THE CHEMICAL AND GENETIC BASIS OF TOMATO FLAVOUR

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ABSTRACT

Tomatoes often lack the characteristic good taste that consumers expect. In recent years, the fruit and vegetable industry has focused on traits such as yield, colour, and fruit size; however, flavour has been largely forgotten, at least for varieties that are grown in bulk and are cheap to purchase. Tomato volatiles are one of the most important flavour contributors along with sugars and organic acids. Over 400 volatiles are present in tomato, but only about 30 are considered critical to tomato flavour. The purpose of this study was to identify volatile Quantitative Trait Loci (vQTL) using publicly available *Solanum pennellii* and *Solanum habrochaites* introgression lines (ILs). Detection of vQTL is an essential milestone for identification of candidate genes involved in tomato flavour.

Identification of vQTL was undertaken by screening the ILs using Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCI-MS) and Gas Chromatography Mass Spectrometry (GC-MS). Additionally key volatiles and their interactions were evaluated in a series of sensory experiments using tomato juice and tomato purée.

The study revealed that, the *S. pennellii* population had several major effects including those on IL1-4, which had a vQTL for C6 volatile E-2-hexenal. It was possible to identify a sub-IL, 1-4-1 that also harboured this vQTL. In this IL1-4-1 region of chromosome 1, 122 genes were present including a possible, candidate gene likely to be involved in lipoxygenase pathway. The sensory experiments showed strong impact of methyl salicylate on other volatiles; E-2-hexenal and citral showed positive effects in purée studies. Determined taste detection thresholds for volatiles in tomato juice and purée were established.

Keywords: volatile, genes, tomato juice, tomato purée, flavour

"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to Heaven, we were all going direct the other way . . ."

Charles Dickens "A Tale of Two Cities"

I dedicate this thesis to my parents

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Abbreviations

Name	Description
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ADH	Alcohol dehydrogenase
ADP	Adenosine 5'-diphosphate
3-AFC	Three alternative forced choice
ANOVA	Analysis of variance
APCI-MS	Atmospheric pressure chemical ionisation mass spectrometry
ASTM	American Society for Testing and Materials
AT	Aftertaste
ATP	Adenosine 5'-triphosphate
BET	Best estimate threshold
BHT	Butylated hydroxytoluene
Br	Breaker
Br+3	Breaker+3
Br+7	Breaker +7
BSM	British Standard Method
CCS	Capsanthin-capsorubin synthase
cDNA	Complementary Deoxyribonucleic acid
CHS	Chalcone synthase
CI	Chemical ionisation
CMB	2-keto-4-methylbutyrate
Cnr	Colourless non ripening
COS	Conserved Ortholog Set marker
CRTL-B	Lycopene β-cyclase
CRTL-E	Lycopene ε-cyclase
CRTR-B	β-ring hydroxylase
CRTR-E	ε-ring hydroxylase
DEFRA	Department for Environment, Food and Rural Affairs
DEPC	Diethylpyrocarbonate, inactivates the RNA enzymes from water
dHPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribonucleic acid
DPA	Days post anthesis
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid, chelating agent
EI	Electron ionisation
EMS	Ethylmethanesulfonate
EPA	Environmental Protection Act/Agency
ER	Endoplasmic reticulum

ESI	Electrospray ionisation
EST	Expressed sequence tags
EtOH	Ethanol
EXLA	Expansin-like A protein
EXLB	Expansin-like B protein
EXPA	α-Expansins
EXPB	β-Expansins
FAOSTAT	Food and Agriculture Organization statistics
FAD	Flavin adenine dinucleotide
FW	Fresh weight
GC-MS SPME	Gas chromatography mass spectrometry with solid phase microextraction
GGDP	Geranylgeranyl diphosphate
GGPS	Geranylgeranyl diphosphate synthase
GM	Genetically modified
GMO	Genetically modified organism
GPA	Generalized Procrustes Analysis
GST	Glutathione-s-transferase
HCN	Hydrogen cyanide
HGA	Homogalacturonan
HPL	Hydroperoxide lyase
HPLC	High-performance liquid chromatography
HPO	Hydroperoxide
H_2O	Water
H_3O^+	Hydronium Ion
ID	Identification/internal diameter
IL	Introgression line
ITAG	International Tomato Annotation Group
LAH	Lipolytic acyl hydrolase
LiCl	Lithium Chloride
LC-MS	Liquid chromatography-mass spectrometry
LCY-B	Lycopene β-cyclase
LCY-E	Lycopene ε-cyclase
LOX	Lipoxygenase
MALDI-TOF-MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
MAS	Marker assisted selection
MeSA	Methyl salicylate
Met	Methionine
MG	Mature green
MSG	Monosodium glutamate
mRNA	Messenger Ribonucleic acid
MTA	5'-methylthioadenosine
MTR	5'-methyltirobose
m/z	Mass to charge ratio
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information

NDF	Nutrient dense food
NMR	Nuclear magnetic resonance
nor	non-ripening
Nr	Never-ripe
NSY	Neoxanthin synthase
PB	Phosphate buffer
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
PE	Protein extraction
PG	Polygalacturonase
Pi	Orthophosphate
PL	Pectate lyase
PME	Pectin methylesterase
Ppi	Pyrophosphate
PR	Pathogenesis related protein
PSY	Phytoene synthase
QTL	Quantitative trait locus
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphism
SA	Salicylic acid
SAM	S-adenosyl-methionine
SIM	Selected ion mode
SNP	Single nucleotide polymorphism
SPME	Solid phase microextraction
SSR	Simple sequence repeat marker
SSTE	Sodium chloride-sodium dodecyl sulphate-Tris-EDTA buffer
Sub-IL	Sub introgression line
TBG	Tomato β-galactosidase
TCA	Tricarboxylic acid cycle
TE	Tris-EDTA buffer
TGRC	Tomato Genetics Resource Center
TILLING	Targeting Induced Local Lesions in Genomes
TRCS	Taste receptor cells
USDA	United States Department of Agriculture
UV	Ultraviolet light
VDE	Violaxanthin de-epoxidase
VIGS	Virus induced gene silencing
VIS	Visible light
vQTL	volatile Quantitative Trait Locus
XET/XTH	Xyloglucan endotransglycosylase/hydrolase
ZDS	ζ-carotene desaturase
ZEP	Zeaxanthin epoxidase
β-Gal	β-galactosidase

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CHAPTER 1:

Introduction and literature review

1.1 Tomato History

Tomato (*Solanum lycopersicum*) is the most important fruit crop in the world by volume consumed. *Solanum* alongside *Capsicum* and *Nicotiana* represent economicaly important comodities (Langer and Hill, 1999). Domestication of tomato is relatively recent, within the past 400 years. *S. lycopersicum* is thought to have originally been domesticated in maize fields by ancient Mexicans, with Peru as the centre of diversity for the genus. Cultivated tomatoes were introduced to Europe, after the discovery of America, and they originated from Andean region in west and south part of this continent (Davies and Hobson, 1981). Initially tomato was known as pomid'oro and mala aurea (golden apple) and sometimes as poma amoris (love apple). The modern name "tomato" originated from Nahua Tongue of Mexico were they used to call this fruit "tomatl" (Goodenough, 1990).

In comparison with small wild type species, the European tomatoes were much larger (Rick, 1978). *Solanum* is subdivided into 2 subgenera, the Eriopersicon and Eulycopersicon. The cultivated tomato has been a subject of narrowing of the germplasm caused by genetic bottlenecks and selection. The major utilization of tomato germplasm for crop improvement in the past 20 years has been the use of wild species as sources of genetic variation. This has led to an important application of wild species introgressions and as a result, an increase in molecular genetic variation in and around regions that have been introgressed from wild species. Wild species have been used as sources of variation for disease and insect resistances or tolerances, abiotic

stress tolerances, and for fruit quality (Robertson 2007). Characteristics of some tomato species are presented in Table 1.1.

Table 1.1 Origins and characteristics of cultivated tomato and wild species. Two Subgenera of genus *Solanum* are presented (from Taylor 1986, Goodenough, 1990; Hobson and Grierson, 1993).

Subgenera	Species	Characteristics	Origin
Eulycopersicon Normal fruits with red or red-yellow colour	*S. lycopersicum* (cultivated tomato)	Self-pollination is automatic	Mexico, central and southern America
	S. lycopersicum var. cerasiforme (cherry tomato)	Resistance to fungus and root rotting	Central and southern America, the region of Andean foothills
	S. pimpinellifolium (currant tomato)	Resistance against diseases, improved composition and colour	Ecuador and north part of inter-Andean regions in Peru
	S. cheesmaniae	Fruit stalks are jointless, tolerance for salt	Galapagos islands
Eriopersicon Hairy, white-green with purple stripes	*S. peruvianum*	Resistance to disease and pests, vitamin C content	High Andes in Peru and some coastal regions
	S. habrochaites	Resistance to disease and pests, tolerance for low temperature, improved fruit colour	Peru and Ecuador, high altitudes
	S. chilense	Cold, virus drought and disease resistance	Northern Chile and southern part of Peru
	S. chmielewskii	Improved composition, fruit colour and total solids	Peruvian Andes, high region
	S. parviflorum	Improved composition, fruit colour and total solids	Peruvian Andes, high region

1.2 Economic importance and nutritional value of tomato

Tomato is known as protective food, because of its special nutritive value and because of its wide spread production. Tomato is the most important vegetable crop cultivated for its fleshy fruits and has a global value in excess of $31B (FAOSTAT, 2008). It is considered as important commercial and dietary vegetable crop. This commodity is a short duration crop and gives high yield, it is important from economic point of view and hence area under its cultivation is increasing day by day. Tomato is widely used fresh, as well as in processed and preserved products, like ketch-up, sauce, chutney, soup, paste, purée. Tomato is a rich source of minerals, vitamins and organic acids, essential amino acids and dietary fibres. It is also a rich source of vitamin A and C; it also contains minerals like iron, phosphorus. Nutritional value of tomatoes is presented in Table 1.2.

In the United States tomato accounts for 14.5% of the economic value of fresh market vegetable production and 50.7% of the economic value of processed production of vegetables (Robertson 2007). United States is the second biggest tomato producer after China. Details of tomato production per country are presented in Table 1.3.

Table 1.2 Nutritional value of tomato. Data compiled from USDA National Nutrient Database for Standard Reference, Release 24 (2011) http://www.nal.usda.gov/fnic/foodcomp/search/

Constituent	Compositional amount per 100g (in mg)
Water	94520
Protein	880
Fructose	1370
Glucose (dextrose)	1250
Calcium, Ca	10
Iron, Fe	0.27
Magnesium, Mg	11
Phosphorus, P	24
Potassium, K	237
Sodium, Na	5
Zinc, Zn	0.17
Copper, Cu	0.059
Manganese, Mn	0.114
Fluoride, F	0.0023
Vitamin C, total ascorbic acid	13.7
Thiamine	0.037
Riboflavin	0.019
Niacin	0.594
Pantothenic acid	0.089
Vitamin B-6	0.08
Folate, total	0.015
Choline, total	6.7
Betaine	0.1
Carotene, beta	0.449
Carotene, alpha	0.101
Lycopene	2.573
Lutein + zeaxanthin	0.123
Vitamin E (alpha-tocopherol)	0.54
Tocopherol, beta	0.01
Tocopherol, gamma	0.12
Vitamin K (phylloquinone)	0.0079
Fatty acids, total saturated	28
Fatty acids, total monounsaturated	31
Tryptophan	6
Threonine	27
Isoleucine	18
Leucine	25
Lysine	27
Methionine	6
Cysteine	9
Phenylalanine	27
Tyrosine	14
Valine	18
Arginine	21
Histidine	14
Alanine	27
Aspartic acid	135
Glutamic acid	431
Glycine	19
Proline	15
Serine	26

Table 1.3 Worldwide tomato production in 2008. Data from FAOSTAT, unofficial figures (http://faostat.fao.org/site/339/default.aspx).

Rank	Country	Production in thousand tonnes
1	China	33938.708
2	United States of America	13718.2
3	Turkey	10985.4
4	India	10303
5	Egypt	9204.1
6	Italy	5976.91
7	Iran (Islamic Republic of)	4826.4
8	Spain	4049.75
9	Brazil	3867.66
10	Mexico	2936.77
11	Russian Federation	1938.71
12	Uzbekistan	1930
13	Nigeria	1701
14	Ukraine	1492.1
15	Greece	1338.6
16	Morocco	1312.31
17	Tunisia	1170
18	Syrian Arab Republic	1163.3
19	Portugal	1147.6
20	Chile	977

Within last ten years tomato production steadily increased. It was an effect of growing economic importance of this crop as well as capability of tomato production through whole year (fields, high tunnels and glasshouses). In the last decade the production incresed over 32 million tonnes worldwide in comparison with FAOSTAT data from 1999 (Figure 1.1).

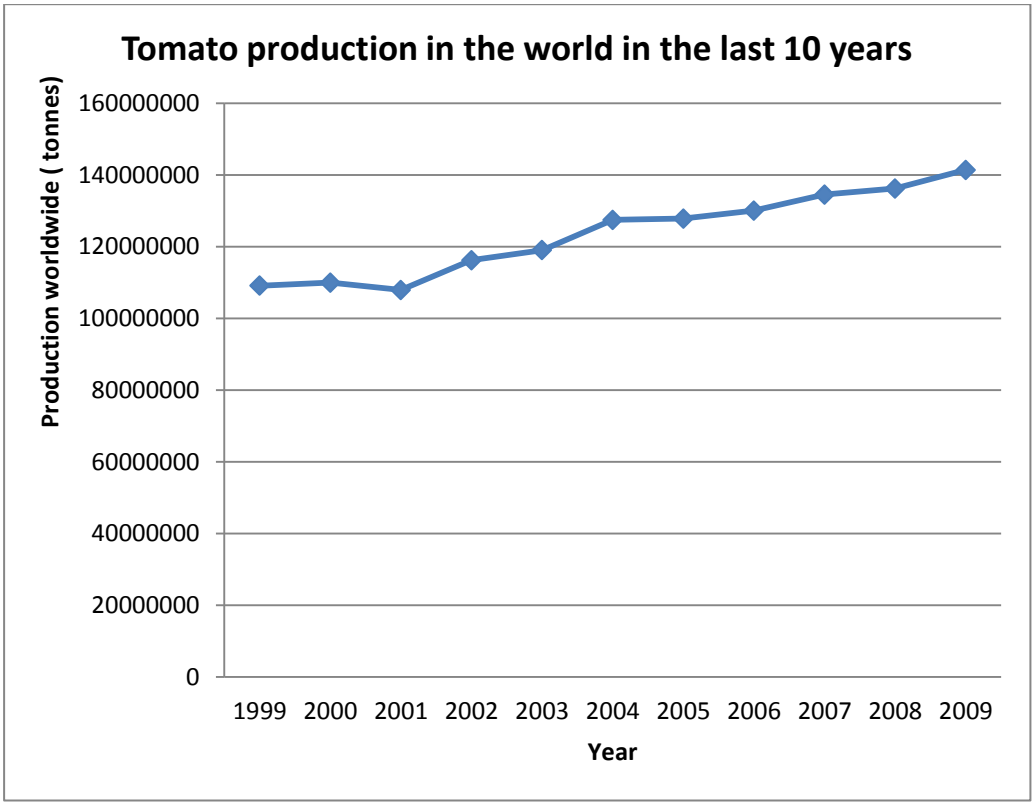


Figure 1.1 Tomato worldwide production in the last decade. Figure drawn using data from FAOSTAT (http://faostat.fao.org/site/567/default.aspx#ancor).

1.3 Tomato biology

Tomatoes are diploid with, like most of Solanaceous species, a basic chromosome number of 12. Nowadays the majority of commercial varieties of tomatoes are grown throughout the year. Tomato plants are capable of growing up to 2 meters height, but struggle to carry the weight of the fruits at this height. Young plants are naturally growing straight, but from a commercial

point of view, determinate varieties are more preferable. Determinate varieties have a predetermined number of stems, leaves, and flowers hardwired into their genetic structure. The development of these plants follows a well-defined pattern. First, there is an initial vegetative stage during which all the stems, most of the leaves, and a few fruit are formed. This is followed by a flush of flowering and final leaf expansion. Finally, during the fruit-fill stage, there is no further vegetative growth. The tomato fruits ripen, the leaves senesce and die. Commercial growers favour this type of tomato, because all the fruits can be mechanically harvested at once. Indeterminate tomato plants continue to grow, limited only by the length of the season. These plants produce stems, leaves, and fruit as long as they are alive. Inflorescences that contain 4 to 12 flowers are created from their leaf axils, very often after the plant would produce 9 leaves. Flowers show a high degree of self-pollination (Rick 1978).

There are thousands of tomato varieties and their classification can be difficult, but in general we can identify three different ways of classification. Classification based on genetic line distinguishes two groups of tomatoes. Heirloom tomatoes are strains that have been reproduced for generations without cross-breeding. The other group, are hybrid tomatoes and these are a cross between two different varieties. Hybrids are cultivated both commercially and in the home garden. Second classification is based on the length of time during which tomato plant produces the fruits, we identify here determinate and indeterminate tomatoes. The last method of classification is based on the fruit shape. Using the last method of classification, we can differentiate tomatoes into four groups, which are presented in Table 1.4.

Table 1.4 Tomato varieties based on fruit shape.

Variety	Characteristics	Example
Classic tomatoes	This is a round variety and is the most popular type of tomato. They are good for salads, grilling, baking or frying and used as a cooking ingredient for soups and sauces.	Ailsa Craig
Cherry or cocktail tomatoes	These are much smaller than the traditional classic tomato. Cherry tomatoes are the smallest and cocktail tomatoes slightly larger. Both are very sweet and have a concentrated flavour. Most cherry tomatoes are red, but golden, orange, and yellow varieties are also available. Cherry tomatoes are delicious eaten whole and raw, or cooked. Cocktail tomatoes can be halved for salads, and are great for grilling.	Sweet 100, Gardener's Delight, Santorini, Tomaccio
Plum and baby plum tomatoes	These have a distinctive oval shape. Their flesh is firm and they have less liquid in the centre. They are the natural choice for pizzas and pasta dishes and their fleshy texture makes them ideal for the barbecue. The smallest, baby plum types are sometimes called mini plum and are popular among children	Roma VF, San Marzano, Amish Paste, Costoluto Genovese
Beef tomatoes	These are larger than the traditional round tomato. Their size and shape make them excellent for stuffing and baking whole. There is a range of beef tomatoes available, varying in their shape and texture.	Beefmaster VFN, Beefsteak VFN, Big Beef, Brandywine, Bucking Bronco, Cherokee Purple, Marmande, Mortgage Lifter

1.4 Morphology of the tomato fruit

Tomato fruit is attached to the rest of the plant by the pedicel at the receptacle, where also sepals that are the remaining of the flowers, are also attached (Figure 1.2A). The pericarp tissue includes the outer pericarp, septa known also as radial pericarp, which separate the locules and the inner pericarp (Figure 1.2B).

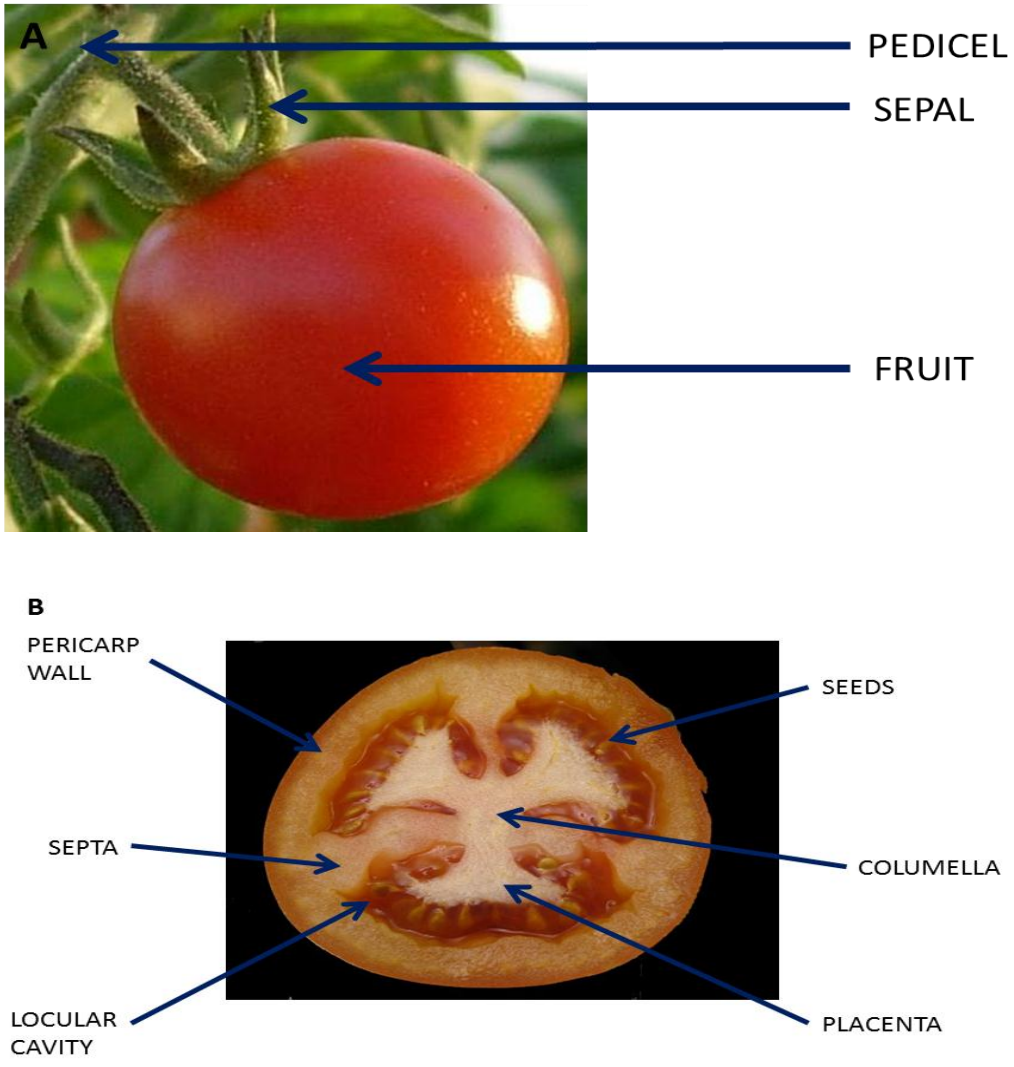


Figure 1.2 Morphology of tomato fruit. Tomato fruit develops from the flower ovary; and is attached to plant by the pedicels, where sepals remaining from the flower structure are also attached (A). Longitudinal-section through tomato fruit revealing internal tissues (B).

