTIN Inc.) was used for cell disruption with a pressure setting between 15 and 20 kpsi. The homogenate was centrifuged at 10000 g for 30 min at 4°C and the supernatant
was filtered (0.2 μM Minisart®, Sartorium stedim biotech) before loading onto a 1-mL HiTrap FF column (GE Healthcare) using a peristaltic pump. Proteins were eluted from the Ni²⁺ resin with an imidazole gradient of 0–0.5 M using fast protein lipid chromatography (FPLC, Äktaprime plus, GE Healthcare). The eluate-fractions were selected according to their OD₂₈₀ in the range of 350–500 mM imidazole and Western Blot analysis was performed (Trans-Blot Semi-Dry Cell, Bio-Rad Laboratories), using anti-V5 (Invitrogen) as primary and anti-mouse IgG alkaline phosphatase conjugated IgG (H+L) (Promega) as the secondary antibody. Proteins were visualised using the 1-STEP™ NBT/BCIP alkaline phosphatase detection reagent (Pierce) according to the manufacturer’s instructions. Recombinant, semi-purified protein could not be clearly identified on Coomassie-stained polyacrylamid gels because of its low content (no overexpression in S. cerevisiae!) and the presence of similar-sized proteins after FPLC purification.

5.2.4 Functional characterisation

5.2.4.1 Enzyme assay and kinetic studies

To test for AAT-activity of the semi-purified extract, the combined fractions (350–500 mM Imidazole) were assayed using GC-MS after HS-SPME. Proteins were quantified according to Bradford (1976). In a total volume of 1 mL, 300μl (100μg) total protein extract was mixed with assay buffer (20 mM Na₂H₂PO₄ at pH 8, 1M NaCl, Complete™ protease inhibitor tablet-EDTA-free, Roche), alcohol mix (methanol, ethanol, propanol, butanol) containing 1 mM each, 5 μM of each internal standard ([D5]ethyl butyrate, ethyl 3-([D3]-MeS)propionate) and acyl-CoA mix containing 100 μM of each CoA-substrate (acetyl-CoA, propionyl-CoA, butanoyl-CoA, hexanoyl-CoA, octanoyl-CoA, benzoyl-CoA, 2-MeSacetyl-CoA and 3-MeSpropionyl-CoA) to start the reaction. These substrates were chosen because the corresponding esters were reported to occur in ripe kiwifruit. The samples were incubated at 30ºC for 1 h and kept on ice for 5 min before adding 100μl of 10% SDS (w/v) solution, which had been confirmed to stop the enzyme reaction. The samples were immediately vortexed for 30 sec, frozen in liquid nitrogen and stored at -20ºC until needed. Samples containing boiled protein and semi-purified, non-induced protein extract of each transformant were tested as negative controls. Different assay times (15 min – 3 h) were tested to ensure optimal peak size and a linear increase in ester production rate (data not shown) and incubation for 1 h was found to be appropriate. In order to screen for preferred AAT-substrates of the recombinant protein, firstly an acyl-
CoA mix was tested with each alcohol individually and secondly each individual acyl-CoA was tested with a mixture of four alcohols. All samples were measured in duplicates.

Because AAT-activity remained stable for a maximum of 24 hours, the semi-purified extract from a total of 1.2 L of yeast culture was combined for enzyme kinetic studies. The ACE from semi-purified protein was defined as the initial slope of the enzyme reaction rate under limiting substrate conditions and mathematically responds to \( V'_{\text{max}} K_m^{-1} \) (where \( V'_{\text{max}} \) is \( V_{\text{max}} \) of the semi-purified protein of unknown concentration). To calculate the enzyme kinetic parameters separately, acetyl-CoA substrate concentrations in the 3 mM range were necessary for AT16 using butanol. For our study, it was not possible to use alkanoyl-CoA substrates at these elevated concentrations because of the comparatively large reaction volume of the enzyme assay. Furthermore, these concentrations are not likely to be physiological and we therefore analysed the ACE in order to compare the substrate preferences of recombinant enzymes for different substrates. The ACE was fitted as described in Section 4.2.5.4.

**5.2.4.2 Quantitative GC-MS analysis**

Optimised conditions according to Section 4.2.5.2 were applied. The GC oven temperature programme was 40°C for 2 min, to 200°C at 5°C min\(^{-1}\), and held 2 min.

Authentic reference compounds were used for ester identification and quantification using a standard dilution series (1–10µM \( R^2 \geq 0.985 \)) in assay buffer with 1% SDS (w/v). The repeatability of eight samples containing equal amounts of standard compounds was within 10% relative standard deviation if peak areas of esters with \( C_2-C_4 \) acyl-moiety were corrected against \([D5]\)ethyl butyrate and the remaining ones against ethyl 3-[D3]-MeSpropionate as internal standards. Because reference compounds were unavailable for propyl hexanoate, propyl octanoate and propyl benzoate, those compounds were quantified in equivalents of their corresponding ethyl esters.