1.5 Fruit development and ripening.

Fruit growth leads to changes associated with different biochemical processes. These changes are presented in Figure 1.3. Fertilisation of the tomato flower leads to the development of the ovary. In the ovary occurs the process of cell division and tissue differentiation followed by seed development and early growth of embryo. Later on the ovary develops into the pericarp. Fruit development starts with cell division (0-10 days post anthesis (DPA)) to reach the immature green stage. The number of cell divisions, as well as duration of this particular phase, may differ among fruits, and have an impact on a final fruit size (Gillasby *et al.,* 1993). Then, there is a longer phase of cell expansion (10-35 DPA) resulting in the fully developed mature green fruit. After cell expansion, the next step is fruit ripening (35-60 DPA), when the fruit changes colour from green to orange and later on to red (Carrari *et al.,* 2006). The first change in colour is a result of transformation of chloroplasts into chromoplasts, and decreasing chlorophyll concentration, followed by an increase of β-carotene, responsible for the orange colour. Red colour is caused by high concentration of lycopene. Major biochemical changes during this stage are an increase in; respiration, increase in ethylene production, fruit acidity changes, and starch decreases and sugar content increases (Ranič 2010). The last stage, after red ripe, is the process of senescence, which involves degradation of cellular tissue and organelles and finally leads to the death of the cells.

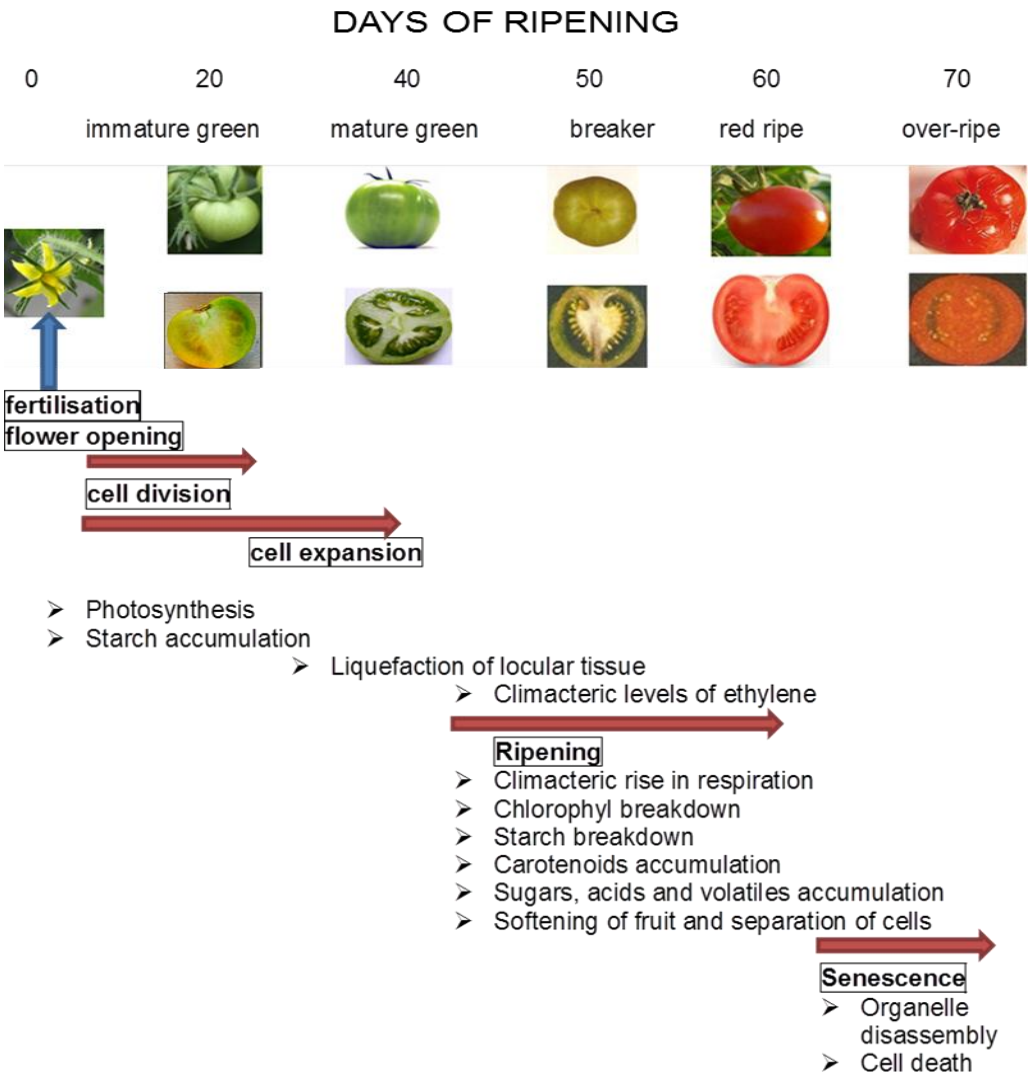


Figure 1.3 Development and ripening of tomato fruit.

Ethylene, the plant phytohormone plays a very important role in fruit development. Climacteric fruits, such as apples, pears and tomatoes are linked with a ripening-related increase in respiration and ethylene synthesis, for rapid coordination and synchronisation of ripening. On the contrary, non-climacteric fruits like strawberries, grapes or citrus, lack this respiratory burst and are insensitive to ethylene (Cara *et al.,* 2008).

Synthesis of ethylene was described in work that lead to definition of YANG cycle (Yang and Hoffman 1984). In this cycle (Figure 1.4), enzyme S-adenosyl-methionine (SAM) synthase, catalyses adenosylation of the sulphur atom of methionine. SAM is later metabolized to 5'-methylthioadenosine (MTA), which is incorporated into the methionine cycle, to recover the sulphur atom and 1-aminocyclopropane-1-carboxylic acid (ACC). There are two key factors that control tomato fruit ripening, these are enzymes involved in ethylene biosynthesis, ACC synthase (ACS) and ACC oxidase (ACO). Expression of genes encoding these enzymes has been widely studied. Cervantes (2002) suggested that auxin treatment and stress conditions might affect this and as a result, enhance ACS. It is believed that the ACS-ACO pathway is the main route for biosynthesis of ethylene. However, lipoxygenase throughout its activity and in the same conditions, might be an extra source of ethylene (Sheng and Wainwright, 2002). ACC is the first compound that is involved in ethylene biosynthesis. The enzyme, that catalyses this reaction, is ACC synthase (ACS), which is known to be pyridoxal phosphate dependent. In the presence of oxygen, ACC is converted to ethylene by ACC oxidase (ACO), known originally as ethylene-forming enzyme.

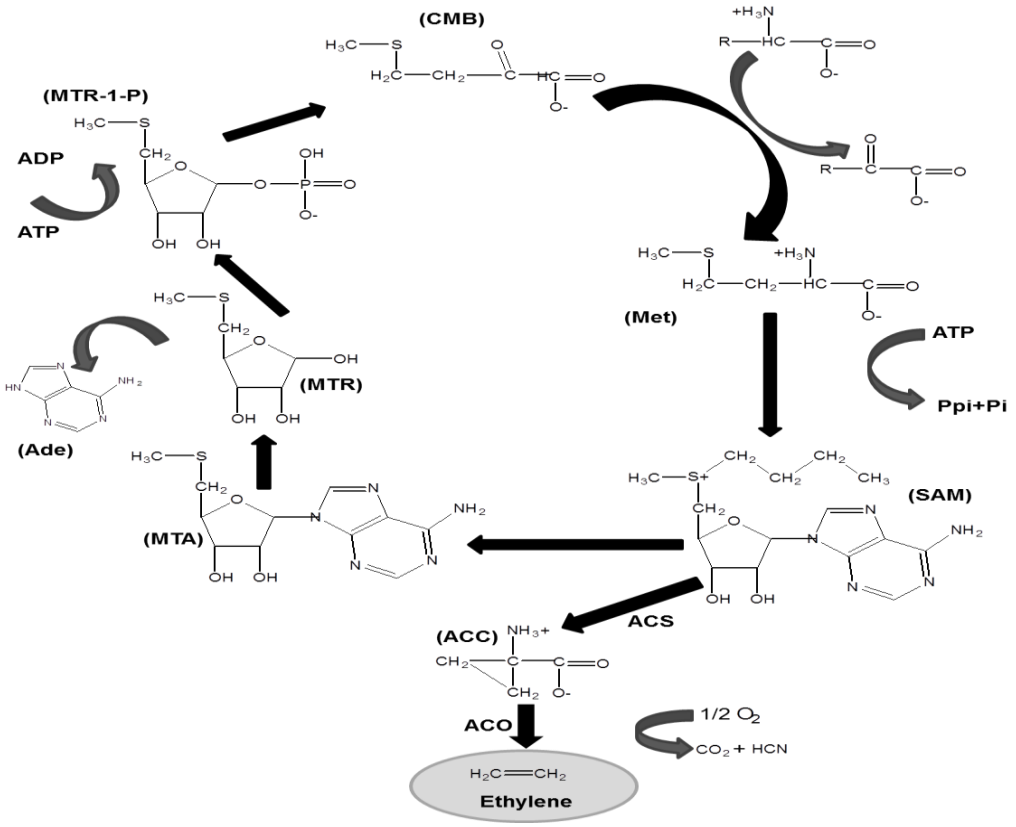


Figure 1.4 Ethylene biosynthesis pathway in relation to methionine cycle ACC: 1-aminocyclopropane-1-carboxylic acid; ACS: ACC synthase; ACO: ACC oxidase; Ade: adenosine; ADP: adenosine 5'-diphosphate; ATP: adenosine 5'-triphosphate; CMB: 2-keto-4-methylbutyrate; CO_2: Carbon dioxide; HCN: Hydrogen cyanide; Met: Methionine; MTA: 5'-methylthiadenosine; MTR: 5'-methylthioribose, MTR-1-P: 5'-methylthioribose-1-phosphate; O_2: Oxygen; Ppi: Pyrophosphate; Pi: Orthophosphate; SAM: S-adenosyl methionine (redrawn from Chaves *et al.,* 2006 and Yang and Hoffman 1984).

In the methionine biosynthesis pathway, there are a number of genes responsive to ethylene during fruit ripening, but ACS and ACO genes have been characterised very extensively (Zegzouti *et al.,* 1999). Nine genes encoding ACS have been identified. These genes are: *LeACS1A*, *LeACS1B*, *LeACS2-8* (Cara *et al.,* 2008). Four of these genes are expressed differentially during fruit ripening: *LeACS1A*, *LeACS2*, *LeACS4* and *LeACS6* (Nakatsuka *et al.,* 1998; Barry *et al.,* 2000). Barry *et al.,* (2000) proposed a model (Figure 1.5) explaining differential regulation of these genes during pre-climacteric and

climacteric ethylene production. The pre-climacteric phase was called system 1, while the climacteric phase was called system 2. LeACS6 is considered the main gene responsible for ethylene synthesis in green fruit (System 1); however, *LeACS1A* is present in these tissues. During the transition stage, expression of *LeACS1A* and *LeACS4* is induced and is dependent on the presence of the RIN MADS-box transcription factor (Vrebalov *et al.,* 2002). *LeACS2* induction leads to production of autocatalytic ethylene (System 2). High ethylene in System 2 results in negative feedback on the System 1 and therefore reduced expression of *LeACS1A* and *LeACS6* occurs.

In tomato, five genes have been found that encode the ACO enzyme (*LeACO1-5*). Three of them *(LeACO1*, *LeACO3*, *LeACO4*) are differentially expressed in the fruit. *LeACO1* and *LeACO4* are accumulating during the immature stages and their expression level changes at the beginning of climacteric phase and ripening. *LeACO3* expression is induced, but it is transitory at the breaker stage. Expression of *LeACO1* and *LeACO4* is sustained during ripening.

ACS and ACO genes have been found and described in many other fruits including, but not limited to melon (Yamamoto *et al.,* 1995; Lassere *et al.,* 1996), apple (Dong *et al.,* 1992; Ross *et al.,* 1992; Sunako *et al.,* 1999), banana (Liu *et al.,* 1999; Huang *et al.,* 1997), pear (Lelievre *et al.,* 1997), kiwi (Whittaker *et al.,* 1997), peach (Tonutti *et al.,* 1997; Tatsuki *et al.,* 2006) and persimmon (Nakano et al., 2003).

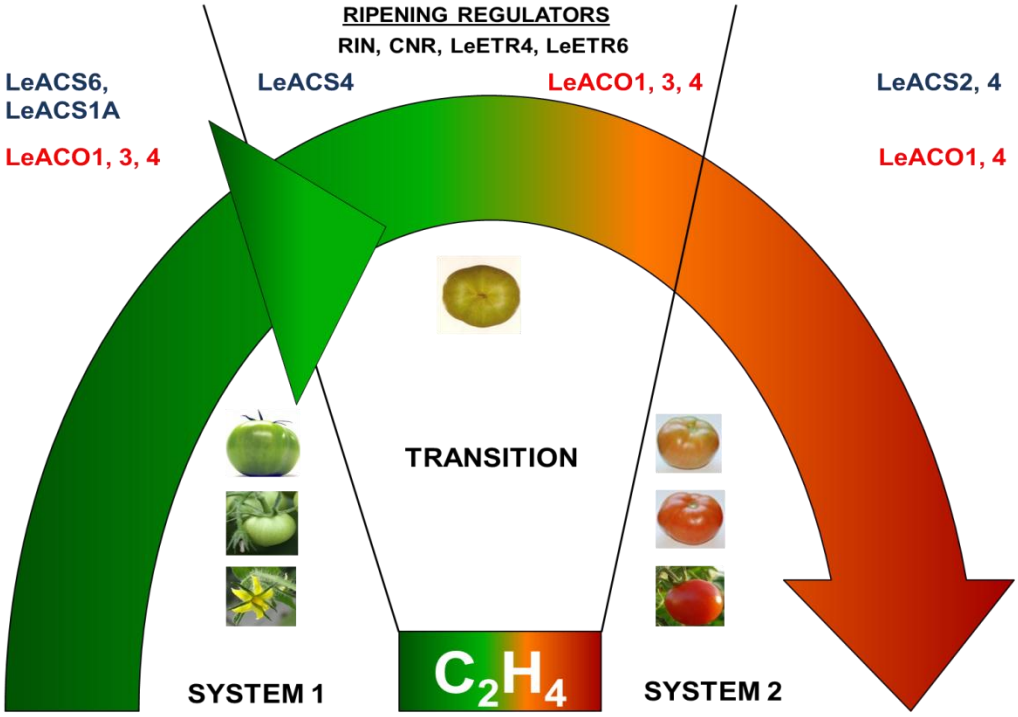


Figure 1.5 A model of ethylene regulation proposed by Barry *et al.,* 2000. During development (System 1) auto-inhibitory ethylene is synthesized by *LeACS1A*, *LeACS6* and *LeACO1, 3, 4*. At transition stage, ripening regulators are actively involved. *LeACS4* is induced; autocatalytic ethylene increases and has a negative effect on System 1. *LeACS2,* 4 and *LeACO1, 4* are then responsible for high ethylene production in System 2 (redrawn from Cara *et al.,* 2008).

Ethylene receptors were identified in studies involving *Arabidopsis thaliana* mutants (Cara *et al.,* 2008). Dominant gain-of-function mutations in ethylene receptors led to reduced sensitivity to ethylene, loss-of-function mutants in two or more genes gave a constitutive ethylene response. Ethylene receptors are disulphide-linked dimers. They are integral membrane proteins, endoplasmic reticulum (ER)-associated; and show similarity with bacterial two-component regulators that include up to three domain termed sensors, the kinase and receiver (Bleecker *et al.,* 1998). Sensor domain is localized in the N-terminal region of the protein. Its role is associated with perception of ethylene molecule, dimerization, and binding the necessary copper cofactor (Wang *et al.,* 2003; Schaller *et al.,* 1995). Auto phosphorylation of histidine residue from

ATP is catalysed by the kinase domain, but sub-domains that define the catalytic core of histidine kinase (HK), are not conserved in all plant ethylene receptors (Cara *et al.,* 2008). The histidine-phosphate group, is transferred to an aspartate residue on the receiver domain, and this domain become active after signalling. The receiver element can be found in the same sensor or HK protein, but it can also be a separate protein (Klee 2004). In tomato, there are six receptors responsible for perception of ethylene and they are divided into two sub-families. There are three members of each sub family. LeETR1, LeETR2 and LeETR3 (also called NR-Never Ripe) are included in sub-family 1. LeETR3, LeETR5 and LeETR6 are members of sub-family 2 (Klee 2004; Lashbrook *et al.,* 1998; Tieman *et al.,* 1999; Wilkinson *et al.,* 1995). Expression of the ethylene receptors in tomato has been successfully detected in all tissues that have been analysed; however, these tissues show distinct expression patterns throughout development and in response to differing environmental stimuli. *LeETR1* and *LeETR2* are expressed at constant levels in all tissues throughout development, while *NR*, *LeETR4*, *LeETR5*, and *LeETR6* are highly expressed in reproductive tissues (flowers and fruits). Significant increase of NR, LeETR4 and LeETR5 was observed in ripening fruits (Klee 2004).

1.5.1 The Tomato cell wall

Plant cells need to be firm in order to support the plant and provide the shape for tissues and fruits. This difficult task is achieved by the cell wall, an important part of plant cells. Dicotyledonous plants (Fisher and Bennet, 1991), have a primary cell wall that consists of 30% cellulose, 30% hemicellulose, 35% pectin and 5% protein. Fruit ripening leads to a series of biochemical events that alter the unripe hard, green, acid tasting tissue into one with plesant appearence, sweet taste, fragrant aroma and soft texture. Changes in

fruit firmness during ripening are a result of cell wall disassembly (Brummell 2006). Cell wall disassembly during ripening process is mostly caused by actions of polysacharide modifying enzymes secreted into the cell wall space; however, the non-enzymatic mechanism can also be involved, mostly by action of free radicals (Dumville and Fry 2003).

In tomato, there are number of cell wall modifying enzymes to include: polygalacturonase (PG), pectin methylesterase (PME), pectate lyase (PL), β-galoactosidase (β-Gal), endo-1,4-β-glucanase, xyloglucan endotransglycosylase/hydrolase (XET/XTH) and expansin. A cell wall model is presented in Figure 1.6.

Inhibition of PG in tomatoes leads to improvement of some physical properties that impact postharvest characteristics of the fruit, to include improved shelf life, improved pathogen resistance and increased viscosity of both juice and paste (Kramer *et al.,* 1992; Langley *et al.,* 1994), but other quality attributes like colour, pH or Brix are not affected (Powell *et al.,* 2003). Recent research indicates that softening is not driven primarily by PG action, as was initially assumed. It was indicated that in some cases fruit with reduced PG activity were actually firmer than controls (Brummell and Labavitch, 1997). In addition, a tomato line with an insertion in the PG gene, resulted in gene inactivation, but the fruits showed normal softening (Cooley and Yoder, 1998). Antisense suppression of PME mRNA abundance and reduction in activity by 90% in tomato has little effect on fruit firmness (Tieman *et al.,* 1992). Antisence PME fruits possess reduced shelf life (Thakur *et al.,* 1996; Tieman *et al.,* 1995).

Absence of softening has been reported in tomato mutants *nor* (non-ripening) and *Nr* (never-ripe). These mutants have similar PME activity like

normally ripening genotypes (Harriman *et al.,* 1991). In contrast with these mutants, there is *Cnr* (colourless non ripening mutant), a non ripening phenotype (Thompson *et al.,* 1999), with firm fruits that have reduced cell-to-cell adhesion and lack long un-methylesterified homogalacturonan (HGA). In addition, the unesterified HGA in *Cnr* seems to have a different block structure that may affect calcium binding and cell adhesion. Morover, PME isoform assciated with ripening is not active in *Cnr* fruits (Orfila *et al.,* 2002). Biochemical function of pectate lyase genes has not been experimantally proved and their function was assumed on sequence conservation in relation to pathogen PL (Benítez-Burraco *et al.,* 2003). The significance of PL was recognised recently, after isolation of PL gene from ripe strawberry (Medina-Escobar *et al.,* 1997) and banana (Dominguez *et al.,* 1997).

In ripening tomato fruits, three isoforms of β-galactosidase were identified (Pressey, 1983). Seven tomato β-galactosidase genes (TBG) were found to be expressed during ripening (Smith and Gross, 2000). TBG1 antisense suppressed tomato plants showed a reduction in mRNA accumulation to 10% of normal levels, but this did not affect total β-Gal (Carey *et al.,* 2001). TBG 1, 2, 3 and 5 are present in *rin*, *nor* and *Nr* tomato mutants that fail to soften and they show a similar pattern of expression to wild type. However, reduced levels of TBG3 resulted in lower level of enzyme activity, modified cell wall composition and lowered rate of detoriation observed in fruits. TBG4 may be involved in softening in contrast to TBG6, which is present in mutants but not detected in wild fruits (Smith and Gross 2000). In tomato two endonucleases were found to increase in abundance during ripening. These were LeCel1 and LeCel2 (Gonzalez-Bosch *et al.,* 1996).

Antisense supression of these particular genes, did not affect fruit softening in terms of detectable differences (Brummell *et al.,* 1999). The

expression of a ripening-related XET gene was down-regulated in antisense fruits (Brummell and Harpster 2001; De Silva *et al.,* 1994). These authors reported that there were no changes in softening and postharvest behaviour in the transformed tomato lines. In contrast, non softening *rin* mutant had a lower endotransglycosylase activity in comparison with wild type (Maclachlan and Brady 1992, 1994).

Fruit specific and ripening related expansins were identified in tomato (Rose *et al.,* 1997). There are at least seven expansin isoforms with different and specific expression patterns, but only LeExp1 is specific for the fruit and also is ripening related (Brummell *et al.,* 1999). Fruit that were supressed in LeExp1 still showed softening, but at a lower rate than controls. In contrast, over-expression lines had fruits that soften more rapidly especially in early ripening (Brummell *et al.,* 1999). The recent discoveries in terms of gene manipulation of members of candidate enzyme families in transgenic fruits lead to conclusion that no single cell wall enzyme is responsible for changes in texture during ripening. It is rather a complex action between various enzymes (John *et al.,* 1997; Moscatiello *et al.,* 2006).

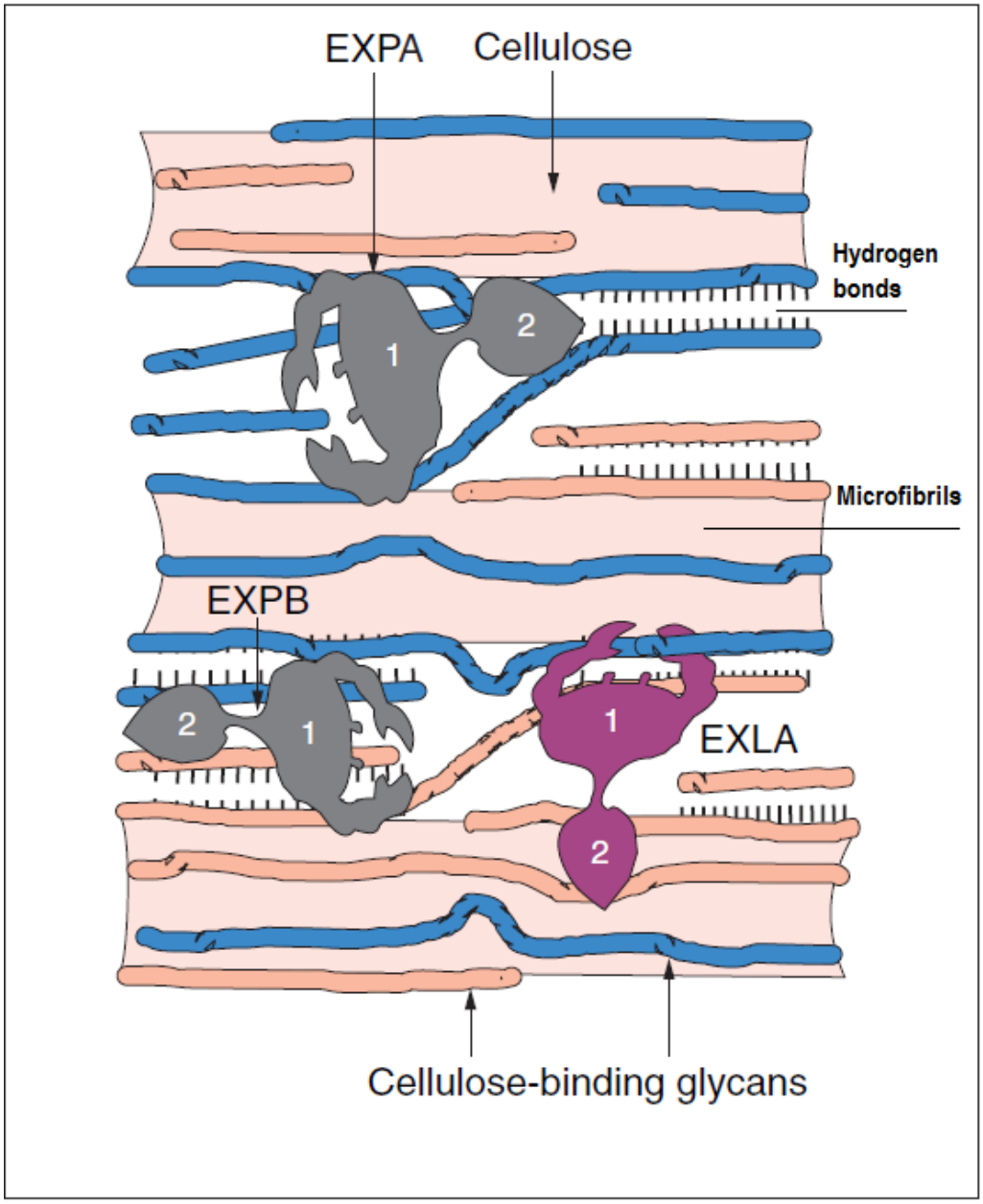


Figure 1.6 Plant cell wall model and its loosening by expansins. The cell wall consists of a scaffold of cellulose microfibrils (shaded areas) to which are bound various glycans such as xyloglucan or xylan. They create a flexible, loadbearing network based on hydrogen bonds. Extension of the cell wall entails movement and separation of the cellulose microfibrils. α-Expansins (EXPA) may promote such movement by inducing local dissociation and slippage of xyloglucans on the surface of the cellulose, while β-expansins (EXPB) work on a different glycan, perhaps xylan, to achieve a similar result. Expansin-like A (EXLA) and expansin-like B (EXLB) proteins are predicted to be secreted, to the cell wall, but their activity has not yet been fully characterised. 1 – domain 1, 2 – domain 2. Modified and adapted from Sampedro 2005.

1.5.2 Fruit colour

Green tomato fruits accumulate chlorophyll in the thylakoid membranes in the chloroplasts. The ripening process executes a change and chloroplasts turn into chromoplasts, accumulating caroteniods, lycopene and β-carotene. Figure 1.7 shows the chloroplast to chromoplast transition, where formation of carotenoid storage structures is presented and remodelling of internal membrane system occurs.

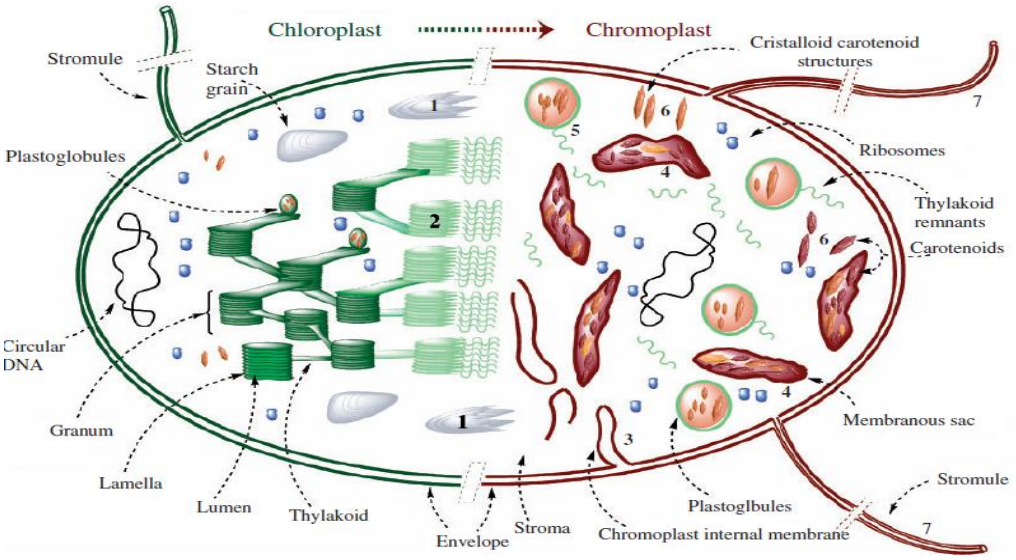


Figure 1.7 A schematic representation of the chloroplast–chromoplast transition. 1-starch granules, 2-grana and thylakoids, 3-inner membrane envelope, 4-membraneous sac, 5-plastoglobules, crystalloids with carotenoids, 7-stromules. Modified and adapted from Egea *et al.,* 2010.

Carotenoids are involved in the colour change in tomato fruits from green to orange. The source of red ripe is lycopene. These compounds are created from phytoene in carotenoid biosynthetic pathway, shown on Figure 1.8.

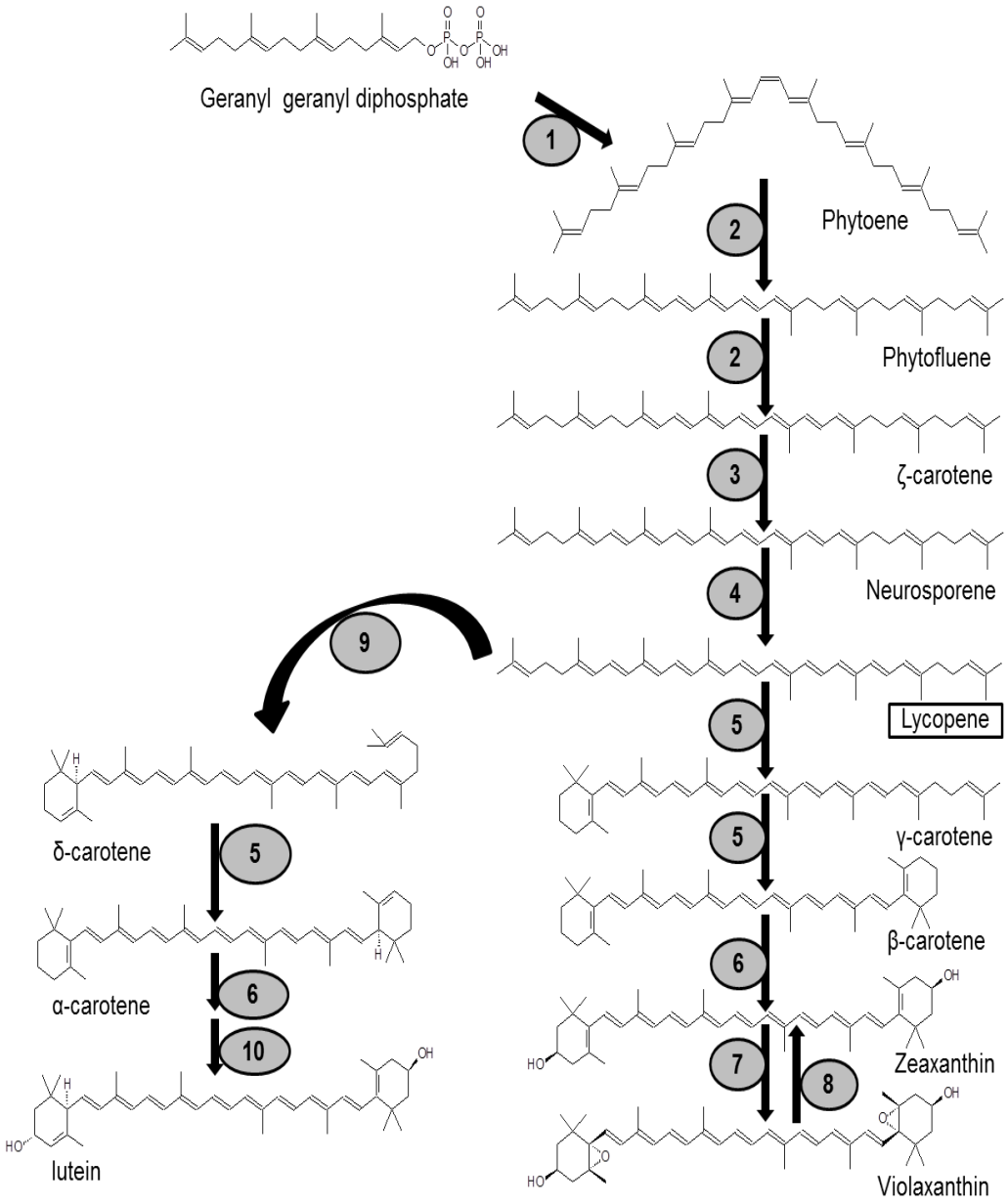


Figure 1.8 The carotenoid biosynthesis pathway in tomato. Enzymes: 1 - Phytoene synthase (PSY); 2 - Phytoene desaturase (PDS); 3 - ζ-carotene desaturase (ZDS); 4 - ζ-carotene desaturase (ZDS); 5 - Lycopene β-cyclase (CRTL-B) ; 6) β-ring hydroxylase (CRTR-B); 7) Violaxanthin de-epoxidase (VDE); 8) Zeaxanthin epoxidase (ZEP); 9) Lycopene ε-cyclase (CRTL-E); 10) ε-ring hydroxylase (CRTR-E). The structures are drawn in *trans* configuration. Drawn based on Ronen *et al.,* 2000.

There are number of genes responsible for control of the carotenoid pathway. Phytoene synthase (PSY) results from the expression of two genes *PSY-1* and *PSY-2*. The first one encodes the fruit-ripening-specific isoform, the other one is located in green tissues, including mature green fruit and it is not responsible for carotenogenesis in ripening fruit. (Fraser *et al.,* 1999). Two structurally and functionally similar enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), convert phytoene into lycopene, via ζ-carotene. The isolation of a carotene isomerase gene from *Synechocystis* (Breitenbach *et al.,* 2001) and tomato (Isaacson *et al.,* 2002) lead to establishment of the mechanism where cis-trans isomerizations occur during the desaturation of phytoene into lycopene. The cyclization of lycopene creates a series of carotenes that have one or two rings of either the β- or ε- type. Lycopene β-cyclase (LCY-B/CRTL-B) is responsible for a two step reaction that leads to β-carotene, while lycopene ε-cyclase (LCY-E/CRTL-E) creates one ε-ring to produce δ-carotene. It is assumed that α-carotene is formed by the action of both enzymes. These enzymes in tomato, show a large amount of structural resemblance and both contain Flavin adenine dinucleotide/Nicotinamide adenine dinucleotide (FAD/NAD)- binding sequences at the amino termini. Suprisingly tomato contains two lycopene β-cyclases, LCY-B and also CYC-B, a chromoplast-specifc cyclase (Ronen *et al.,* 2000). They show a 53% identity at the amino acid level. In addition, CYC-B shows a far greater identity to capsanthin-capsorubin synthase (CCS) of pepper, leading to assumption of a common ancestral gene (Ronen *et al.,* 2000; Hirschberg, 2001). Another carotenoid enzyme, neoxanthin synthase (NSY) from tomato is closely related to LCY-B and CCS (Bouvier *et al.,* 2000). This enzyme catalyses the conversion of violaxanthin to neoxanthin.

A number of genes in carotenoid pathway affects fruit ripening. During ripening, at breaker stage, mRNA levels of *PSY-1* and *PDS* significantly increase (Fraser *et al.,* 1994; Corona *et al.,* 1996). mRNAs of both lycopene cyclases (LCY-B and LCY-E) go down (Pecker *et al.,* 1996; Ronen *et al.,* 1999). This indicates that transcriptional regulation is involved in the accumulation of lycopene in tomato fruit. Differential gene expression has also been linked with the accumulation of δ-carotene in fruits of the *Delta* tomato mutant, which results from increased transcription of *LCE-E* (Ronen *et al.,* 1999) and in the formation of β-carotene rather than lycopene in the high-β mutant caused by the up-regulation of the *Cyc-b* gene (Ronen *et al.,* 2000). The high pigment (*hp*) locus in tomato also affects the levels of total carotenoids. Investigation of the *hp-2* mutant revealed that it is involved in phytochrome signalling pathways (Mustilli *et al.,* 1999). While the gene expression at the transcriptional level is a main regulatory mechanism that is responsible for carotenoid development in chromoplasts, it is not unique. This pathway may also be controlled by feedback inhibition by its end-products. Inhibition of lycopene cyclization in tomato leaves causes increased expression of both *PDS* and *PSY-1* (Giuliano *et al.,* 1993; Corona *et al.,* 1996). In support of this hypothesis are the studies were carotenoid biosynthesis inhibitors in treated tissue accumulated more total carotenoids than controls (Bramley, 1993).

1.5.3 Generation of flavour

Flavour is a complex trait that can be regarded as a combination of taste, odour and general mouth feel (Seidman, 1979). Volatile compounds that are detected in the nose, are responsible for aroma, while non-volatile compounds, presented in the mouth, perceived by the tongue and surrounding tissue, are responsible for taste (Taylor, 1996; Taylor and Linforth, 2000).

Taste buds in the mouth contain taste receptors. These taste receptors contain microvilli, a part of the receptor, where food dissolved in the saliva is binding. Five classes of taste receptors have been identified: sour, sweet, salty, bitter and umami. If translated from Japanese, it means “delicious”. Although the unique flavour for umami has not been identified, this receptor is regarded as a flavour enhancer and is commonly associated with food additive, monosodium glutamate (MSG), and also with the amino acid glutamate (German and Stanfield, 2005).

1.5.4 Taste perception

Taste receptors in the mouth (Figure 1.9a) are capable of responding to a stimulus from different chemicals, but receptors that are located in different regions in the mouth are more sensitive to specific tastes (German and Stanfield, 2005).

Early studies indicated specific regions on the tongue responsible for taste perception (Boring 1942, Collings 1974). Sweetness is perceived in the process of binding organic molecules to the receptor and most sensitive region was reported to be at the top of the tongue. The organic molecules responsible for sweet sensation in tomatoes are fructose and glucose. Bitter taste is perceived at the back of the tongue and nitrogenous compounds are generating this particular sensation. Bitterness is also related to avoidance response, mainly because, a lot of natural poisons have a bitter taste (Sandell and Breslin 2006). Sourness and saltiness were reported to be perceived on the sides of the tongue. However, receptors responsible for perception of saltiness are located closer to the anterior part of the tongue. Salty taste is caused by sodium ions, on the contrary, hydrogen ions are responsible for sourness. In case of tomato, sourness is a result of citric and malic acids present in the fruit (Mahakun *et al.,* 1979).

More recent work by Chandrashekar and colleagues (2006) suggests that, contrary to popular belief, the location of different tastes is not specific to the regions on the tongue, it is rather a case of receptors being present on all areas of the tongue (Figure 1.9b) and also presence in the gut (Sternini 2006; Jang *et al.,* 2007).

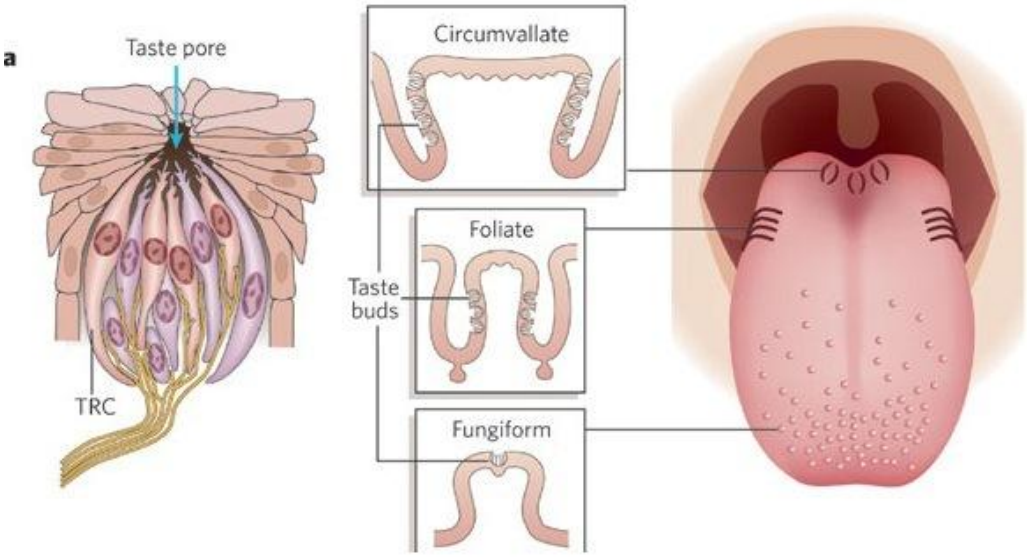


Figure 1.9a Taste buds, shown on the left are composed of 50 to 150 taste receptor cells (TRCS) and they are distributed across different papillae. Circumvallate papillae are found at the back of the tongue and contain thousands (in humans) of taste buds. Foliate papillae are found at posterior lateral edge of the tongue and contain dozen to hundreds of taste buds. Fungiform papillae contain one or few taste buds and are found in the anterior two thirds of the tongue. TRCS project microvillae to the apical surface of the taste bud, where they form, the “taste pore”, this is a place of interaction with tastants (Modified and adapted from Chandrashekar *et al.,* 2006).

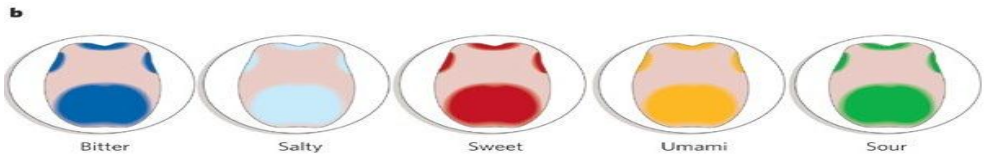


Figure 1.9b Taste perception. Recent functional and molecular data reveals, that in oposition to popular belief, there is no “tongue map”: responsiveness to the five basic moadlities – bitter, sour, sweet, salty and umami – are present in all areas of the tongue (Modified and adapted from Chandrashekar *et al.,* 2006).

German and Stanfield (2005) reported several mechanisms responsible for transmition of signals from the taste receptors to the brain. Receptors responsible for sweetness, bitter and umami use a G-protein signalling cascade (Figure 1.10a), salty and sour receptors open voltage-gated channels and bitter receptors can use both voltage or protein signalling cascades (Figure 1.10b, c).

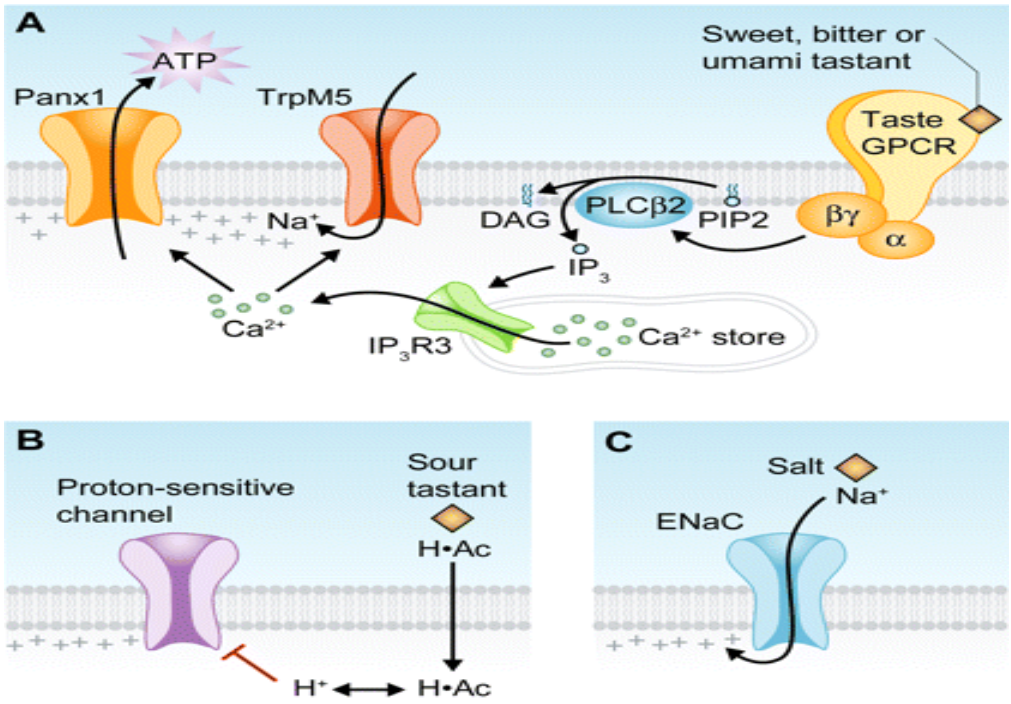


Figure 1.10 Mechanisms describing how five taste qualities are transduced in taste cells. (A) In receptor (Type II) cells, sweet, bitter, and umami ligands bind taste GPCRs, and activate a phosphoinositide pathway that elevates cytoplasmic Ca^{2+} and depolarizes the membrane via a cation channel, TrpM5. The combined action of elevated Ca^{2+} and membrane depolarization opens the large pores of gap junction hemichannels, resulting in ATP release. A dimer of T1R taste GPCRs (sweet, umami) is shown here. T2R taste GPCRs (bitter) do not have extensive extracellular domains and it is not known whether T2Rs form multimers. (B) In presynaptic (Type III) cells, organic acids (HAc) penetrate through the plasma membrane and acidify the cytoplasm where they dissociate to acidify the cytosol. Intracellular H+ is believed to block a proton-sensitive K channel (as yet unidentified) and depolarize the membrane. Voltage-gated Ca channels would then elevate cytoplasmic Ca^{2+} to trigger exocytosis of synaptic vesicles (not depicted). (C) The salty taste of Na+ is detected by direct permeation of Na+ ions through membrane ion channels, including ENaC, to depolarize the membrane. Modified and adapted from Chaudhari and Roper 2010.