**5.2.5 Phylogenetic analysis**

Eleven previously reported plant AAT genes from GenBank along with four *Actinidia* AATs (AT16 [HO772640], AT9 [HO772637], AT1 [HO772635], AT17 [HO772638]), isolated from *A. chinensis, A. deliciosa and A. eriantha* were analysed. Amino acid alignments of predicted proteins were constructed using Clustal X (Thompson et al., 1997). The presence of an active site histidine residue embedded in the HXXXD motif was checked in alignments. Phylogenetic analysis was carried out using the PHYLIP
suite of programs and bootstrap analysis was conducted from 1000 bootstrap replicates using Seqboot (Felsenstein, 1993). Distances were calculated using Protdist, and the Fitch method was used to construct the unrooted tree. Treeview (v.1.6.6) was used to display results (Page, 1996). The GenBank protein accessions used in the alignment are included in Fig. 5.5.

## 5.3 Results and Discussion

### 5.3.1 Identification of ripening-related ATs using qRT-PCR analysis

We hypothesised that enhanced volatile ester production during kiwifruit ripening (Günther et al., 2011; Günther et al., 2010; Wang et al., 2011; Young and Paterson, 1985) was linked to increased expression of flavour-related AATs. Therefore, qRT-PCR analysis of *Actinidia* EST-contigs (Crowhurst et al., 2008) (all full-length-clones except for AT15) with putative AAT and AT-function was performed using RNA isolated from *A. delicosa* ‘Hayward’ and *A. chinensis* ‘Hort16A’. Because (MeS)alkanoate ester biosynthesis appeared to be dependent on ethylene-regulated AAT expression in *A. chinensis* as described in Chapters 2 and 3, we investigated the expression of AT-contigs in fruit that were softened to eating ripeness either with or without ethylene treatment. Ratios of the relative amounts of transcripts from ripe versus unripe fruit are displayed in Fig. 5.2A and 5.2B. Six of these tested contig sequences, derived from three different *Actinidia* species (*A. delicosa, A. chinensis, A. arguta*) (AT18, AT2, AT15, AT1, AT17, AT16), were suggested to function as AATs and are shown to be phylogenetically closely related (Crowhurts et al., 2008). In *A. chinensis* ‘Hort16A’ (Fig. 5.2B) transcript levels of these six sequences was shown to increase during fruit ripening (Section 3.3.4) and respond to ethylene in all *A. chinensis* genotypes (Section 4.3.4). These findings were confirmed for ‘Hort16A’ in this study and relative transcripts were 10 (AT18) to 550 (AT15) fold increased in ripe compared with unripe fruit, and 20 (AT16) to 2000 (AT15) fold after ethylene treatment. Except for AT16, these same contigs were also more highly expressed in *A. delicosa* ‘Hayward’ (Fig. 5.2A) showing 30 (AT1) to 100 (AT18) fold increased accumulation in ripe ethylene treated and untreated versus unripe fruit. Interestingly, transcript levels of AT16, which was isolated from *A. chinensis*, were only two-fold increased in ripe compared with unripe *A. delicosa* ‘Hayward’ fruit and even 2-fold decreased after ethylene treatment. It has been suggested that AT1, AT17 and AT16 (Crowhurst et al., 2008) are orthologues, and it appears likely that ethylene regulation of AT16 diverged upon speciation. Transcripts of AT10 and AT12, isolated from *A. eriantha*...
and *A. deliciosa* with “unknown” function (Crowhurst et al., 2008) accumulated approximately 5-fold in ripe ‘Hort16A’ but 15 and 40-fold, respectively, in ethylene-treated ‘Hayward’ fruit.

**Fig. 5.2:** Fold difference in transcript accumulation of Actinidia-derived contigs with proposed acyltransferase function in ripe compared with unripe fruit. **A** Actinidia deliciosa ‘Hayward’; **B** Actinidia chinensis ‘Hort16A’; **C** Actinidia chinensis ACO-RNAi (T1) fruit. Black bars display the transcript level ratio of ripe fruit without ethylene treatment to unripe fruit and grey bars display the ratio of ethylene-treated, ripe fruit to unripe fruit. The prefix indicates the species from which the sequence was originally isolated from (Aa: *A. arguta*, Ac: *A. chinensis*, Ad: *A. deliciosa*, Ae: *A. eriantha*, Ap: *A. polygama*). The error bars represent the standard deviation of the average of four technical replicates. Relative transcript levels are displayed on a log₁₀ scale. Vector NTI® software was used to align the contig sequences and to generate the phylogenetic tree (horizontal axis).
This suggests that the steady-state transcript levels of these sequences are likely to be ripening-related in *A. deliciosa*. Interestingly, transcript accumulation of *AT14*, *AT7*, *AT8* and *AT23* (isolated from *A. deliciosa* and *A. chinensis*) in ‘Hayward’ and ‘Hort16A’ fruit after ethylene treatment was lower than in unripe fruit or absent. This suggests that this ripening hormone suppressed the expression of the corresponding genes, which therefore do not appear to be involved in ethylene-stimulated fruit ester biosynthesis.