1.5.5 Tomato taste

Tomato taste is achieved by a balanced combination between sugars, organic acids and their interactions in tomato fruit (Stevens *et al.,* 1977a). Reducing sugars (glucose and fructose) and sucrose are main factors responsible for sweetness in tomato. Sourness is due to organic acid content, mostly malic and citric acids. High sugars and acids are necessary for the best flavour and are widely accepted by consumers (Hobson, 1981). Stevens and collegues (Stevens *et al.,* 1977b) reported an effect of locular tissue on flavour. It was proven that differences in composition between pericarp and locular tissue have a massive effect on flavour.

Tomato cultivars with large locular tissue, with high concentration of acids and sugars, have been reported to have a good flavour quality. In ripe tomato mostly glucose and fructose was found (Winsor, 1966), with contribution up to 50% of total soluble solids. Citric acid was found to be the main organic acid in ripe tomatoes and during maturation it level increases (Carangal *et al.,* 1954).

1.5.6 Aroma

Creation of odour can be defined as a complex quality sensation caused by a mixture of volatiles. Ripe fruit contains a significantly high number of volatiles, mainly due to breakdown of some macromolecules and generation of volatiles *de novo*. These volatiles might be aldehydes, alcohols, ketones and esters and their precursors are mostly amino acids, carotenoids and fatty acids.

1.5.6.1 Tomato volatiles

Based on chemical structures we can separate three sources for tomato volatiles. These are amino acids, carotenoids and lipids. Volatiles in

tomato may serve as a cue to link the fruit with its nutritional components (Goff and Klee, 2006).

Over 400 volatile compounds have been identified in tomato, but no single compund has been directly related to tomato flavour (Buttery and Ling 1993). These volatiles are usually concentrated in the pulp and locular gel of tomatoes, but not in the skin and seeds. (Buttery *et al.,* 1988). This specific localisation could be explained by the fact that the locular gel contains the seeds, and concentrating volatiles in this specific region could greatly faciliate seed dispersal.

Most of the volatiles are produced after fruit will reach ripeness, this may be caused by a lack of substrate availability or it may be due to localisation of the enzymes responsible for volatile production in different compartements. These enzymes start producing volatiles after tissue disruption (Buttery and Ling, 1993). The tricarbolic acid cycle (Figure 1.11) (Carrari and Fernie 2006) is a major biochemical cycle involved in production of amino acid related volatiles and compounds related to shikimate pathway.

The impact of individual volatile compounds on flavour can be ranked according to their log odour units. Log odour unit is expressed as a ratio of measured concentration of the compound to its detection threshold. Usually compounds with low detection thresholds are recognized by the smell better than the high thresholds. Detection limits can vary, depending on the solution that was used during evaluation (Tandon *et al.,* 2000). During measurements of tomato volatiles in different media like water, methanol/ethanol/water solution and deactivated tomato homogenate, it was reported that the detection threshold increased from water, to alcohol and homogenate. This was due to the fact that viscosity and polarity of the solutions changed, and

solution mediums had different affinities for volatile compounds. If the ratio between concentration and detection threshold of a compound is greater than one, the volatile is said to have a positive log odour unit and positively contributes to flavour (Tandon *et al.,* 2000). Table 1.5 presents some volatiles from freshly ripened tomatoes.

Table 1.5 Concentrations of volatiles, odour thresholds and log odour units in fresh ripe tomatoes (Buttery, 1993). Log odour units are defined as the relative odour contribution in fruit. Odour threshold is the minimum physical intensity detected by sensory analysis. Odour unit is calculated by dividing the concentration of a compound (in ppb) by compound's detection threshold (in ppb).

Compound	Log odour units	Odour threshold (ppb in water)	Concentration (ppb in fresh tomato)
Z-3-Hexenal	3.7	0.25	12,000
β-ionone	2.8	0.007	4
Hexanal	2.8	4.5	3,100
β-damascenone	2.7	0.002	1
1-penten-3-one	2.7	1	520
3-methylbutanal	2.1	0.2	27
E-2-hexenal	1.2	17	270
2-isobutylthiazole	1.0	3.5	36
1-nitro-2-phenylethane	0.9	2	17
E-2-heptenal	0.7	13	60
phenylacetaldehyde	0.6	4	15
6-methyl-5-hepten-2-one	0.4	50	130
Z-3-hexenol	0.3	70	150
2-phenylethanol	0.3	1,000	1,900
3-methylbutanol	0.2	250	380
Methyl salicylate	0.08	40	48
Geranylacetone	-0.02	60	57
β-cyclocitral	-0.2	5	3
1-nitro-3-methylbutane	-0.4	150	59
Geranial	-0.4	32	12
Linalool	-0.5	6	2
1-penten-3-ol	-0.6	400	110
E-2-pentenal	-1.0	1,500	140
Neral	-1.2	30	2
Pentanol	-1.5	4,000	120
Pseudoionone	-1.9	800	10
Hexanol	-1.9	500	7

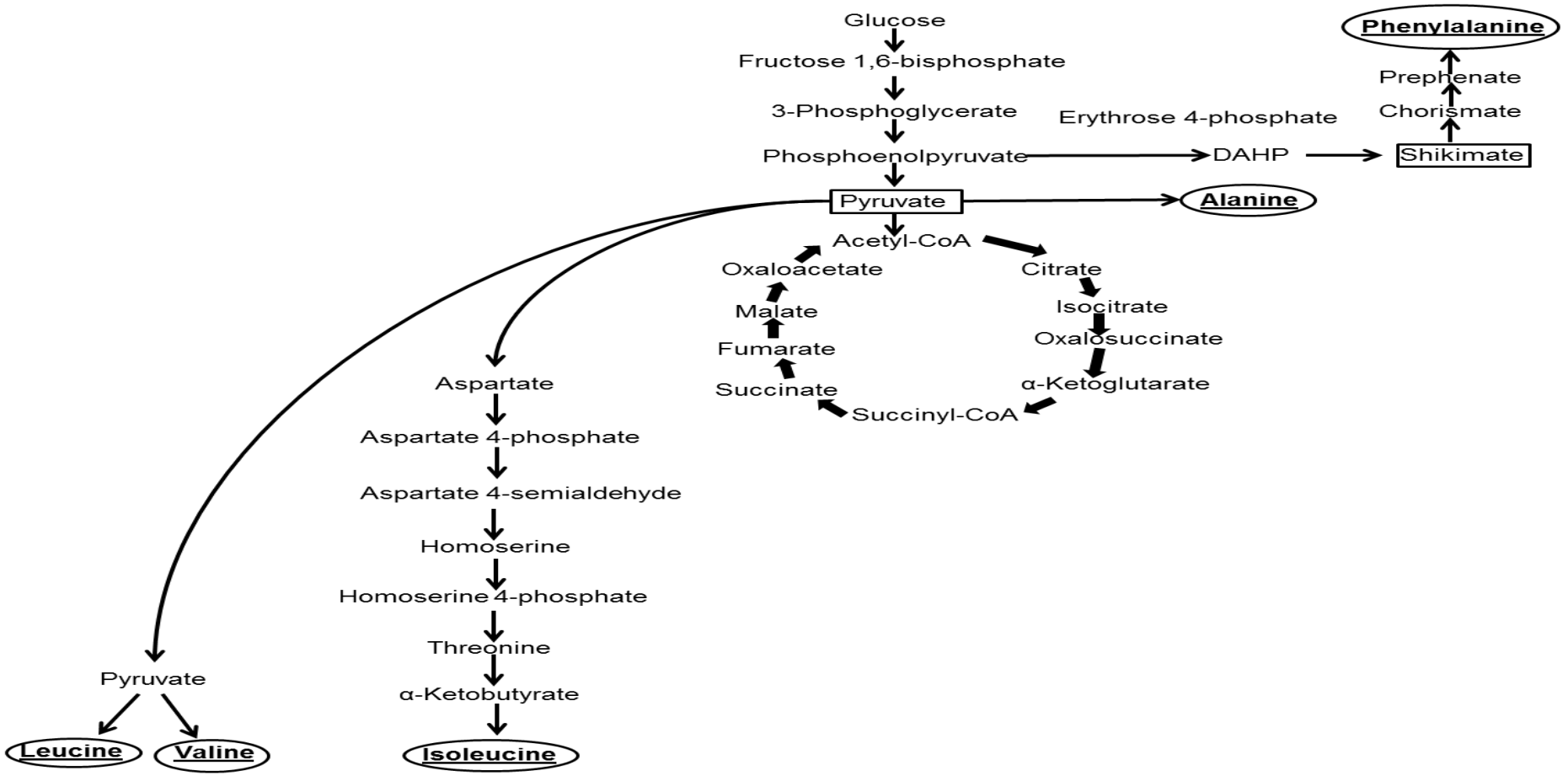


Figure 1.11 TCA cycle. Pyruvate and shikimate are major precursors (shown in boxes) of amino acids (in oval). Drawn based on Carrari and Fernie 2006.

1.5.6.2 Tomato aroma

Tomato aroma is influenced by compounds that have an impact on flavour, mostly aldehydes, ketones and some alcohols. These chemicals are produced in plant tissues during ripening by biosynthetic pathways. Secondary volatile compounds are generated in small quantities in intact cells, but are formed at high rates during cell disruption by crushing, cutting and slicing (Goodenough, 1990). Process of lipid oxidation is a major biogenesis pathway for the creation of volatiles in tomato fruit after maceration (Boukobza *et al.,* 2001; Boukobza and Taylor, 2002; Leahy and Roderick, 1999). Volatiles are compounds that have low molecular weights and therefore evaporate quickly at room temperature.

After inhalation they dissolve in the mucus and are carried by olfactory binding proteins to the olfactory receptor cells located in the nasal cavity. Volatiles bind to olfactory receptors and then a G-protein signaling cascade is activated (Mombaerts, 1999). Olfactory receptor cells are neurons and are connected with the olfactory bulb located in the brain. The olfactory cortex and limbic system are the two regions of the brain that receive olfactory signals. The olfactory cortex is responsible for perception and discrimination of the smells, limbic system is associated with emotions related to the smells. However, in comparison with taste receptors, responding to all classes of taste, each class of olfactory receptors responds to unique set of volatiles (Hallem *et al.,* 2004). Moreover, the olfaction process may be more complex, due to the fact that one volatile compound is caplable of activating more than one type of receptor. This complexity of aroma perception, is caused mainly by the fact that there is a large number of genes encoding olfactory receptors. About 500 to 750 genes encode olfactory receptors in humans, in mice this

number is higher reaching 1000, it is more than immunoglobulin and T-cell receptor genes (Moembarts, 1999).

1.5.6.3 Aroma precursors

Amino acid related volatiles

Metabolism of amino acids is the source of aliphatic and branched chain alcohols, acids, esters and carbonyls (Wyllie et al., 1996), all contributing to tomato flavour. Formation of these chemicals occurs during ripening process in the intact tomato fruit, there is little evidence for the creation of these compounds after maceration. Buttery and colleagues (Buttery *et al.,* 1987) suggested that these compounds are generated between breaker and ripe stages. The main amino acid sources of aroma compounds in tomato are: alanine, isoleucine, leucine (Figure 1.12) phenylalanine (Figure 1.13) and valine. Figure 1.14 presents the pathway for methyl salicylate, derived from a shikimate pathway. Table 1.6 shows amino acids derived volatiles.

Table 1.6 Amino acids related volatiles in fresh tomato fruits (from Buttery and Ling, 1993)

Amino acid	Volatile compound	Concentration (ppb in tomato)
Alanine	Acetaldehyde	800
Valine	1-nitro-2-methylpropane	5
Leucine	3-methylbutanol	150-380
	3-methylbutyric acid	200
	3-methylbutanal	27-65
	3-methylbutylnitrile	13-42
	1-nitro-3-methylbutane	59-300
	2-isobutylthiazole	36-110
Isoleucine	2-methylbutanol	100
	2-methylbutyric acid	5
Phenylalanine	Phenylacetaldehyde	15-18
	2-phenylethanol	1000
	1-nitro-2phenylethane	17-54
	Phenylacetonitrile	3-8

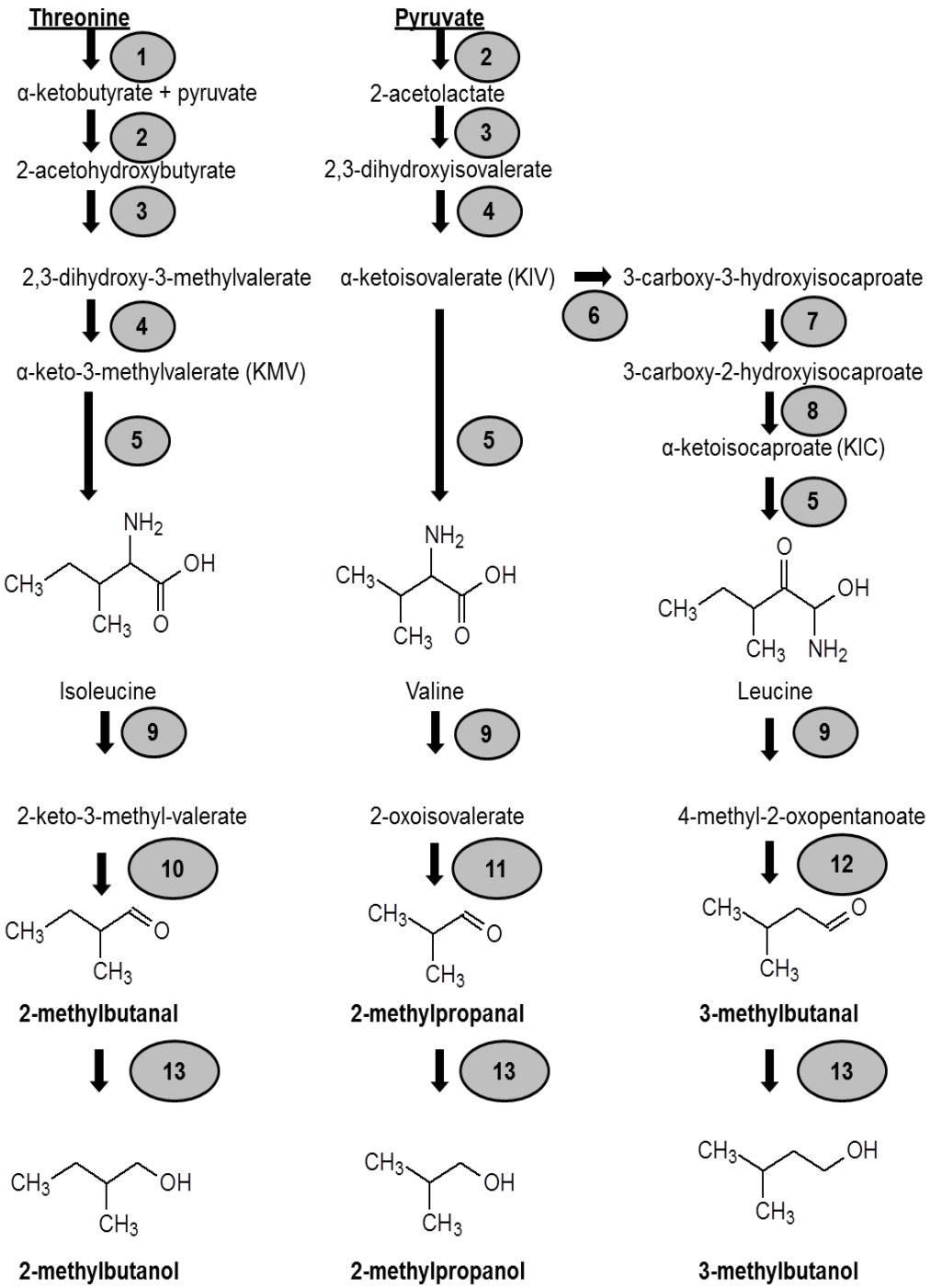


Figure 1.12 Amino acids pathway leading to formation of some volatiles. The chemical structures of the amino acids and volatile compounds are drawn. Enzymes: 1 - Treonine deaminase, 2 - Acetolactate synthase, 3 - Acetolactate isomeroreductase, 4 - Dihydroxy-acid dehydratase, 5 - Branched-chain aminotransferase, 6 - 2-isopropyl malate synthase, 7 - 2-isopropyl malate isomerase, 8 - 2-isopropyl malate dehydrogenase, 9 - Oxoglutarate aminotransferase, 10 - 2-oxo acid decarboxylase, 11 - Keto isocaproate decarboxylase, 12 - α-Ketoisovalerate decarboxylase, 13 - Alcohol dehydrogenase. Pathway drawn based on Kochevenko and Fernie 2011; Maloney *et al.,* 2010.

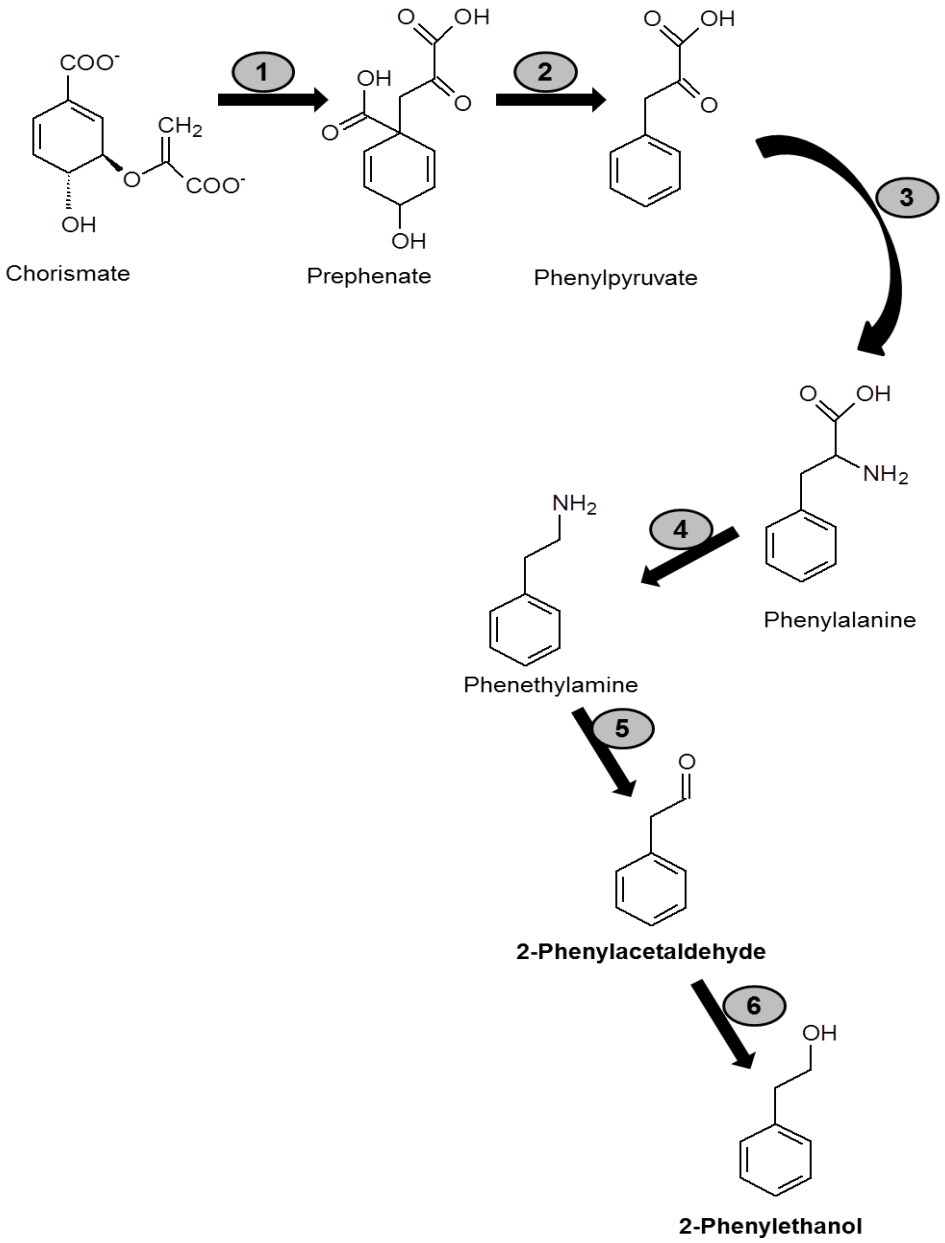


Figure 1.13 Biochemical pathway for phenylalanine derived volatiles (2-phenylacetaldehyde and 2-phenylethanol). Enzymes: 1 - Chorismate mutase, 2 - Prephenate dehydratase, 3 – Aminotransferase, 4 - Aromatic aminoacid decarboxylase, 5 - Monoamine oxidase, 6 - Phenylacetaldehyde reductase. Drawn based on Tieman *et al.,* 2006; Tieman *et al.,* 2007).

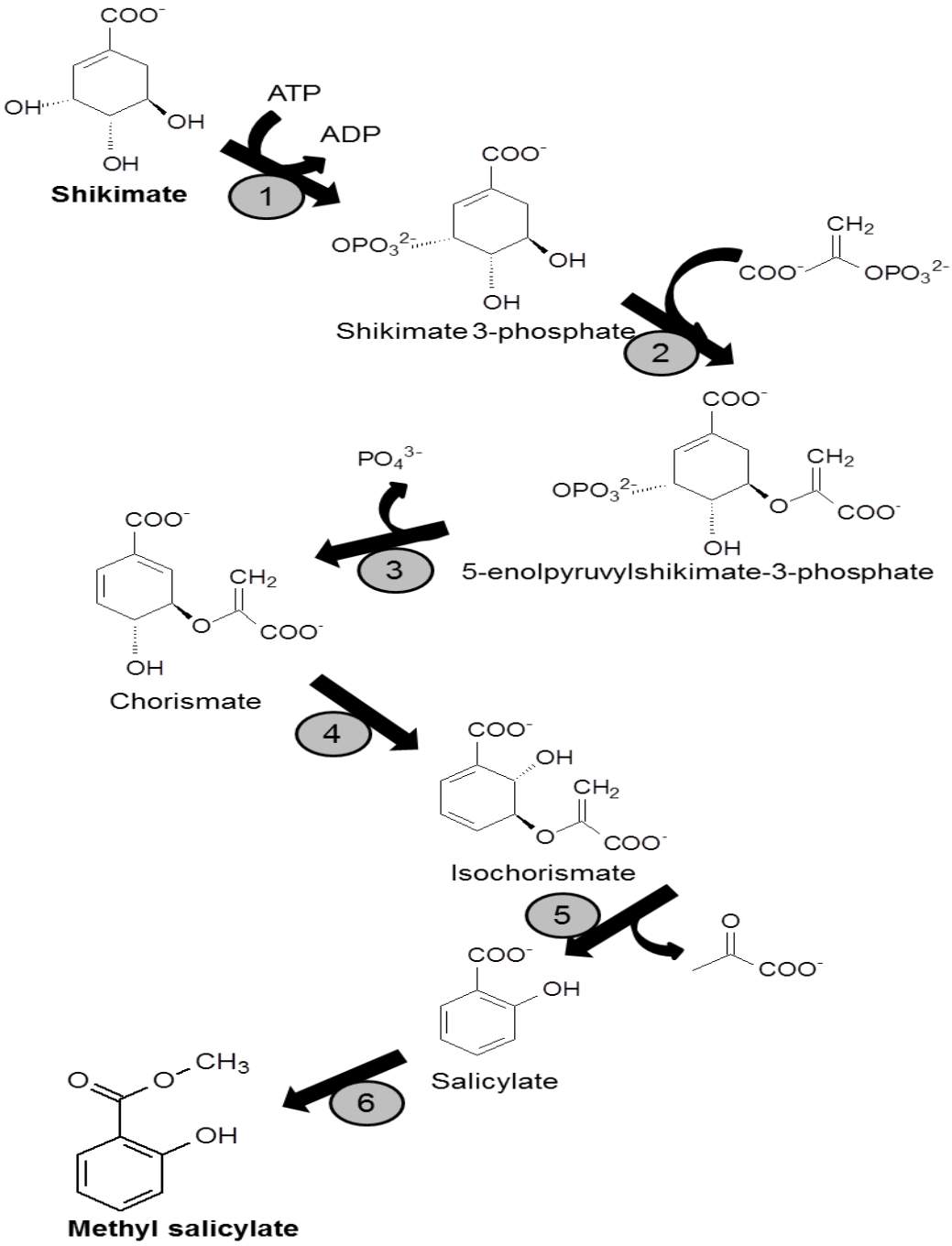


Figure 1.14 Shikimate pathway leading to creation of methyl salicylate. Enzymes: 1 - Shikimate kinase, 2 - 3-Phosphoshikimate1-carboxyvinyl transferase, 3 - Chorismate synthase, 4 - Isochorismate synthase, 5 - Isochorismate pyruvate lyase, 6 - Salicylic acid carboxyl methyltransferase. Drawn based on Kerbach *et al.,* 2005; Tieman *et al.,* 2010

Terpene related compounds

In most cases terpenoids are stored in plants as glycosides and therefore are non-volatile. They are usually present in leaves, in plastids and other parts of the plant, but they are not so common in tomato fruit. In Table 1.7 the terpenoids volatiles are shown. Lewinsohn and colleagues (Lewinsohn *et al.,* 2001) suggested geranyl pyrophosphate as an intermediate precursor that is converted to many monoterpens such as linalool and 8-hydroxylinalool. Biosynthesis pathway of geranial is presented on Figure 1.15.

Table 1.7 C10 and C 15 terpenoid volatiles in blended tomato leaves and fruit (from Buttery and Ling, 1993).

Compound	Concentration (ppb in leaves)	Concentration (ppb in fruit)
α-Pinene	100	<1
(+)-2-Carene	1700	<1
Limonene	1000	<1
β-Phellandrene	8000	<1
Linalool	<10	2
Neral	<5	2
(-)-α-capaene	<5	12
Caryophyllene	350	<1
Humulene	250	<1
Carophyllene epoxide	64	<1

Table 1.8 Lycopene and β-Carotene concentration in different tomato products. Modified and adapted from Ryan *et al.,* 2009.

	Lycopene (µg/100g)		β-Carotene (µg/100g)		Total (µg/100g)	% Total Lycopene	% Total β-Carotene
Tomato product	**Mean**	**SE**	**Mean**	**SE**			
Raw tomatoes	1716	181	538	47	2254	76	24
Canned tomatoes	3740	436	651	88.5	4391	85	15
Ketchup	3397	246	772	105	4169	81	19
Relish	2989	174	400	59.3	3389	88	12
Tomato juice	2530	182	554	30.2	3084	82	18
Bolognese sauce	2115	185	935	14.4	3050	69	31
Mixed vegetable juice	1366	106	3311	13.5	4677	29	71

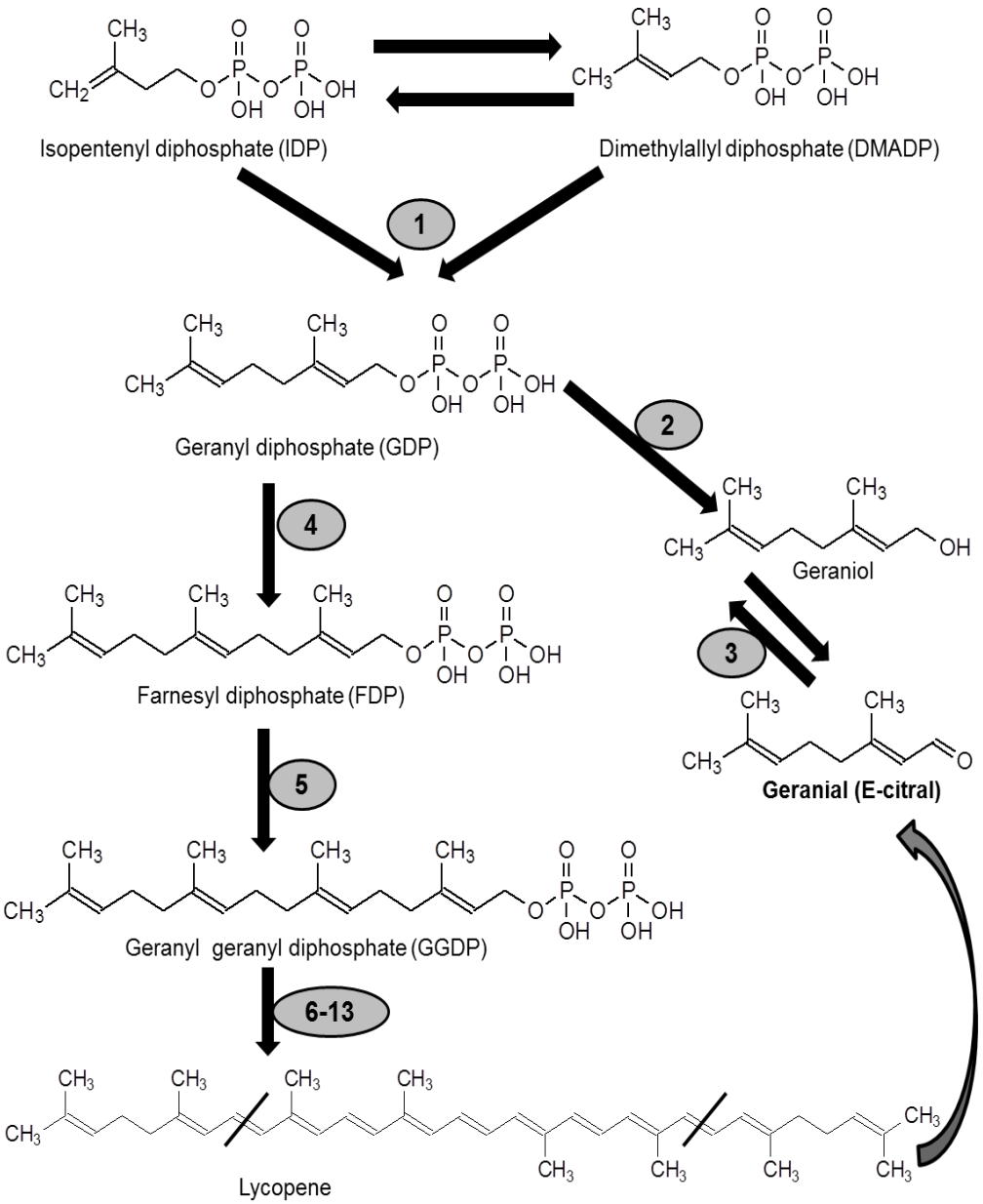


Figure 1.15 Biochemical pathway leading to formation of geranial. Enzymes: 1 - Geranyl diphosphate synthase, 2 - Geraniol synthase, 3 - Geraniol dehydrogenase, 4 - Farnesyl diphosphate synthase, 5 - Geranyl geranyl diphosphate synthase, 6 - Prephytoene synthase, 7 - Phytoene synthase, 8 - Phytoene desaturase, 9 - Phytofluene desaturase, 10 - Carotene isomerase, 11 - Carotene desaturase, 12 - Neurosporene desaturase, 13 - Prolycopene isomerase. Alternative pathway indicated by grey arrow. Black lines show cleavage sites in lycopene for creation of geranial. Drawn based on Lewinsohn *et al.,* 2005.

Lycopene is the source of C10 oxygenated terpenoids in blended fresh fruits. Buttery and Ling (Buttery and Ling, 1993) suggested here terpenoids like linalool, neral, geranial. In fully ripened tomato carotenoids consist of 60% of lycopene and 20% of β-carotene (Bramley 1997) although in different tomato products it may vary (Table 1.8, Ryan *et al.,* 2009). Buttery and Ling (Buttery and Ling, 1993) suggested that terpenes and terpenes-like compounds are formed through oxidative decomposition of carotenoids. 6-methyl-5-hepten-2-one and geranylacetone are the main volatiles generated from degradation of lycopene. Thermal breakdown of lycopene leads to formation of branched ketonic volatile compounds. β-carotene is a source of C9 to C13 cyclic compounds, they are shown in Table 1.9

Table 1.9 Tomato volatiles derived from carotenoids (from Buttery and Ling, 1993)

Compound	Concentration, ppb (intact fruit)	Concentration, ppb (macerated fruit)
Open chain		
6-methyl-5-hepten-2-one	100	210
6-methyl-5-hepten-2-ol	8	8
Geranylacetone	20	330
Pseudoionone	11	6
Cyclic		
2, 2, 6-trimethylocyclohexanone	<5	<5
β-Cyclocitral	3	5
β-Damascenone	<5	<5
β-Ionone	11	18
Epoxy-β-ionone	<5	<5

Compounds related to lignin

Phosphoenol pyruvate and D-erythrose phosphate are precursors of shikimic acid in plants. Shikimate is later converted into chorismate, which is an intermediate, in aromatic compounds that are related to lignin derived compounds such as cinnamaldehyde and benzaldehyde (Fisher and Scott, 1997). Kazeniac and Hall (Kazeniac and Hall, 1970; Ishida *et al.,* 1998; Yilmaz 2001) have found that there are other lignin-related chemicals, to include eugenol, guaiacol and methyl salicylate.

Fatty acid derived volatiles

Metabolism of fatty acids may be very beneficial for plant development. Phospholipases release free fatty acids from phospholipids (Laxalt and Munnik, 2002), but lipases can also release free fatty acids from triglycerides. These fatty acids may be involved in pathways of lipid oxidation, plant defense as well as creation of flavour compounds (Feussner *et al.,* 1997). Lipoxygenases (LOX) are involved in the generation of C6 volatile alcohols and aldehydes in tomato, through lipid oxidation pathways (Figure 1.16) Linoleic (C18:2) and linolenic (C18:3) acids are the main substrates of lipoxygenase pathway. The sequential action of enzymes initiated by lipolytic acyl hydrolase (LAH) leads to release of free fatty acids. These fatty acids are then oxidised to create acids hydroperoxides (HPOs) by LOX. HPOs are later cleaveged to generate short chain aldehydes and oxo-acids and this process is possible via fatty acid hydroperoxide lyase (HPL) activity (Yilmaz 2001).

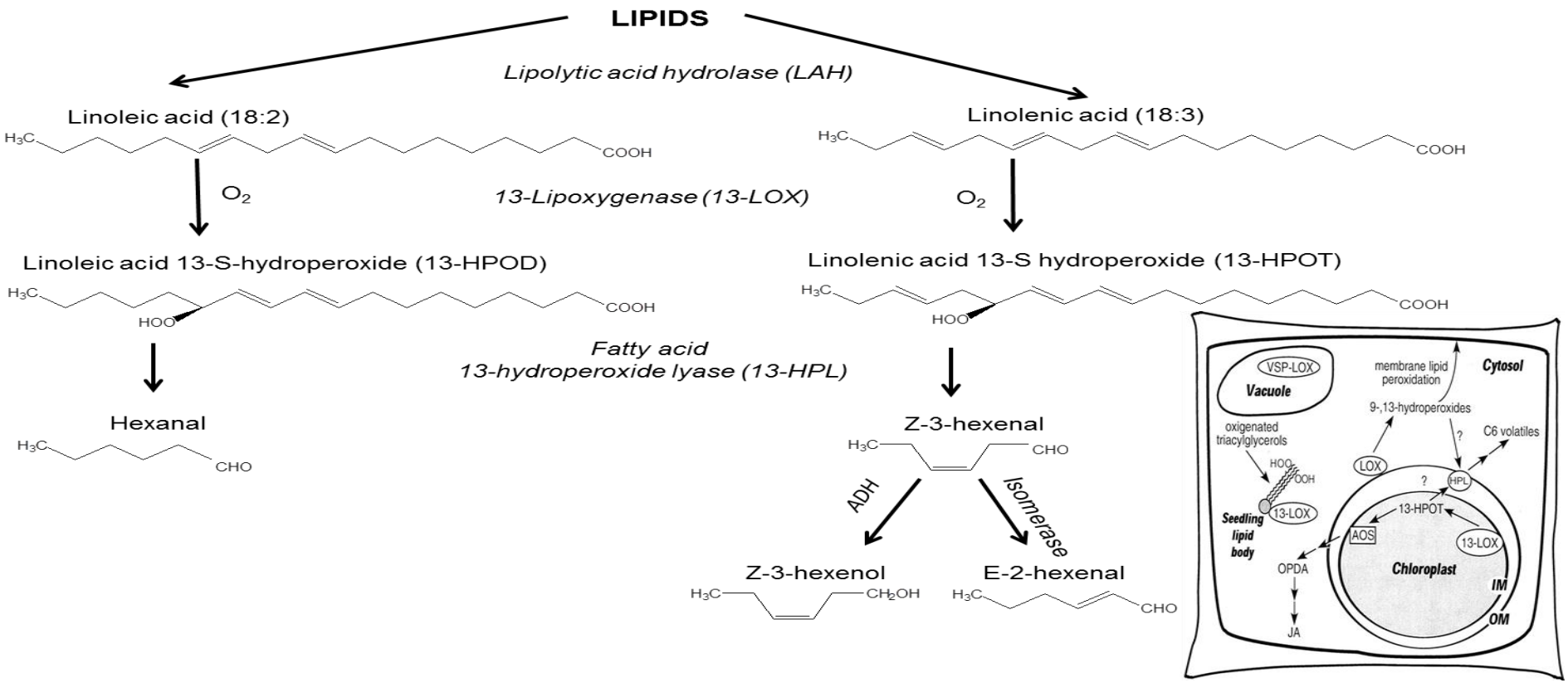


Figure 1.16 C6 volatiles formed in plants through lipoxygenase pathway (redrawn from Matsui *et al.,* 2001) and proposed localisation of the pathway (modified and adapted from Porta and Rocha-Sosa 2002). Lipids are hydrolysed to free fatty acids (linoleic and linolenic), which are then later converted to relevant hydroperoxides with aid of lipoxygenase. The next step in the pathway via lyase activity, leads to creation of C6 aldehydes (hexanal and Z-3-hexenal).

Tomlox

LOX genes have been isolated from tomato fruits; they were named *TomloxA*, *TomloxB* (Ferrie et al., 1994), *TomloxC*, *TomloxD* (Heitz *et al.,* 1997) and *TomloxE* (Accession No AY008278). However, Griffiths and colleagues (Griffiths *et al.,* 1999) reported that only three genes are expressed at significant levels through ripening period, and they may have different functions. These genes are *TomloxA*, *TomloxB* and *TomloxC*. *TomloxC* is a chloroplast targeted lipoxygenase isoform, it can use as substrates both linoleic and linolenic acids and generate C6 volatiles (Chen *et al.,* 2004). *TomloxD* was found in leaves and its levels increased in case of wounded leaves (Heitz *et al.,* 1997). *TomloxD* may be involved in defence signalling as a response to pathogen and herbivore attack (Hu *et al.,* 2011).

HPL

Hydroperoxide lyase (HPL) is the other enzyme involved in lipoxygenase pathway. It is responsible for the cleavage of a C-C bond very near to a hydroperoxide group of polyunsaturated fatty acids to form short chain aldehydes. It was reported that the majority of the HPLs are substrate specific and use 13-hydroperoxides of α-linolenate and linoleate; however, in cucumber and melon, HPLs can modify both 9-hydroperoxide as well as 13-hydroperoxide (Grechkin, 2002). HPL purified from bell pepper fruits, has a heme protein which spectrophotometric properties are similar to cytochrome P-450 (Shibata *et al.,* 1995; Matsui *et al.,* 1996).

ADH

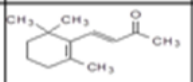
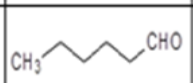
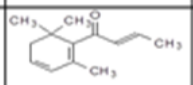
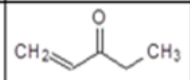
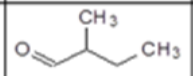
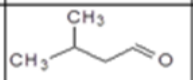
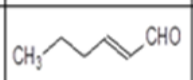
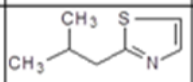
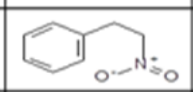
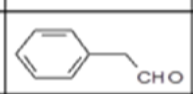
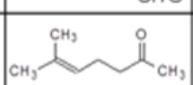
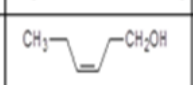
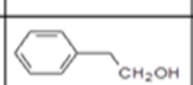
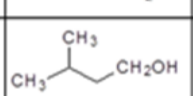
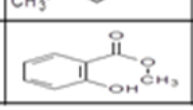
In tomato, an important task in conversion of aldehydes to alcohols in ripening fruit is performed by alcohol dehydrogenase (ADH) (Prestage *et al.,* 1999, Speirs *et al.,* 1998). There is a multigene family for ADH and three ADH genes were discovered. ADH1 is expressed in pollen, seeds, as well as young

seedlings; however, ADH2 was found, to be accumulated in different parts of tomato to include ripe fruit (Chen and Chase, 1993). Speirs and colleagues (Speris *et al.,* 1998) genetically manipulated levels of ADH2 in ripening tomato fruit and proved that such a manipulation, affects the balance between some of the flavour aldehydes and alcohols and that increased ADH2 were giving fruits with more intense, characteristic ripe fruit flavour. Experiments conducted on transgenic plants failed to show significant differences in alcohol levels in comparison with control fruits, but down regulation revealed, that there were higher aldehyde to alcohol ratios (Prestage *et al.,* 1999).

C6 volatiles

C6 volatiles are the major compounds identified in macerated tomato fruit (Buttery, 1993). Over 400 chemicals contribute to tomato flavour, but only a combination of 10 major volatiles were considered very close, in terms of flavour, to a sliced, fresh, ripe tomato (Buttery, 1993). These compounds are: Z-3-hexenal, E-2-hexenal, hexanal, 1-penten-3-one, 3-methylbutanal, Z-3-hexenol, 6-methyl-5-hepten-2-one, methyl salicylate, 2-isobutylthiazole and α-ionone. Chemical structures, concentration in some of tomato fruits and odour thresholds are presented in Table 1.10.

Table 1.10 Selected volatile compounds, structures and their precursors (Adapted from Goff and Klee 2006). Shown are volatile chemicals positively contributing to tomato flavour. The rank order of volatiles is based on the work of Buttery and Ling. Odour thresholds in parts per billion in water (ppb) are taken from Leffingwell and associates.

Volatile	Structure	Precursor	Pathway	Odour threshold (ppb)
Z-3-Hexenal		Linolenic acid	Lipoxygenase	0.25
β-Ionone		β-carotene	Carotenoid	0.007
Hexanal		Linoleic acid	Lipoxygenase	5
β-Damascenone		Neoxanthin	Carotenoid	0.002
1-Penten-3-one		Linolenic acid	Lipoxygenase	1
2-Methylbutanal		Isoleucine	Amino acid	1
3-Methylbutanal		Leucine	Amino acid	0.2
E-2-Hexenal		Linolenic acid	Lipoxygenase	17
2-Isobutylthiazole		Unknown	Unknown	3.5
1-Nitro-2-phenylethane		Phenylalanine	Phenylalanine	2
E-2-Heptenal		Fatty acid	Lipoxygenase	13
Phenylacetaldehyde		Phenylalanine	Phenylalanine	4
6-Methyl-5-hepten-2-one		Carotenoid	Carotenoid	2000
Z-3-Hexenol		Linolenic acid	Lipoxygenase	70
2-Phenylethanol		Phenylalanine	Phenylalanine	750
3-Methylbutanol		Leucine	Amino acid	120
Methyl salicylate		Phenylalanine	Salicylic acid	40

1.5.6.4 Temporal differences in volatile release

Boukobza *et al* (2001) have observed a temporal difference in the release of tomato volatiles when fruits are macerated. Two types of release behaviour occurred. Some compound showed very fast release immediately after maceration, with maximum concentration around the first 30s (Figure 1.17a). The concentration of other compounds, increased steady, reaching maximum concentration after 2 min (Figure 1.17b). Major volatiles that belong to the first group are as follows: 2-isobuthylthiazole, 6-methyl-5-hepten-2-one, methylbutanal, methylbutanol, 1-penten-3-ol. Major volatiles belonging to the second group are hexenal, hexanal and hexenol, 1-penten-3-one. Fast and dynamic release of the first group of volatiles suggests that they were preformed prior to maceration. The second release pattern involved compounds, which were derived from the lipid oxidation pathway (Boukobza *et al*. 2001).