Because kiwifruit exhibit a sharp peak in climacteric ethylene production (Whittaker et al., 1997), non-ethylene treated ripe fruit were likely to produce at least traces of this hormone. To provide evidence for the role of ethylene on *AT* expression in kiwifruit we monitored the steady-state transcript levels of their contigs in an ACO-silenced *Actinidia chinensis* RNAi-line (T1) that did not produce detectable amounts of climacteric ethylene (Atkinson, *submitted*). This RNAi line was deficient in volatile ester production unless treated with exogenous ethylene (Atkinson, *submitted*). It is demonstrated in Fig. 5.2C that little or no transcription of *AT18*, *AT2*, *AT15*, *AT1*, *AT17* and *AT16* was detectable from soft ACO-silenced kiwifruit but 130 (*AT16*) to 3400-fold (*AT17*) increased levels were observed after ethylene treatment, thus confirming ethylene regulation of these genes in *A. chinensis*. In ethylene-treated ACO-silenced T1 kiwifruit (Fig. 5.2C) transcript accumulations of five further sequences, (*AT14*, *AT3*, *AT22*, *AT10*, *AT12*) were 13 (*AT14*) – 55-fold (*AT22*) higher compared with non-treated fruit but only up to 3-fold increased in comparison to unripe fruit. Finally, transcript levels of *AT7* and *AT8* decreased during ripening and especially in response to ethylene as previously seen for ‘Hayward’ and ‘Hort16A’ kiwifruit (Fig. 5.2A, 5.2B). This suggests a potential role of these latter contigs for fruit development, which was supported by their accumulation in developing *A. chinensis* ‘Hort16A’ fruit and a further decline during fruit maturation (Fig. S5.1). In contrast, *AT18*, *AT2*, *AT15*, *AT1*, *AT17*, *AT16*, *AT10* and *AT12* were defined as ripening-related contigs due to increased transcript levels in ripe fruit and in response to ethylene as observed for ACO-RNAi, ‘Hort16A’ and ‘Hayward’ fruit. A flavour-related AAT-function was previously proposed for *AT18*, *AT2*, *AT15*, *AT1*, *AT17*, *AT16* (Crowhurst et al., 2008) but also for *AT9*, *AT23* and *AT22* which exhibited slightly increased transcript levels in ethylene-treated *A. chinensis* ACO-silenced (Fig. 2C) but not in ‘Hort16A’ or ‘Hayward’ fruit.
5.3.2 AAT-activity was confirmed for *Actinidia* ESTs using recombinant expression.

The recombinant expression of proposed kiwifruit AATs in yeast yielded soluble proteins at their predicted weights (AT1, 51 kDa; AT16, 51.98 kDa; AT9, 47.67 kDa; supplemental data, Fig.S5.2A-C) that were similar to the predicted or actual sizes of most plant-derived ATs from the BAHD superfamily (St-Pierre B, 2000). The recombinant enzymes were then tested for AAT-function by measuring their ability to produce esters from a mixture containing alcohol and acyl-CoA substrates (Fig. 5.3). This experimental set-up was chosen to imitate a potential *in vivo* situation in which different substrates are available at the same time. From this mixture the major ester formed by AT1 (Fig. 5.3A) and AT16 (Fig. 5.3B) was butyl benzoate (53-55 %), and by AT9 (Fig. 5.3C) was butyl acetate (45.6 %). The general profile of esters produced by recombinant AT1 and AT16 was similar. This is perhaps not too surprising given that AT1 which was isolated from *A. deliciosa* and AT16 from *A. chinensis* are likely orthologues (Crowhurst et al., 2008). However, butyl butyrate production by AT1 was 4-times higher than by AT16, whereas butyl 3-MeSpropionate formation was 5-fold lower (Fig. 5.3A and 5.3B). Acetyl-CoA and propionyl-CoA were barely used as substrates for volatile ester production (less than 1.5%) by AT1 and AT16, but AT9 in contrast, predominantly formed acetate and propionate esters (Fig. 5.3C). Benzoate esters were not detected from recombinant AT9 and its ester profile comprised only 0.1% butyl 2-MeSacetate. Owing to their striking differences in ester production, AT16 and AT9 were chosen for further functional studies.
Fig. 5.3: Volatile esters produced by recombinant alcohol acyltransferases expressed in *Saccharomyces cerevisiae*. Levels of individual esters are displayed as the average % of total esters produced from a mixture of alcohol and acyl-CoA substrates (n=2). A AT1; B AT16; C AT9. 1 Methyl acetate; 2 Methyl propionate; 3 Methyl butyrate; 4 methyl hexanoate; 5 methyl octanoate; 6 methyl 2-(methylsulfanyl)acetate; 7 methyl 3-(methylsulfanyl)propionate; 8 methyl benzoate; 9 ethyl acetate; 10 ethyl propionate; 11 ethyl butyrate; 12 ethyl hexanoate; 13 ethyl octanoate; 14 ethyl 2-(methylsulfanyl)acetate; 15 ethyl 3-(methylsulfanyl)propionate; 16 ethyl benzoate; 17 propyl acetate; 18 propyl propionate; 19 propyl butyrate; 20 propyl hexanoate; 21 propyl octanoate; 22 propyl 2-(methylsulfanyl)acetate; 23 propyl 3-(methylsulfanyl)propionate; 24 propyl benzoate; 25 butyl acetate; 26 butyl propionate; 27 butyl butyrate; 28 butyl hexanoate; 29 butyl octanoate; 30 butyl 2-(methylsulfanyl)acetate; 31 butyl 3-(methylsulfanyl)propionate; 32 butyl benzoate