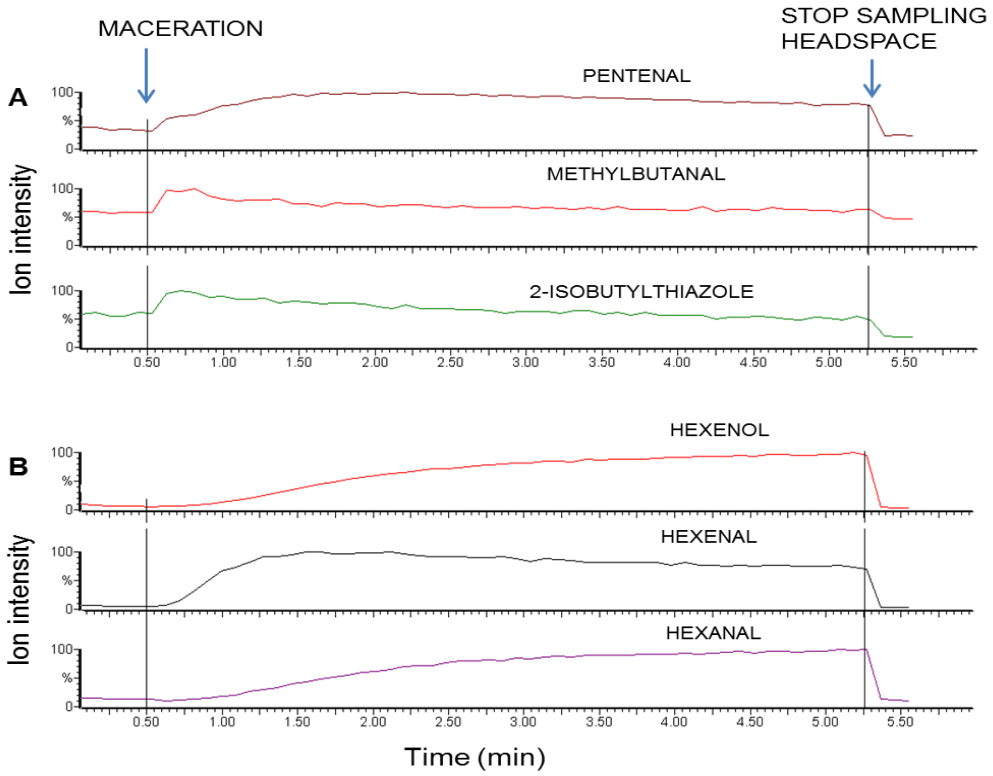


Figure 1.17 Time course release of six different volatile compounds from tomato after maceration of the tissue (t=0.50 min); intensity expressed on relative scale. (A) - Compounds showing very fast release, immediately upon maceration with maximum concentration after around 30s; (B) - compounds with steady release, reaching maximum concentration after 2 min. Data was taken for M82D control fruit.

1.6 Metabolomics

1.6.1 Metabolomics overview

Knowledge of the biosynthetic pathways for the production of critical nutrients (minerals, vitamins, fibres, antioxidants) is of essential importance (Carrari and Fernie 2006). The model system such as *Arabidopsis thaliana* may be a good starting point in terms of regulatory mechanisms that affect development and also ripening (Liljergen *et al.,* 2004). Arabidopsis system, does not reflect very well on development and ripening in other species, to include the tomato (Carrari *et al.,* 2006), even the species of the same family

may be subject of remarkable developmental changes (Fernie and Willmitzer, 2001)

Tomato (*Solanum lycopersicum*) has arisen as the principal experimental model for study the development and ripening of fleshy fruits (Giovannoni, 2004; Fernandez *et al.,* 2009). This reflects its economic importance and many favourable genetic characteristics, to include small genome size (950 Mb), a moderately short life cycle, and routine transient and stable genetic transformations (Osorio *et al.,* 2011).

The development and maturation of tomato fruits have been extensively studied and resulted in the identification of specific genes that participate in ripening (Vrebalov *et al.,* 2002; Manning *et al.,* 2006; Wang *et al.,* 2009; Karlova *et al.,* 2011). Moreover, much has been learned about the substantial changes in both primary and secondary metabolism that accompany tomato fruit ripening, however much of these data have not been directly related to regulatory events (Carrari and Fernie, 2006) and often are related to specific pathways. Tomato development has also been studied at the levels of transcriptome (Alba *et al.,* 2005; Carrari *et al.,* 2006; Wang *et al.,* 2009), proteome (Faurobert *et al.,* 2007; Page *et al.,* 2010), and metabolome (Roessner-Tunali *et al.,* 2003; Carrari *et al.,* 2006; Fraser *et al.,* 2007). In addition, up until now, only few approaches have combined multilevel strategies (Carrari *et al.,* 2006; Enfissi *et al.,* 2010), and those that have, are limited with regard to genotype, developmental stage, and the number of measured factors.

Metabolomics approach can aid in identification of correlation between transcript and metabolites. Carrari and colleagues (2006) established that sugar phosphates, organic acids, and pigments are all highly correlated to

specific ripening associated transcripts. This study has substantial biotechnological implications because the manipulation of cellular levels of transcripts is within feasible target.

1.6.2 Technology for metabolomics analysis

The metabolomics involves use of high throughput profiling and fingerprinting methods (Fiehn *et al.,* 2000a; Glassbrook and Ryals, 2001) capable of measuring the absolute or relative amounts of all metabolites. The great diversity of chemical properties in addition to wide scale of concentration these compounds presents a significant challenge because the methodologies used need to be robust and replicable allowing reliable sample comparison (Glassbrook *et al.,* 2000).

Rapid and general screening methods provide a “fingerprinting” library. Included here are: atmospheric pressure ionization (API-MS), NMR spectroscopy, and other methods such as Raman spectroscopy and Fourier transform infra-red spectroscopy, all of which provide complementary information. These techniques can be arranged as ‘high-throughput’ and are appropriate for differentiation and classification of samples (Halket *et al.,* 2004). Direct infusion API-MS has been used with success to classify a variety of samples (Castrillo, *et al.,* 2003; Overy *et al.,* 2004). In addition, Fourier transform mass spectrometry can provide even higher mass resolution and has been used to determine the relative amounts of over 6000 metabolites in ripening strawberries using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) with recording of both positively and negatively-charged ions (Goodenowe, 2001; Aharoni *et al.,* 2002).

Comprehensive metabolomics analysis can be conducted using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass

spectrometry (LC-MS), nuclear magnetic resonance (NMR) or matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). These techniques provide a detailed chromatographic profile of the sample and consequently measurements of the relative or absolute amounts of the components. The number of components measured will depend on the resolution of the chromatographic system and the specificity of the detection technique (Halket *et al.,* 2004). LC-MS allows for a high separation power of high-performance liquid chromatography (HPLC), separating compounds of high molecular weight, in contrast to GC-MS. Usually HPLC is combined with ultraviolet and visible light (UV/VIS) or a diode array detector. However coupling HPLC with MS introduces further selectivity, good detection and structural information of separated compounds (Kopka *et al.,* 2004). In LC-MS, compounds are introduced into a MS by electrospray ionisation (ESI), transferring compounds eluting from HPLC into a gas phase suitable for mass analysis. NMR uses magnetic properties of isotopes, with ability to detect a broad range of metabolites based on the fact, that hydrogen, carbon; phosphorous, oxygen and nitrogen are detectable using NMR technology (Bligny and Douce 2001). Software for NMR is well developed. It enables rapid processing of the obtained spectral data with convenient metabolite identification (Kopka *et al.,* 2004). The MALDI-TOF-MS was effectively used for acquisition of mass spectra for carotenoids, with sensitivity of pmol range (Fraser *et al.,* 2007).

1.6.3. Metabolomics and the tomato

Metabolomics analysis of tomato plants lead to several discoveries. Profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato lead to identification of high pigment line (hp-1) with elevated levels of chlorogenic acid, rutin and carotenoids (Long *et al.,* 2006). Metabolic

analysis of carbohydrates in tomato introgressions lines (IL, see next section for ILs description) lead to identification of line with increased soluble solids (Baxter *et al.,* 2005). Moreover metabolite profiling also indicated a major regulator of tomato ripening, APETALA2a (Karlova *et al.,* 2011). It was also associated with QTL (Schauer *et al.,* 2006). Potentially promising changes in tomato wild species (leaves and fruits) were also identified, that may affect stress response and have an impact on nutrition (Schauer *et al.,* 2005). The significant part of plant metabolome consist of volatile compounds and the number of individual volatiles identified in different plants is approximately 2000 (Dudareva *et. al.,* 2006). Profiling of volatile compounds in tomato lead to recognition of a role for different glycoconjugation related to emission of phenylpropanoid volatiles (Tikunov *et al.,* 2010). Large scale profiling of tomato fruit volatiles was also possible using non-targeted data analysis (Tikunov *et al.,* 2005). Analysis of pleiotropic ripening mutation in tomato suggested that the biggest variation between different tomato mutants, were the result of differences in the compounds (hexanal, E-2-hexenal, Z-3-hexenal) derived from the lipoxygenase pathway (Kovács *et al.,* 2009).

1.6.4 Experimental design for chemical and genetic basis of tomato flavour

The study of tomato flavour was undertaken using multidisciplinary approach, summarized in the body of this thesis. The workflow of the project is presented on Figure 1.18, highlighting the methodology and timescale for the project. Direct mass spectrometry known as atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was initially used as a fingerprinting technique to analyse a set of tomato introgressions lines (ILs). This procedure was performed over two years of study. Verification of compounds and quantification was undertaken using gas chromatography-mass spectrometry with solid phase micro extraction (GC-MS SPME). The data from these

experiments indicated important compounds that were further studied using sensorial analysis, to correlate the findings with consumer acceptance and establish the impact of volatiles in different tomato related matrices. The microarray approach, in combination with other molecular biology techniques, was used to identify a region on tomato chromosome (known as quantitative trait locus (QTL)) with a potential to improve flavour. QTL is a stretch of DNA linked or containing the genes underlying the quantitative trait. QTL analysis, has become an important tool that helps biologists to resolve the genetics of complex species. In order to begin a QTL analysis, required are two or more strains of organisms that differ genetically with regard to the trait of interest. Secondly, genetic markers that distinguish between these parental lines are needed. Molecular markers are preferred for genotyping, because these markers are unlikely to affect the trait of interest.

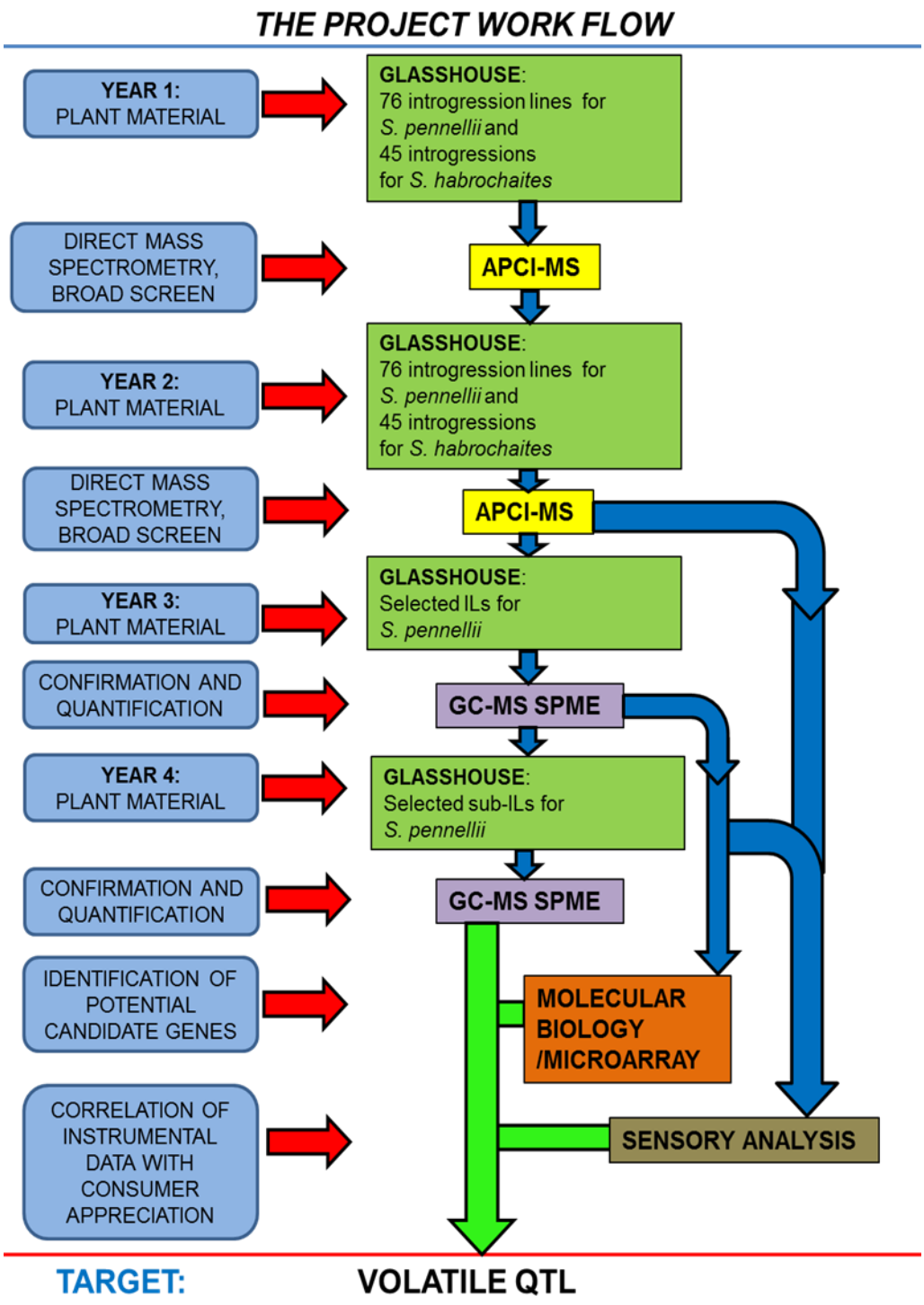


Figure 1.18 Project workflow. APCI-MS was performed during two years of experiments, followed by GC-MS SPME. The outcome was then used in a series of sensory experiments and in addition, the identified ILs were subject of molecular biology/microarray approach to identify tomato flavour QTL.

1.6.4.1 APCI-MS

APCI-MS was chosen to screen two tomato populations over two years of study because this system has several advantages. The direct mass spectrometry is robust, and sample analysis is simple. With a method presented in this thesis, it was possible to analyse up to 50 tomato fruits per day. Tomato fruit was macerated using specifically adapted blender connected to the APCI interface via a transfer line and heated probe. Compounds from the headspace (in the gas phase) entered the atmospheric region with aid of a gas from compressed air tank. In the atmospheric region ionisation occurred due to reactant ion. APCI works by initial formation of a reactant ion from water created by discharge from the corona pin (4kV). This ion can then transfer its charge to other molecules like in the reaction below:

$$\mathbf{H_3O^+ + R \rightarrow [R+H]^+ + H_2O}$$

Where:

H_3O^+ - hydronium ion

R - molecule

$[R+H]^+$ - protonated molecule

H_2O - water

This type of ionisation is considered soft, which means that there is very little or no fragmentation of the molecules. Aroma compounds mixtures will appear as single ions where the mass to charge ratios (m/z) for each are either one mass unit above the actual molecular weight of the compound.

Once the ions have been created, they travel into the region of a mass analyser, a quadrupole in this case, which consists of four parallel stainless

steel rods. An electrostatic field then causes the ions to oscillate with amplitude that is unique for each ion according to their mass to charge ratio (m/z). The mass spectrometer can be set beforehand in order to scan through all the possible electrostatic fields, e.g. in mixture analysis. Alternatively, just one ion mass can be selected in advance to be monitored by the mass analyser.

APCI-MS is a very stable analytical system. The maceration device allowed analysis of whole fruits and imitated the process of chewing a tomato, therefore being similar to the tomato consumption. The biggest disadvantage of this system is lack of ability to distinguish between isomers (position or stereoisomers) or the compounds with the same molecular weight. This happens because the isomers or compounds with the same molecular weight have the same molecular ion and mass to charge ratio (m/z). However, the cone voltage can be increased to subsequently enhance the degree of fragmentation of molecules with similar mass and therefore helping in their identification. The differences in isomerisation are presented on Figure 1.19

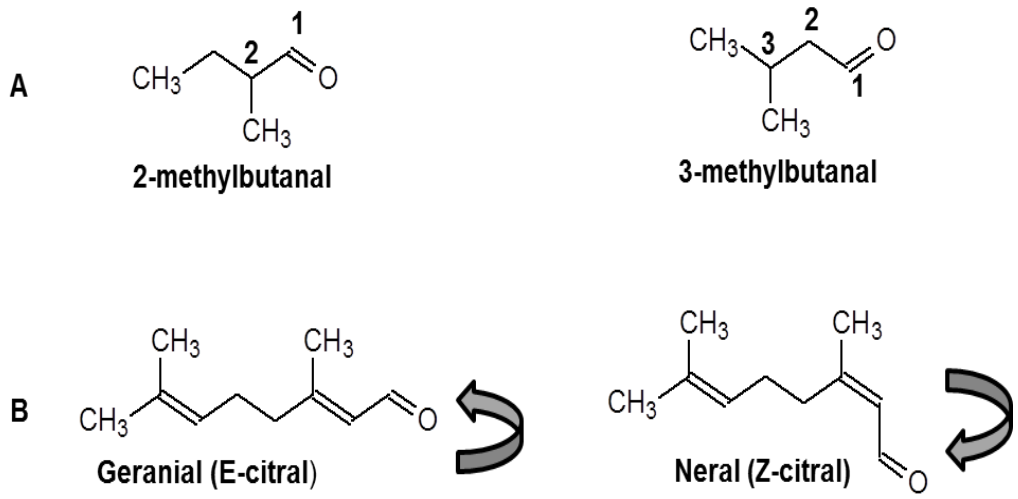


Figure 1.19 Different types of isomerisation relevant to APCI-MS. Structural (in this case positional) isomers, compounds differ in the position of functional group (A). Stereoisomers (in this case geometric/configuration isomers) compounds differ in the orientation of functional group (B). Numbers 1 to 3 refer to position of a carbon within the compounds structure, functional group is CH_3. Grey arrows indicate the change of orientation of functional group (functional group is COH).

1.6.4.2 GC-MS SPME

Gas chromatography-mass spectrometry is a common analytical tool with wide applications in biological research. GC can separate volatiles with high resolution, but it is not eligible for their identification. MS delivers structural information leading to identification, however do not provide separation. Only the combination of these two devices gives enough power for separation, identification, and quantification (Settle 1997).

In analytical gas chromatography, samples are introduced into GC-MS via injector port, kept hot (200°C) to retain compounds in the gas phase. Carrier gas (helium) also enters injector and is responsible for transport of compounds through the GC system. Volatiles move from hot injector into a cold column, and there they are separated due to a temperature gradient (40-260°C), because a temperature is gradually ramped up (via GC program).

Compounds leave the stationary phase and enter the gas phase when it is energetically convenient for them. Later, they are transferred into mass spectrometer (quadrupole), where molecules enter a high vacuum region. There compounds are bombarded by high-energy electrons and as a result fragmentation occurs. Created ions are than introduced to the analyser where an electric field isolates them based on their mass.

The two popular approaches for analysis of volatile compounds are either solvent or non-solvent (headspace) based. However, solvent extractions require a lot of sample preparation time and may interfere with volatile content, due to sampling procedures. Headspace technique is simpler and can be effectively related to real volatile composition in the tomato fruit. Non-solvent volatile extractions became very popular (Tikunov *et al.,* 2005; Tikunov *et al.,* 2010) after establishment of SPME technique (Pawliszyn, 1997). In the solid phase micro extraction, volatile compounds from specifically macerated tomato sample, were introduced into a GC injector using a SPME fibre via headspace extraction (Figure 1.20). The fibre is a form of reversed short chromatography column with extracting phase outside, very efficient for volatile compounds.

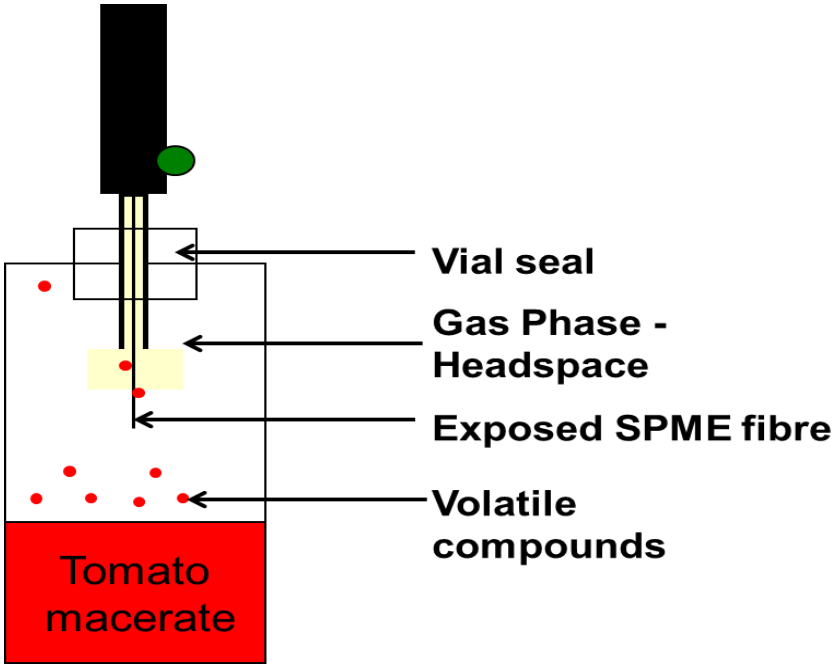


Figure 1.20 Extraction of volatile compounds from tomato macerate. Compounds migrate from the macerate into the headspace and then into absorbing, exposed fibre. Fibre is then desorbed into a GC-MS injector.

1.6.4.3 Molecular biology approaches towards identification of tomato volatile QTL

Microarray technology advanced from Southern blotting. In Southern procedure, a fragmented DNA is attached to a substrate and then probed with a known gene or fragment (Maskos and Southern 1992). Currently, microarrays are used for comparative gene expression (Moore *et al.,* 2005), to correlate compounds concentrations with enzyme activities (Di Matteo *et al.,* 2010).

The basic principle of microarray technology is the hybridization between two DNA strands, where complementary nucleic acid sequences pair together and form hydrogen bonds between complementary base pairs. The more of complementary base pairs the better bonding between two strands. Only successfully bonded strands are hybridized. As a result, fluorescently labelled target sequences that bind to the probe sequence are highly

dependent on hybridization indicated by fluorescence signal intensity. Hybridization may be affected by number of paired bases, conditions, and washing steps. The details of microarray technology are presented in Figure 1.21

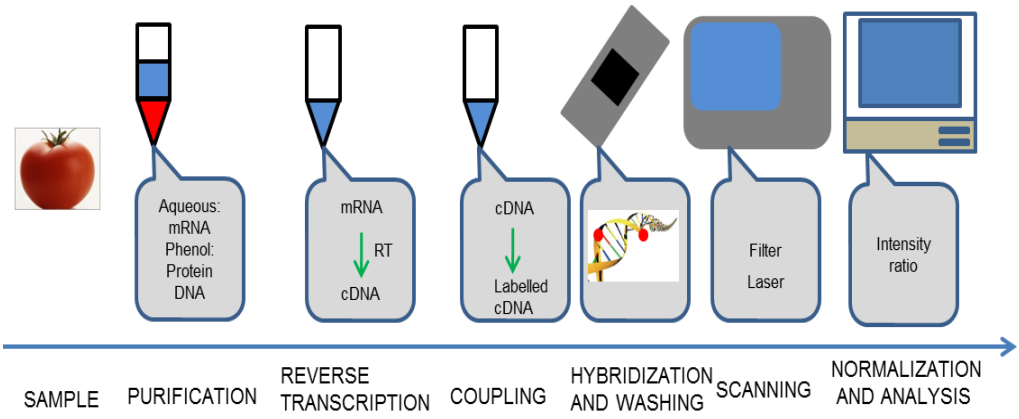


Figure 1.21 A schematic of microarray technology. Tomato sample is subject of purification; mRNA is isolated and converted to cDNA via reverse transcriptase. cDNA is later labelled with probes, hybridized and scanned. Results are evaluated using computer software.

1.6.4.4 Sensory analysis of tomato juice and tomato purée.

Sensory evaluation is a discipline that employs principles of experimental design and statistical analysis to the use of human senses (sight, smell, taste, touch, and hearing) for the purposes of evaluating consumer products. Sensory methods require panels of human assessors, who test the products, and record their responses. Application of statistical analysis to the obtained results provides interpretative and comprehensive information about the tested products. Using information from both APCI-MS and GC-MS SPME it was possible to nominate candidate volatiles and test their interactions in different matrices.

There are number of methods used for establishment of detection thresholds, but only two will be introduced here. Ascending limits is a fast method of determining threshold (Figure 1.22B), however, two errors can occur; the errors of habituation and the errors of anticipation. The error of habituation occurs when subjects develop a habit of responding to a stimulus (Lawless and Heymann 2010). The error of anticipation occurs when subjects prematurely report seeing the stimulus before the threshold has been reached. Clear instructions, demonstrations, and practice runs can reduce the errors of habituation. Errors of anticipation can be minimised by changing the starting intensity for each trial.

A variation of the method of limits is the staircase method (Figure 1.22A) which involves both the ascending and descending limits in a trial (Lawless and Heymann 2010). Stimulus intensity is gradually increased (ascending) until the subject reports the stimulus. At this point, the intensity value is recorded and the stimulus intensity is then progressively reduced (descending), until the subject does not report the stimulus. Threshold is considered the average of several of these reversal points. Threshold estimates using this method are also prone to the errors (habituation and anticipation) and consequently, multiple simultaneous staircases are used to minimise such errors.

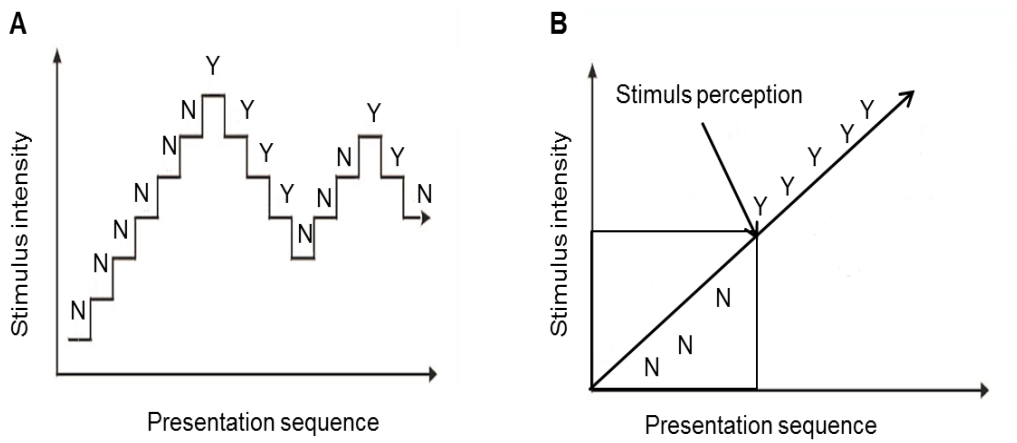


Figure 1.22 Common detection thresholds methods. Staircase method (A) involves both ascending and descending steps, while ascending method (B) is linear. Y-yes, stimulus is perceived, N – no, stimulus is not perceived.

In this study, an ascending method was chosen, due to its simplicity and quickness. To overcome error of habituation and anticipation, additional method was selected to confirm and verify established thresholds, known as a triangle test. In this test, two presented samples are controls and the third sample is the tested sample. Panelists were asked to identify the tested sample.

In addition to detection thresholds, hedonic evaluation and flash profile were used. Hedonic test provides information about panelists likes and dislikes of a product. They are not intended to evaluate specific characteristics, such as saltiness, or sweetness focusing on an overall perception. The test is subjective and includes comparison (with a reference sample), and scoring the results on the scale. The flash profile is an alternative sensory analysis technique adapted from Free Choice Profiling to understand the sensory positioning of products. Subjects select their own terms to describe and evaluate a set of products simultaneously, and then rank the products for each attribute that they individually create. The focus is

on the descriptive terms, not on the hedonic terms. The Flash profile is also both less time and money consuming, providing a good overview of tested samples.

1.7 Strategies for crop improvement

Currently food manufacturing is met by rigorous production of agricultural commodities. In addition, usage of large land area and efficient farming, provide sufficient quantities of food. However, current world population of nearly 7 billion (http://www.census.gov/main/www/popclock.html) with current problems affecting agriculture like pollution, fresh water availability or fuel availability, may have a substantial impact on the future crops. Domestication of crops leads to intensive selection process, and as a result, some important traits have been lost. Modern plant breeding reduced the levels of genetic variation (Fu 2007) and therefore remains a threat for the future crops. Several methodologies can be applied to overcome this problem to include here genetically modified organisms, use of available and future mutation populations and harvesting natural variation.

1.7.1 Genetically modified tomatoes

Genetically modified organism (GMO) is on organism, with altered genetic material using genetic engineering technology. The DNA may come from different source resulting in new set of genes. In some cases, DNA from different species can be successfully used to improve the important traits. In plant organisms, *Agrobacterium* is widely used to transfer genetic material to plants via horizontal gene transfer. The genes required in the plant are cloned into a plant transformation vector. It comprises the T-DNA region of the disarmed plasmid, together with a selectable marker (usually antibiotic

resistance) to enable selection for plants after successful transformation. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA included into their genome will die.

Genetically modified fruits with reduced polygalacturonase (PG) activity (<1% of normal PG), showed increased storage life and capability for withstanding transportation while the firmness was not affected (Schuch *et al.,* 1991). Manipulation of alcohol dehydrogenase (ADH) levels in ripening tomato fruit had an effect on the ratio of alcohol and aldehyde volatiles. With increased ADH activity, the amount of alcohols increased as well (Speirs *et al.,* 1998). Wang *et al.* (1996) have reported the introduction of a functional yeast Δ–9 desaturase gene into tomato. The introduced gene affected unsaturation of fatty acids in the fruit, which resulted in increased abundances of several of the volatiles discussed below. In fruit from one transgenic plant, hexanal and Z-3-hexenal concentrations were 2.7-and 2-fold higher, respectively, than levels in control fruit, whereas hexanol and Z-3-hexenol concentrations were both increased about 4-fold relative to controls. The tomato flavour can be improved by early diversion of terpenoid pathway in plastids, with increased geraniol content, but at the expense of reduced lycopene (Davidovich-Rikanati *et al.,* 2007; Mollet *et al.,* 2008).

The current EU legislation requires food products to be labelled as containing GM if they possess an unintended GM content of 0.9% or more (http://www.parliament.uk/documents/post/postpn211.pdf; 2004). The GM products are therefore allowed on the market, but a bad publicity behind GM (Bonny 2003; Hewlett *et al.,* 2008) makes this technology unprofitable.

1.7.2 Targeting Induced Local Lesions in Genomes (TILLING)

Targeting Induced Local Lesions in Genomes (TILLING; McCallum et al., 2000) was a new reverse genetics tool. Traditional chemical mutagenesis is followed by high-throughput screening for point mutations. Wide use of chemical mutagenesis for forward genetics in many species indicates that it can also be successfully used in TILLING (Henikoff *et al.,* 2004). Furthermore, as far as organism is concerned, TILLING is no different from traditional mutation breeding, so genetically modified organism issues do not arise. Overall, TILLING is an attractive strategy, with applications in functional genomics, but also in agriculture (Henikoff *et al.,* 2004). The strength and potency of this reverse genetic strategy has been validated by its successful application in several plants to include: *Arabidopsis thaliana* (Greene *et al.,* 2003; Till *et al.,* 2006; Martin *et al.,* 2009), pea (Dalmais *et al.,* 2008), wheat (Slade *et al.,* 2005), rice (Wu *et al.,* 2005; Till *et al.,* 2007), barley (Caldwell *et al.,* 2004), maize (Till *et al.,* 2004), soybean (Cooper *et al.,* 2008), *Lotus japonicus* (Perry *et al.,* 2003), sorghum (Xin *et al.,* 2008), tomato (Rigola *et al.,* 2009; Gady *et al.,* 2009; Minoia *et al.,* 2010).

The original TILLING method used a commercially available denaturing HPLC (dHPLC) for mutation detection (Henikoff *et al.,* 2004), but the methodology was difficult to scale up, so a novel approach using enzymatic mismatch cleavage (Oleykowski *et al.,* 1998) was developed and LI-COR gel analyser system (Middendorf *et al.,* 1992) proved to be efficient for robust experimentation. Successful and robust use of TILLING, on *Arabidopsis* population was undertaken by Colbert and colleagues (2001). The description of methodology in Arabidopsis is presented on Figure 1.23 and described below.

For TILLING Arabidopsis, seed are mutagenized using ethylmethanesulfonate (EMS). The resulting M1 plants are self-fertilised and the M2 individuals are used to prepare the DNA samples and screened for mutations, while the seeds are inventoried for possible later use (Colbert *et al.,* 2001). The DNA samples are pooled and arrayed in microtier plates. These pools are then amplified using gene-specific primers. The amplification products are then incubated with CEL I endonuclease, a member of the S1 nuclease family of single stranded nucleases (Oleykowski *et al.,* 1998). CEL I cleaves the 3' side of the mismatches in heteroduplexes between wild type and mutant DNA, leaving the duplexes intact (Henikoff *et al.,* 2004). The cleavage products are then electrophoresed using LI-COR gel analyser system and a commercially available image processing program (Adobe Photoshop, Maidenhead, UK) is used to inspect the gel readout. Distinctively different double-end labelling of the amplification products leads to rapid visual confirmation because the mutations are detected on complementary strands, and can be discriminated from amplification artefacts.

After detection of a mutation in a pool, the individual DNA samples are subject to screening to identify the individual plant carrying the mutation. This procedure verifies the location of a mutation to within ±10 bp for PCR products of 1 kbp in size. This approach facilitates the sequencing because the mutation exists within small interval (Henikoff *et al.,* 2004)

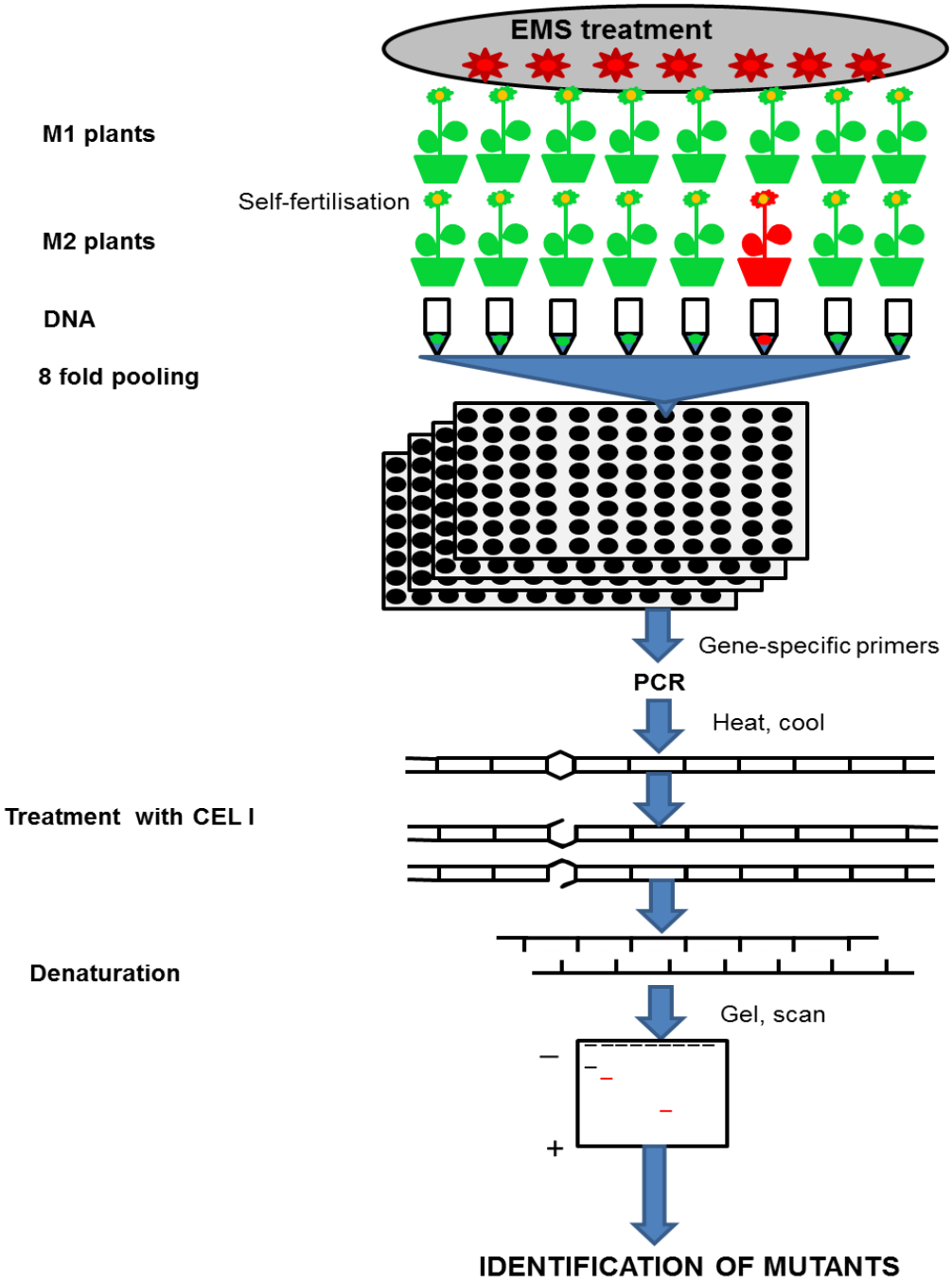


Figure 1.23 High-throughput TILLING. The ethylmethanesulfonate (EMS) treated plants self-fertilise and the DNA material from M2 plant population is pooled. Samples are amplified with gene specific primers and treated with CEL I enzyme. The cleaved products are then analysed on LI-COR gel system and the mutations are identified based on different amplification products sizes. Drawn based on Colbert *et al.,* 2001.

The TILLING allows the efficient detection of mutations, but in addition, this technology is perfect for discovery of natural polymorphisms: CEL I can cut with a partial efficiency, therefore allowing multiple mismatches in DNA duplex (Henikoff *et al.,* 2004). Examination of unknown homologous DNA by heteroduplexing to a known sequence exposes the number and the position of polymorphic sites. Nucleotide changes, small insertions, and deletions are identified, to include some repeat number of polymorphisms. This approach is called ECOTILLING (Comai *et al.,* 2004). This TILLING method can be accomplished with reduced cost in comparison with full sequencing strategy. Plates containing arrayed ecotypic DNA are screened instead of pools of DNA from mutagenized plants. The detection process is on gel with nearly base pair resolution and in addition, background pattern is uniform across the lanes which means that bands of the same size can be effortlessly matched leading to discovery and genotyping SNPs in a single step (Comai *et al.,* 2004).

1.7.3 Harvesting natural variation

Tomato has been a model plant for QTL mapping, the low level of variation affects a number of agronomically important traits. Currently, the narrow genetic basis of the cultivated tomato has emphasized the value of exotic germplasm for improvement of this crop.

A vast untapped resource of genetic variation from crop wild species-relatives is available. A green-fruited species, *S. pennellii* is genetically related to *S. lycopersicum* and has evolved in terms of morphology, chemical compounds including secondary metabolites, reproduction systems and its responses to stress. Although there are ecological differences between these two varieties, *S. pennellii* produces fertile hybrids with *S. lycopersicum* because they are sexually compatible (Lippman, 2007).

Introgression lines or ILs are set of lines where each contain a single homozygous restriction fragment length polymorphism (RFLP)-defined chromosome segment. RFLP markers are short DNA sequences developed in a process called restriction digest. In RFLP analysis, the DNA sample is digested by restriction enzymes. The obtained restriction fragments are separated according to their lengths by gel electrophoresis. These segments were introduced from the green-fruited species (*Solanum pennellii*) within the background of the cultivated tomato variety *Solanum lycopersicum* (Figure 1.24).

The ILs are nearly isogenic to the recipient genotype, and therefore all the genetic variation that differentiate them can be associated with the introgressed segment. They are produced through a series of backcrosses and using selected markers (Eshed and Zamir 1994). These IL's were created through successive introgression backcrossing and marker assisted selection to generate a set of recurrent parent lines with single introgressed segments (Eshed and Zamir, 1994).

To investigate the genetic and molecular basis of flavour volatile production, a genetically diverse but well-defined *S. pennellii* IL population can be used. These chromosomal regions from related wild species lead to increased genetic diversity without losing any desirable traits from a wild variety of crop (Zamir, 2001). The Zamir *S. pennellii* population consist of 76 lines (Figure 1.24) that contain regions of the wild-green fruited *S. pennellii* genome introgressed into the *S. lycopersicum* cv. M82 background to create 107 marker-defined bins. An example of the marker-defined bins on chromosome 1 is shown in Figure 1.25. These introgressed regions specific to each IL, allow identification of QTL and their subsequent introgression into elite crop lines. ILs represent a complete coverage of wild species genome.

This population represents a great potential for genetics and molecular biology to dissect quantitative traits and improve crops.

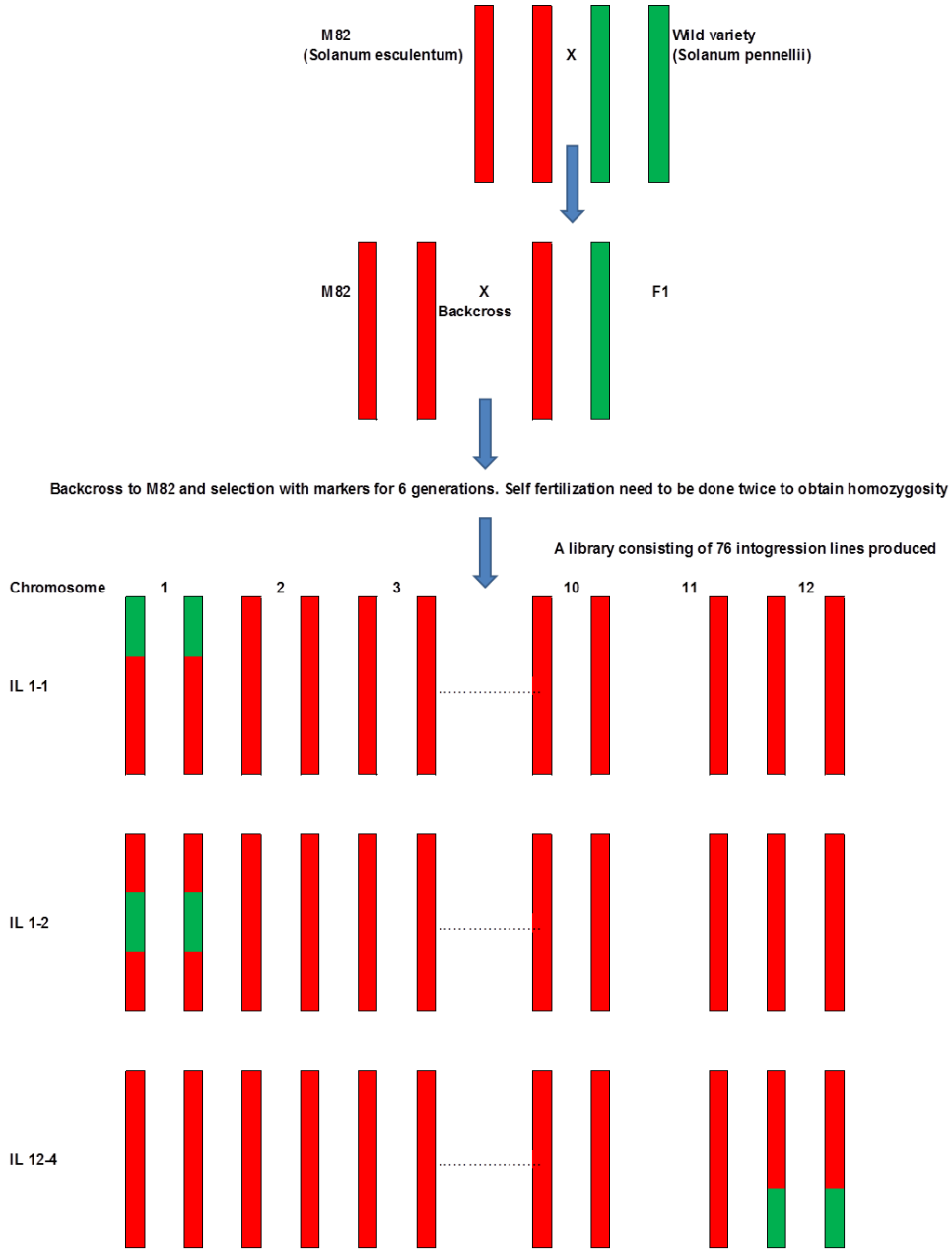


Figure 1.24 Cultivated variety (*S. lycopersicum*, in red) is crossed with wild species (*S. pennellii*, in green). The first generation is then backcrossed to M82 parent for 6 generations and with aid of marker assisted selection as well as relevant phenotype, the specific library consisting of 76 different ILs was created. Homozygosity was obtained by self-fertilization. This procedure was performed twice.