5.3.3 Functional characterisation revealed benzoyltransferase activity for AT16 and acetyltransferase activity for AT9.

The catalytic properties of recombinant AT16 and AT9 were evaluated in two ways. First, preferred substrates were screened, using either individual alcohols with a mixture of eight different CoA-thioesters (Fig. 5.4A, 5.4C) or individual acyl-CoAs with a mixture of four alcohols (Fig. 5.4B, 5.4D). These substrate combinations were chosen according to those esters previously found in *kiwifruit* (Friel et al., 2007; Günther et al., 2010; Wang et al., 2011; Young and Paterson, 1985). As presented in Fig. 5.4A, AT16 utilised C$_1$-C$_4$ straight chain alcohols but individual alcohols appeared to influence the acylation reaction carried out by the enzyme. Each acyl-CoA substrate tested in this study was utilised for ester formation with butanol, whereas methyl esters were only formed with benzoyl- and 2-MeSacetyl-CoA. Generally, the number and amount of volatile esters produced by AT16 increased with increasing carbon-chain length of the alcohol acceptor molecule (Fig. 5.4A). From a mix of alcohols, AT16 primarily catalysed butyl ester
formation (Fig. 5.4B), with the highest enzyme activities observed for butanol with benzoyl-CoA and (MeS)alkanoyl-CoAs. This indicates a preference for the latter two substrates. In comparison, AT9 produced butyl acetate and butyl propionate with the highest catalytic activities (Fig. 5.4D), suggesting the preferred conversion of butanol with acetyl-CoA and propionyl-CoA. In contrast to AT16, benzoyl-CoA was utilised only in the absence of alternative acyl-CoA substrates by AT9 (Fig. 5.4D) and 3-MeSpropionate ester formation was not detected at all (Fig. 5.4C, 5.4D). In general, AT9 appeared to be more selective towards alcohols with an even-number of carbon atoms (Fig. 5.4C), except for propanol with acetyl-CoA, and ethyl esters were primarily formed with hexanoyl- and octanoyl-CoA.

**Fig. 5.4:** Specific activity of recombinant alcohol acyltransferases (AAT) from kiwifruit. Panels A and B display the ester production rate of AT16, while panels C and D show AAT-activity of AT9. Recombinant protein was incubated with individual alcohol substrates and a mix of acyl-CoAs (A, C) or with individual acyl-CoAs and a mix of different alcohols (B, D), respectively. The error bars represent the standard deviation of the average of two replicates.
Next, the ACE was calculated for those substrates leading to highest ester production rates. For AT16 the ACE for benzoyl-CoA was 5-fold higher when using butanol compared with ethanol (Table 5.2). Furthermore, if butanol was present as a co-substrate, benzoyl-CoA was utilised at 15- and 20-fold higher reaction rates than butyl 3-MeSpropionate and butyl 2-MeSacetate, respectively. Even though benzoate ester levels in ripe ‘Hayward’ and ‘Hort16A’ kiwifruit are lower than methyl and ethyl butyrate levels, they form a major part of the volatile ester profiles of these commercial cultivars (Fig. 5.1). AT16 is likely to play a role in the production of benzoate esters and to contribute to (MeS)alkanoate ester synthesis in ripe A. chinensis fruit. The finding that AT16 preferentially produced butyl esters in vitro does not diminish its role in the in vivo synthesis of ethyl esters, which dominate the kiwifruit volatile profiles (Friel et al., 2007; Günther et al., 2010; Takeoka et al., 1986). This may be explained by substrate availability with ethanol, the dominating alcohol present in ‘Hort16A’ kiwifruit (97%, Fig. 5.1). Substrate availability in vivo may also explain the elevated amounts of butyrate esters, but there is limited information about physiological butanoyl-CoA-levels in kiwifruit and it is also possible that other contigs, perhaps AT2 or AT15, which have their highest transcript levels in ripe kiwifruit, encode for proteins with high specificity for this substrate.

AT9, in comparison, also preferred butanol over ethanol with a 70-fold higher ACE for butyl acetate compared with ethyl acetate (Table 5.2). The $V'_{\text{Max}} K_m^{-1}$ value for the formation of the enzyme’s second major ester, butyl propionate, was 2-fold lower compared with butyl acetate. Butyl acetate was reported as the major fruit ester identified in Actinidia eriantha genotypes (Crowhurst et al., 2008), implicating a role for AT9 in ester formation for this species, from which it was isolated. However, considerable amounts of acetate and propionate esters are also produced in the commercial kiwifruit cultivars (Fig. 5.1) and an ortholog of AT9 has recently been discovered in A. chinensis and A. deliciosa (personal communication, Richard Newcomb), suggesting the involvement of this enzyme in the generation of volatile esters in ripe fruit for these commercial cultivars. In comparison with AT16, the ACE of AT9 for 2-MeSacetyl-CoA with butanol (Table 5.2) was 8-fold lower, indicating that this enzyme is not important for the generation of (MeS)alkanoate esters.
Table 5.2: Apparent catalytic efficiencies of recombinant Actinidia alcohol acyltransferases with preferred substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AT16</th>
<th>AT9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA + ethanol</td>
<td>NA</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Acetyl-CoA + butanol</td>
<td>BQT</td>
<td>71.1 ± 1.2</td>
</tr>
<tr>
<td>Propionyl-CoA + butanol</td>
<td>NA</td>
<td>33.5 ± 1.1</td>
</tr>
<tr>
<td>2-(methylsulfanyl)acetyl-CoA + butanol</td>
<td>8.2 ± 0.4</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>3-(methylsulfanyl)propionyl-CoA + butanol</td>
<td>12.9 ± 3.1</td>
<td>NA</td>
</tr>
<tr>
<td>benzyol-CoA + ethanol</td>
<td>27.5 ± 2.0</td>
<td>NA</td>
</tr>
<tr>
<td>benzyol-CoA + butanol</td>
<td>158 ± 6.5</td>
<td>BQT</td>
</tr>
</tbody>
</table>

NA: not analysed; BQT: below quantification threshold.

5.3.4 Phylogenetic analysis and sequence alignment studies

The kiwifruit AATs described here are members of the BAHD superfamily. They share the common features of plant ATs (Crowhurst et al., 2008), including an active site motif HXXXD (amino acids 186-190, Fig. 5.5) and the conserved motif DFGWG (amino acids 455-460, Fig. 5.5) with likely structural function.

Caption Fig. 5.5:

Amino acid sequence alignment of four Actinidia alcohol acyltransferases (AATs) with fruit- or flower-derived AATs from different plant species. Abbreviations for species, acyltransferase (AT) names, and their GenBank accessions are as follows: AdAT1 (Actinidia deliciosa AT; HO772635); AdAT17 (A. deliciosa AT; HO772638); AcAT16 (A. chinensis AT; HO772640); AeAT9 (A. eriantha AT; HO772637); BEAT (Clarkia breweri acetyl-CoA:benzylalcohol acetyltransferase; AF043464; Dudareva et al., 1998); CbBEBT (C. breweri benzoyl-CoA:benzyl alcohol benzoyl transferase; AF500200; D’Auria et al., 2002); BPBT (Petunia x hybrida benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase; AAU06226; Boatright et al., 2004); MpAAT1 (Malus pumila alcohol acyltransferase; AY707098; Souleyre et al., 2005); CMAAT1 (Cucumis melo alcohol acyltransferase; CAA94432; El-Sharkawy et al., 2005); CMAAT2 (C. melo alcohol acyltransferase; AAL77060; El-Sharkawy et al., 2005); CMAAT3 (C. melo alcohol acyltransferase; AAW51125; El-Sharkawy et al., 2005); CMAAT4 (C. melo alcohol acyltransferase; AAW51126; El-Sharkawy et al., 2005); SAAT (Fragaria x ananassa alcohol acyltransferase; AAG13130; Aharoni et al., 2000); VAAT (Fragaria vesca alcohol acyltransferase; AX025504; Beekwilder et al., 2004); BanAAT (Musa acuminata alcohol acyltransferase; AX025506; Beekwilder et al., 2004); SALAT (Papaver somniferum salutaridin 7-O-acetyltransferase; AF339913; Grothe et al., 2001); VpAAT1 (Vasconcellea pubescens alcohol acyltransferase; FJ548611; Balbontín et al., 2010); RhAAT1 (Rosa hybrid acety CoA geraniol/citronellol acetyltransferase; AAW31948; Shalit et al., 2003).
Caption Fig 5.5: See page 89
Two major clades of functionally characterised BAHD AATs were defined using alignment (Fig. 5.5) and phylogenetic (Fig. 5.6) studies of *Actinidia* AATs with flavour- and scent-related AATs from other plant species. Most members from clade 1 prefer benzoyl-CoA and may consequently be classified as benzoyl-CoA: alcohol O-benzoyltransferases while those from clade 2 were previously characterised as acetyl-CoA: alcohol O-acyltransferases (D’Auria, 2006). However, functional data are somewhat incomplete. For example, the catalytic activity for benzoyl-CoA remains to be tested for MpAAT1 and all CmAATs (clade 1, Fig. 5.6). Phylogenetically, AT9 from *Actinidia* and BanAAT appeared to be less closely related to the majority of clade 1 AATs, which is also reflected in their substrate preference for acetyl-CoA (Fig. 5.5). This dissociation is apparent from a lower degree of conservation found in the key motifs of clade 1 AATs and in the novel motifs described below:

The benzoyl-CoA preferring AT16 and its relatives (AT1, AT17 Souleyre et al., 2010, in press) display a HTMSD active site motif (Fig. 5.5, amino acids 186-190,) that can be extended to FAX₁RLNHTMX₂D (amino acids 180-190; with X₁= Leu or Ile and X₂= Ser or Ala) for benzoyl-CoA O-acyltransferases from kiwifruit and other species like papaya (VpAAT1, Balbontín et al., 2010), *Clarkia breweri* (CbBEBT, D’Auria et al., 2002), *Petunia* (BPBT, Boatright et al., 2004), apple (MpAAT1, Souleyre et al., 2005) and melon (CmAAT3, El-Sharkawy et al., 2005). This key motif is different compared with the acetyl-CoA preferring *Actinidia* AAT (AT9) and those from most other plant species in that His₁⁸⁶ and Asp¹⁹⁰ are the only conserved amino acids in this region. The novel motif PLLLIQVT (amino acids 164-171) was identified in close proximity to the active site motif for members of clade 1 (Fig. 5.5) but not for AT9, BanAAT and clade 2 proteins. In addition, AT9 also contains a single amino acid substitution in the highly conserved DFGWG motif (aromatic Trp⁴⁵⁹ is replaced by aliphatic Leu). Furthermore, the conserved Thr₃⁰⁹ that has been shown to impact catalytic AAT activity in *Cucumis melo* (El-Sharkawy et al., 2005) is replaced by a Ser in AT9 but not in AT16, AT1 and AT17 from kiwifruit. The motif YYPLAGRL (amino acids 95-102) at the N terminus of the protein was recently described (Balbontín et al., 2010) and appears to be embedded in a region consisting of 39 amino acids (amino acids 91-130) that are mainly conserved for clade 1 AATs. However, whether these conserved motifs determine substrate specificity of the enzyme would need to be investigated using site-directed mutagenesis experiments combined with functional studies using key substrate combinations.
Fig. 5.6: Un-rooted phylogram of four Actinidia alcohol acyltransferases (ATs) with fruit- or flower-derived AATs from different plant species. The prefix indicates the species from which the sequence was originally isolated from (Ac: A. chinensis, Ad: A. deliciosa, Ae: A. eriantha). This tree is based on the amino acid alignment presented in Fig. S5.3 and percentage bootstrap values (1000 bootstrap replicates) for groupings are given for each branch.

5.4 Concluding remarks

The transcript accumulation of six Actinidia-derived EST-sequences (AT18, AT2, AT15, AT1, AT16, AT17) was ethylene-regulated and specific for ripe ‘Hort16A’ and ‘Hayward’ (except AT16) kiwifruit, as demonstrated by qRT-PCR analysis. We provide evidence that recombinant AT1, AT16 and AT9 exhibit AAT-activity with a broad range of...
alcohol and acyl-CoA substrates leading to the formation of volatile esters commonly found in kiwifruit. In particular, AT16 and AT9 showed a preference for butanol as an alcohol acceptor molecule in combination with most acyl-CoAs, but AT16 was more specific towards benzoyl-CoA and (MeS)alkanoyl-CoAs. In contrast, AT9 displayed a clear preference towards short-chain aliphatic-CoAs, namely acetyl-CoA and propionyl-CoA. On closer evaluation of protein sequence differences, conserved motifs were identified in benzoyl-CoA: alcohol O-benzoyltransferases that may be involved in the CoA-substrate preference and may be useful for the classification of flavour-related AATs in the future.

5.5 Supplementary

**Fig. S5.1:** Transcript accumulation (relative to Ubiquitin conjugating protein 9) of AdAT7 and AdAT8 in Actinidia chinensis ‘Hort16A’ during maturation on the vine and in response to ethylene treatment. Fruit were sampled over a period of 13 weeks from “premature” (w1) until “vine ripe” (w13). Time point w4 represents kiwifruit sampled at commercial maturity with a fruit firmness of 6 kgf. The sample w4et represents a subsample of w4 fruit that was treated with 100ppm ethylene for 24h directly after harvest and kept at 20°C for further 5 days until fruit ethylene production was detected. RNA was extracted after Lopez-Gomez and Gomez-Lim (1992) from five combined fruit. The error bars represent the standard deviation of the average of four technical replicates. RT-PCR data were acquired and analysed as described in Section 5.2.2.
**Fig. S5.2 A-C:** Western Blot hybridisation of semi-purified recombinant protein. For experimental details see Section 5.2.3.2. **A,** AT1; **B** AT16; **C,** AT9.

Imidazole content of each fraction is indicated in the figure (20 uL of each fraction applied).
CHAPTER 6

FINAL CONCLUSIONS AND FUTURE PROSPECTS

6.1 Headspace sampling techniques for the GC-MS analysis of MeS-volatiles

In this study, DHS and HS-SPME have been employed for the extraction of trace MeS-volatiles from the sample headspace. For the quantification of MeS-volatiles from the fruit matrix, DHS appeared to be the more sensitive and reliable method compared with HS-SPME, resulting in a higher spectrum of identified MeS-compounds and improved chromatography. However, the inhibition of fruit enzymes was necessary in order to maintain good repeatability.

Nevertheless, HS-SPME is the quicker approach and proved to be suitable for the analysis of less complex samples, such as enzymatic products. Therefore, a HS-SPME based assay was developed for enzyme activity studies. To the best of my knowledge, this approach has not been used for enzyme kinetic studies before and if coupled to an autosampler, offers an alternative to for example radioactive assays.

The concentration of MeS-volatiles in the sample headspace was clearly influenced by the addition of salts and the ionic detergent SDS, showing “salting-in” or “salting-out” effects. Therefore, it is advisable to thoroughly test the matrix effect of additives on the compounds of interest. The use of (deuterated) internal standards was crucial for good repeatability between samples, and good linearity was obtained using standard dilution series in a synthetic kiwifruit solution that was sampled according to the “real fruit” samples.

6.2 MeS-volatiles in Actinidia chinensis genotypes

In ‘Hort16A’ and related A. chinensis genotypes, MeS-compounds occurred at trace levels. They were primarily abundant in soft, ethylene-producing fruit. Cold storage appeared to reduce the production of all MeS-volatiles, except methional. Interestingly, (MeS)alkanoate esters were detected only in harvested fruit after ripening and not in “vine-ripened” fruit. This was attributed to the lack of ethylene production in these kiwifruit
because it was shown that ethylene treatment triggered the production of MeS-compounds in fruit harvested unripe and after prolonged chilling.

Therefore, fruit of *A. chinensis* genotypes related to ‘Hort16A’ were standardised by ethylene-treatment before MeS-volatile extraction. It was found that the composition of MeS-compounds was similar between these lines but the actual volatile levels differed up to 100-fold. Since the concentrations of individual MeS-compounds were below or at odour thresholds (where available), an aroma impact can be hypothesised solely by synergistic or cumulative effects of MeS-compounds from the same chemical family, for example (MeS)alkanoate esters. These effects, however, will be stronger in genotypes with increased levels of these volatiles. Ethyl 3-MeSpropionate was the dominating (MeS)alkanoate ester present in all *A. chinensis* lines. Dependent on harvest maturity, the second major (MeS)alkanoate ester was methyl 3-MeSpropionate in ethylene-producing “vine-ripened” and ethyl 2-MeSacetaet in fruit harvested unripe. These three compounds have been described as having tropical, sweet and fruity organoleptic properties.

The only MeS-volatile that was continuously present in all samples regardless of their ethylene production and above odour threshold in most cases was methional. Nevertheless, its levels also increased during fruit ripening and in response to ethylene in parallel with other MeS-compounds. Since methional was shown to act as precursor for the formation of MeS-alcohols and probably (MeS)alkanoate esters in microorganisms, a similar role was suggested in *A. chinensis*. According to lactic acid bacteria, the conversion of KMBA into methional is proposed in kiwifruit. KMBA is an intermediate of the ethylene biosynthetic pathway or Yang cycle, thus creating a possible link between increased availability of Yang-cycle intermediates (KMBA) during fruit climacterium and up-regulated methional levels that may enhance the formation of MeS-precursors and consequently MeS-volatiles.

### 6.3 The role of AATs on (MeS)alkanoate ester formation in *A. chinensis*

It was demonstrated for the first time that (MeS)alkanoate esters are synthesised by AATs after feeding cell-free *A. chinensis* extracts with (MeS)alkanoyl-CoAs and aliphatic alcohols. Although major levels of methyl and ethyl esters were identified from fruit samples, AAT activity was maximal using propanol. In regards to ethyl esters, this can be explained with substrate availability *in vivo* because ethanol is the main alcohol
Chapter 6

6.4 The molecular characterisation of AAT-genes and their function

From 26 Actinidia ESTs with putative AT and AAT functions, six sequences were found to be ripening-specific and ethylene-regulated in A. chinensis fruit using cDNA from ‘Hort16A’ and an A. chinensis ACO-RNAi line. Expression of these six contigs increased concomitantly with (MeS)alkanoate ester production, enzyme activity levels and was inhibited by chilling, thus suggesting their biosynthesis being regulated on the transcriptional level. The transcript accumulation of these AAT-contigs differed significantly between A. chinensis genotypes without affecting the ACE towards (MeS)alkanoyl-CoAs from total-protein extracts. It was therefore concluded that multiple AAT-isoforms were responsible for (MeS)alkanoate ester synthesis and this was proven after recombinant expression of three likely Actinidia orthologues. These phylogenetically closely related enzymes utilised a broad range of acyl-CoA donor and alcohol acceptor molecules. However, a clear preference towards benzoyl-CoA and (MeS)alkanoyl-CoAs was found. In contrast, a phylogenetically more distinct AAT primarily exhibited acetyl-CoA: O-acyltransferase activity. Only minor levels of 2-MeSacetates were produced and 3-MeSpropionate formation was not detected at all. The recombinant AATs, however, preferred butanol over ethanol, thus revealing that genotype-specific kiwifruit ester profiles are determined by the concerted expression of individual AATs and substrate availability. Several conserved motifs, including an identical active site region, were identified from the amino acid sequence of fruit and flower-derived benzoyl-CoA: O-acyltransferases, characterised from kiwifruit and other plant species. These enzymes were classified as
members of the same clade and co-evolution of substrate specificity towards the acyl-CoA donor was suggested.

6.5 Outlook

The pioneering work into (MeS)alkanoate biosynthesis and its regulation in kiwifruit is presented in this thesis. With completion of this study, many more questions arose than initially addressed, thus opening up potential areas of future research:

- It will be of significance to research the aroma impact of MeS-volatiles on the characteristic flavour of *A. chinensis*. This can be realised by co-ordinated GC-O and sensory studies, including aroma reconstitution experiments.

- Furthermore, it will be important to investigate whether cold storage affects total ester production of kiwifruit and if so, whether this can be restored by ethylene treatment. To investigate the aroma impact of chilling and ethylene, a sensory panel would need to be used.

- Increased knowledge about the molecular mechanisms of AAT-gene regulation (e.g. transcription factors) may lead to enhanced control over fruit flavour quality.

- However, since the spectrum and levels of volatile (MeS)alkanoate esters also appear to be determined by precursor availability, their biosynthetic pathways need to be further researched and limiting steps up-stream esterification defined.

- Further characterisations of individual AATs, identification of key-motifs responsible for substrate specificity (e.g. site-directed mutagenesis study) and their molecular mapping may lead to the identification of quantitative trait loci that facilitate the targeted breeding of new kiwifruit cultivars.
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Publications and presentations

Peer-reviewed papers


Publications and presentations

Oral conference presentations

- **The impact of ethylene and cold storage on (methylsulfonyl)alkanoate ester biosynthesis in Actinidia chinensis kiwifruit**
  Catrin Günther, Ken Marsh, Laura Nicolau
  
  *University of Auckland, Chemistry Showcase, June 10th 2010, invited speaker*

- **Cold storage affects sulphur ester biosynthesis in Actinidia chinensis ‘Hort16A’ kiwifruit**
  Günther C.S., Nicolau L., Souleyre E.J.F., Newcomb R.D., Marsh K.
  
  *Combio 2009, December 6th-12th 2009, Christchurch, New Zealand*

- **Regulation of sulphur ester synthesis in tropical-flavoured kiwifruit**
  Catrin Günther, Laura Nicolau, Ken Marsh
  
  *12th International Flavour Conference, May 25th-29th 2009, Skiathos, Greece*

- **A short story about the regulation of sulphur-ester synthesis in Actinidia chinensis ‘Hort16A’ fruit**
  Catrin S.Guenther, Ken Marsh, Laura Nicolau
  
  *McGintie’s 2009, February 19th-20th 2009, Whangaparoa Peninsula, Auckland*

Poster presentations

**Cold storage affects sulphur ester synthesis in tropical-flavoured kiwifruit**

Catrin S.Guenther, Ken Marsh, Laura Nicolau

- 15th-19th of November 2009, Postharvest Pacificia 2009, Napier, New Zealand
- 31st of August-1st of September 2009, Queenstown Molecular Biology Plant Satellite Meeting, Queenstown, New Zealand
- 5th-7th August 2009, Kiwifruit Conference, Auckland, New Zealand