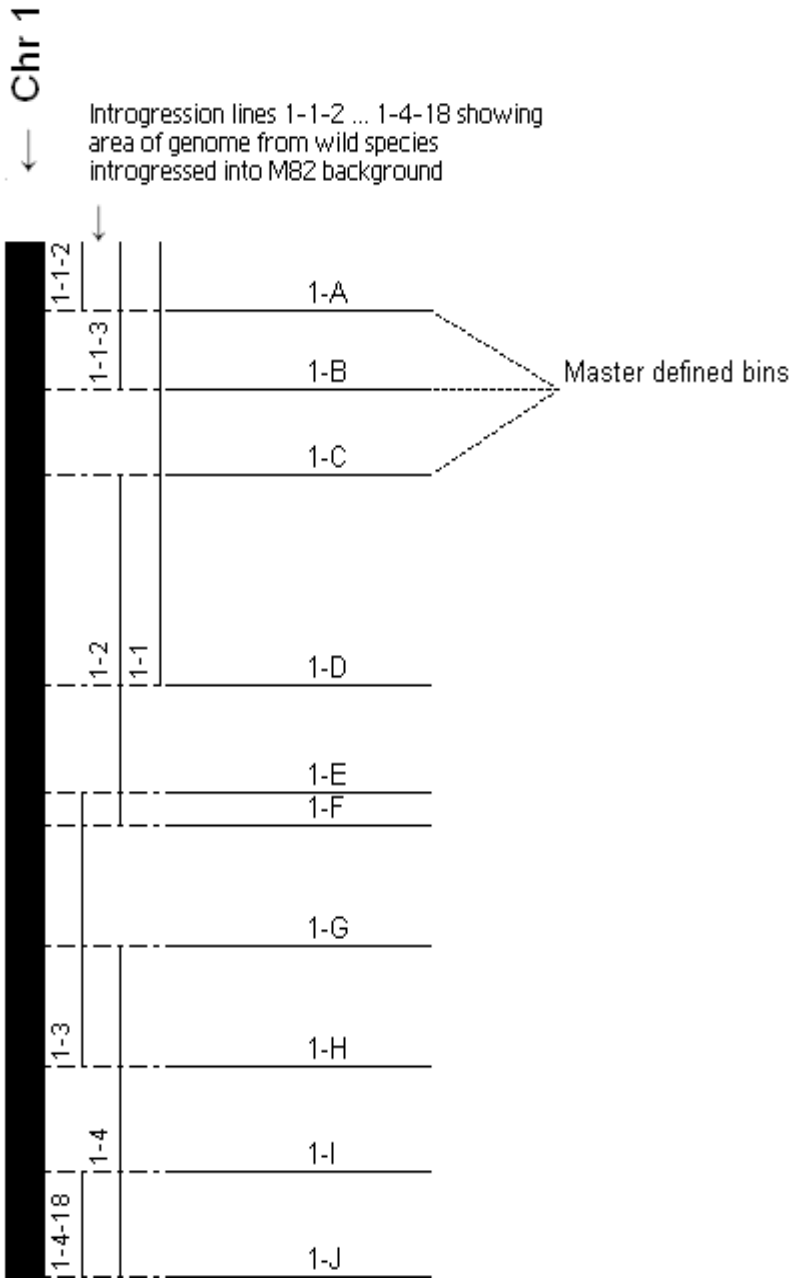


Figure 1.25 Schematic representations of introgressions from *S. pennellii* into the chromosome 1 of *S. lycopersicum*. Letters A to J represent specific overlapping regions called bins, number 1-1-2 to 1-4-18 represent different ILs.

Solanum habrochaites (formerly known *as Lycopersicum hirsutum*) IL's are also available. These lines represent the genome of *S. habrochaites* accession LA1777 in the background of *S. lycopersicum* cv. E6203. Each line contains one to several homozygous chromosome segments introgressed from *S. habrochaites* that are identified by markers. Most of the lines contain a single defined introgression from *S. habrochaites* in the *S. lycopersicum* genetic background and together, the lines provide coverage of more than the 85% of the *S. habrochaites* genome. These lines represent a new tool to uncover the genetic resources hidden in *S. habrochaites* as well as to study the genes responsible for its unique biology. The population includes a core set of 57 lines (LA3913 - LA3969) that provide good genome representation with a minimum number of segments in each line – the majority of them being isogenic (Monforte and Tanksley, 2000).

These three strategies for crop improvement described above lead to identification of quality traits. However, these approaches vary. GM method allows to modify a gene and therefore affecting the plant, while a candidate gene and its pathway is known. Tilling is excellent for creating and characterising novel mutants and new traits. Use of ILs and study of QTL leads to identification of quality traits and new discoveries within uncharacterised or poorly characterised genetic pool. The genetic pool in ILs is limited to available genetic material (in this case 76 *S. pennellii* lines and 57 lines for *S. habrochaites*). Moreover, identification of QTL allows harvesting of natural variation, avoiding difficulties arising from development of GM products and their legislation and publicity issues. ILs have another strong advantage over GM approach because there is no risk of introducing foreign DNA material into the environment. For these reasons ILs were chosen for QTL study and were the subject of this thesis.

1.8 Significance of this work

Improvement of crops has been a frequent process following the domestication of wild species. The development and advance of desired characteristics in agricultural crops, was of great importance specifically for production of food. The progress in enhancement of desired traits relied on genetics and plant breeding and the emphasis was on traits important for manufacturers, but not consumers (i.e. yield, disease resistance, size). This procedure is effective, but slow and has limitations because of the reliance on the limited genetic variability that was found in modern plants (Eshed and Zamir, 1994; Zamir, 2001; Giovannoni, 2006).

However, the breeding focus has changed direction and the strong emphasis is now on compositional characteristics (i.e. colour, flavour, nutrition) which depend on altering the metabolism in the plant. The requirement is in identification of the genes or genetic region associated with the desired trait and altering the metabolic pathway to enhance that trait. New plant breeding approaches have been developed and incorporated into classic breeding programs (Tanksley *et al.*, 1989; Kameswara Rao *et al.*, 2002). DNA-marker assisted breeding in addition to direct gene transfer permitted integration of genes from wider genetic sources.

The International Tomato Genome Sequencing Project was initiated in 2004 by an international consortium that included participants from Korea, China, the United Kingdom, India, the Netherlands, France, Japan, Spain, Italy, and the United States (http://solgenomics.net/). The consortium was created because tomato is the most intensively researched *Solanaceae* genome; it provides the smallest diploid genome for which homozygous inbreds are available. The sequencing of tomato and ITAG Release 2.3 (2011-04-26) official annotations on the SL2.40 genome build by the International

Tomato Annotation Group (ITAG) will facilitate map based cloning of different QTLs and as a result will lead to improvement of quality traits.

Use of tomato introgressions lead to some recent discoveries strongly affecting crop improvement. It was possible to identify a QTL for fruit size, with a single ORFX gene expressed during floral development (Frary *et. al.,* 2000). Other identified QTL include the one for soluble solids (Baxter *et al.,* 2005), yield (Gur *et al.,* 2011), fruit colour (Liu *et al.,* 2003).

In terms of volatile compounds, ILs are very valuable genetic resource. Several loci for different volatiles have been identified for different chromosomes in *S. pennellii* population (Tieman *et al.,* 2006). In addition, two ILs (IL8-2- and IL8-2-1) with elevated levels of phenylacetaldehyde and 2-phenylethanol were identified (Tieman *et al.,* 2006; Tieman *et al.,* 2007). Moreover, bottom of chromosome 4 is altered both by primary metabolites (Schauer *et al.,* 2006) and volatiles (Tieman *et al.,* 2006), The *S. habrochaites* population was also a subject of extensive analysis with total number of 30 QTLs identified affecting emission of one or more volatiles (Mathieu *et al.,* 2009).

This thesis presents a wide range of approaches towards identification of volatile QTL, via metabolomics analysis of volatiles, microarray analysis of selected plant material. In addition, sensory approach provides information about volatile interactions in different matrices. Moreover, the thesis provides information that may be useful for other researchers. APCI-MS can be used with other fruits to measure release of volatile compounds, and it is excellent for screening of high number of samples in different species. The identification and quantification of compounds can be undertaken with GC-MS, while microarray and molecular biology can help in identification of important genes

underlying quality traits. The thesis reveals novel QTLs and introduces potential leads for further investigation under new projects.

AIMS OF THE PROJECT

The aim of this project was to identify and resolve volatile related flavour QTL using tomato IL populations and nominate candidate genes underlying these QTL. The maceration technique coupled to direct MS to monitor the volatile composition of *S. pennellii* and *S. habrochaites* ILs was used. The interactions between volatile components of tomato flavour were evaluated using tomato juice and tomato purée as a base. Information from analytical and sensorial experiments were used to continue genetic work with selected specific targets.

Specific objectives

1. Screening of *S. pennellii* and *S. habrochaites* lines to link volatile production with specific genomic regions using high throughput methods.

2. Comparison of profiles for ILs containing vQTL by Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) and gas chromatography mass spectrometry with solid phase microextraction (GC-MS SPME).

3. Generation of sub lines of IL's to resolve QTL.

4. Nomination of candidate genes underlying QTL by GeneChip experiments. and if time permits then test gene function in fruit systems using virus induced gene silencing (VIGS).

5. Creation of a mixture of selected volatiles in a known matrix and sensory evaluation by a group of trained panelists.

CHAPTER 2:

High throughput screening of tomato volatiles by Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCI-MS)

2.1 Introduction

APCI-MS allows high throughput screening of volatile metabolites in real time. The ability of fast sample analysis, is an advantage for specific volatile compounds in tomato fruit, as this allows them to be measured directly as the tomato is macerated. A list of targets was created by selecting core compounds from the literature (Buttery, 1993) and others of interest to the industrial sponsor. The compounds included: 1-penten-3-one, E-2-heptenal, 2-phenylacetaldehyde, 2-phenylethanol, Z-3-hexenol, pentanal, β-ionone, β-damascenone 2-isobutylthiazole, methyl salicylate, heptanal, limonene, geranylacetone and some other volatiles that might be potentially promising. The methodology was based on earlier tomato work undertaken by Boukobza *et al.,* (2001; 2002). However, this direct mass spectrometry has not been applied before to measure volatile release from tomato ILs.

2.1.1 Maceration of fruits to mimic volatile release on eating

In this research, volatiles were released by using a specifically designed maceration device, which could mimic to some extent the effect of volatile release on eating the tomato fruits, connected to an on-line atmospheric pressure chemical ionisation mass spectrometer. The headspace from above of tomato fruits was measured after maceration over five minute's period. The maceration device was designed to blend single fruits (up to 100g)

rapidly and in constant manner, but without breaking tomato seeds. Tomato seeds contain lipids and may contribute to the products of lipid oxidation and this may lead to creation of artefacts when fruits are highly sheared (Boukobza *et al.,* 2001). The short analysis time allowed us to test fruits within the same day after picking from a glasshouse. The volatiles of interest represent a broad range of compounds with different chemical structure, molecular weight, and reactivity.

2.2 Materials and Methods

2.2.1 Plant material

S. pennellii ILs Dani Zamir's group (Hebrew University, Israel) developed set of 76 *S. pennellii* ILs in the background of *S. lycopersicum* cv. M-82 (Eshed and Zamir 1994). Each line is homozygous for a single chromosome segment (delineated by RFLP markers) introgressed from *S. pennellii,* such that the entire wild species genome is represented in a group of 76 lines. An additional 25 sub-lines have since been added to the collection that provides greater resolution for mapping studies. In addition, the isogenicity of each line has been improved by selection against extraneous *S. pennellii* markers. Seeds from the original lines and sub-lines were obtained from Tomato Genetics Resource Center (TGRC), University of California, Davis, U.S.A). Each of the lines was grown in three randomized plots under standard glasshouse conditions.

S. habrochaites ILs were created in Steve Tanksley's group (Cornel University, N.Y., U.S.A). They represent the genome of *S. habrochaites* accession: LA1777 in the background of S*. lycopersicum* cv. E6203 (Montforte and Tanksley 2000). Each line (there are 45 used in this research) contains one to several homozygous chromosome segments introgressed from *S.*

habrochaites that are identified by markers. These lines provide good genome representation with a minimum number of segments in each line (majority are isogenic).

2.2.2 Growth conditions

ILs as well as control plants were grown in a heated greenhouse using standard cultural practices with regular additions of nitrogen, phosphorus, potassium, fertilizer, and supplementary lightning when required. The glasshouse was under automated control by computer software. Conditions were monitored including temperature, humidity, and radiation both indoor and outdoor. Measurements were taken every 5 min for 24 h, 7 days a week. Temperature was controlled by a heating system and the vents. The vents for regulation of temperature and heating system were also set for optimal conditions for growing plants. Minimum, maximum and average temperature was also recorded.

Growth conditions were different for day and night period. Average temperature during a day was 19°C, relative humidity was 92%, and supplementary radiation was 660J/cm^2 in addition to solar radiation. During night, these values were slightly different. Average temperature was 16°C, relative humidity was 94%, and supplementary radiation was 960 J/cm^2. Higher radiation at night was applied to compensate for solar radiation during daytime. In addition, tomato plants benefit from extended lightning and require 16-18h of light in 24h cycle. Plants were additionally supplemented with calcium to prevent stress disease, known as blossom end rot. Rotted fruits if spotted were removed from the plants, to facilitate transportation of calcium to healthy fruits.

2.2.3 Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS)

APCI sources are simple devices that consist of an inlet and an ionising source, which can be either a radioactive isotope or a corona discharge. Corona discharge is favoured in the laboratory because of the greater dynamic range. The corona discharge energy in APCI-MS system used in our laboratory was set to 4kV. Ions formed were sampled into a standard quadrupole MS that is maintained under vacuum. The APCI process is based on the formation of an initial reactant ion, as the reactant molecules travel through a point-to-plane corona discharge at atmospheric pressure (Taylor *et al.,* 2000).

The reactant ion, is and ion capable of transferring its charge to any molecule with a higher proton affinity. An excellent reagent molecule is water simply because its proton affinity lies between those of the main components of air (oxygen, nitrogen, and carbon dioxide), but below that of most volatile organic compounds. This technology has some advantages. Firstly, water is a requirement for the analysis and not an obstacle. Secondly, none of the air components are ionised however, the wide range of organic compounds, to include volatiles, are a subject of ionisation under appropriate conditions.

APCI ionisation is considered soft which means that the reagent ions have enough energy to ionise the molecule but not to fragment it to any great degree. Most compounds produce RH+ ions. Alcohols dehydrate to give $[M-H_2O+H]^+$ as do aldehydes, although not to the same extent, and the degree of fragmentation can be controlled slightly by manipulating the cone voltage.

This section will follow up by introducing the technical aspects of this direct mass spectrometry. Tomatoes were blended in the maceration device made from a commercial food blender (Kenwood, type BL440; Fig. 2.1). The

blender lid had three Swagelock bulkhead fittings. The left and right were connected to PTFE lines (ID = 3.175 mm) for flushing air through the blender and transfer volatiles to the APCI interface (modified Fisons VG Platform II single quadrupole instrument; Fisons Instruments, U.K.). The intact fruit was placed inside the blender. The headspace was flushed with air (170 ml/min), so that volatiles were removed quickly. On the outlet side, a small (11.5 ml/min) portion of a flow was continuously sampled into the APCI-MS via heated transfer line (0.53 mm ID fused silica tube held at 120°C). The excess of air was released to the atmosphere. The headspace was initially monitored for about 30 s to obtain a baseline measure of volatiles above the intact fruits, then the fruit was blended and monitoring of headspace above the macerate carried out for a further 5 min and 30 s.

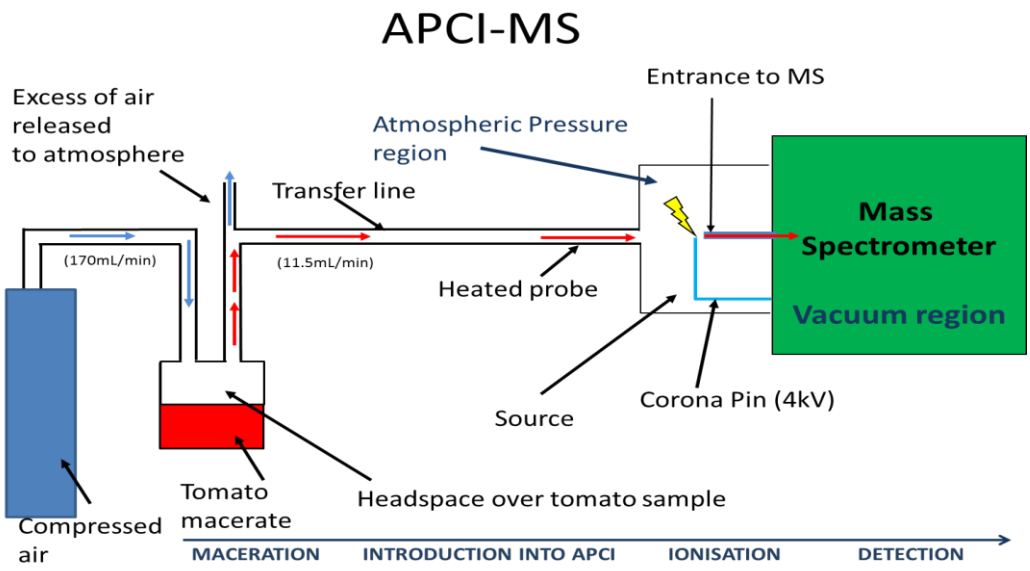


Figure 2.1 Schematic of the maceration device and connections to the APCI-MS. Volatiles from above macerate (headspace) are pushed into a transfer line using compressed air and enter atmospheric region via heated probe. Ionisation occurs in atmospheric region, initiated by the discharge from the corona pin (4kV). The discharge ionises water and creates hydronium (H_3O^+) ions, which transfer charge to compounds entering ionising region. Charged compounds enter high vacuum region were they are separated by their mass.

Each run had a sample number assigned and was created individually for each sample. Because resolution was by mass alone (Taylor, 2000), the equipment was unable to differentiate between positional and stereoisomers and compounds with the same m/z. The sensitivity of this system depends on the type of volatile molecule analysed, with ionization efficiency and the amount of noise at that mass being important factors of overall sensitivity.

Initially samples were monitored in full scan mode and scans were taken between 40 and 200 Da with a cone voltage set to 15 V. Cone voltage is the low voltage bias applied to the sampling cone in the APCI-MS source during analysis. Varying this parameter changes the fragmentation energy imparted to ions. In the case of this study, cone voltage of 15 V was optimal for majority of compounds.

Key ions from traces were selected, in addition, other compounds and their relevant ions were chosen from literature (based on Buttery 1993) an industrial partner research interests, to create a target list. The full target list is presented in Table 2.1.

However, to increase the sensitivity of detection of target compounds, selected ion mode (SIM) was introduced. SIM could offer selectivity in the analysis of aroma compounds, despite the lack of a chromatographic separation. The disadvantage lies in the inability to detect other compounds then specified.

Table 2.1 List of target compounds for APCI-MS. The volatiles were measured via 2 channels with 18 m/z each. Dwell time was 0.10s and cone voltage was 15 V with exception of 2-isobutylthiazole (22 V).

Volatile compound	Molecular mass	m/z
Z-3-Hexenal	98.14	99
β-Ionone	192.3	193
Hexanal	100.16	101
β-damascenone	190.28	191
1-Penten-3-one	84.12	85
2-Methylbutanal	86.13	87
3-Methylbutanal	86.13	87
E-2-Hexenal	98.14	99
2-Isobutylthiazole	141.23	142
1-nitro phenyl ethane	151.16	152
E-2-Heptenal	112.17	113
2-phenylacetaldehyde	120.15	121
6-Methyl-5-hepten-2-one	126.2	127
Z-3-Hexenol	100.16	83
2-Phenylethanol	122.16	123,105
3-Methylbutanol	88.15	71
Methyl salicylate	152.15	153
Acetaldehyde	44.05	45
Ethanol	46.07	47
pentanal	86.14	87
Methylpropanal	72.11	73
Ethyl acetate	88.105	89
1-Penten-3-ol	86.13	69
2,3-dimethylbutanol	102.17	103
2-methyl-2-butenal	84.12	85
Pentanol	88.15	71
2-Methylbutanol	88.15	71
E-2-pentenal	84.1	85
Hexanol	102.17	85
Nitropentane	117.15	118
Heptanal	114.1	115
5-ethyl-2-(5H)-furanone	112	113
Limonene	136.24	137
1,4-dichlorobenzene	147	148
Phenol	94.11	95
Linalool	154.25	137,155
Guaiacol	124.137	107,125
Pantolactone	130.14	131
Hexanoic acid	116.158	117
Neral (Z-citral)	152.24	153
1-ethyl-2-nitrobenzene	151.16	152
Geranial (E-citral)	152.24	153
Triacetin	218.21	219
Eugenol	164.2	147,165
Geranylacetone	194.32	195

Figure 2.2 shows a typical chromatogram (Figure 2.2A) for a tomato fruit sample and spectrum with major tomato volatiles is also presented (Figure 2.2B).

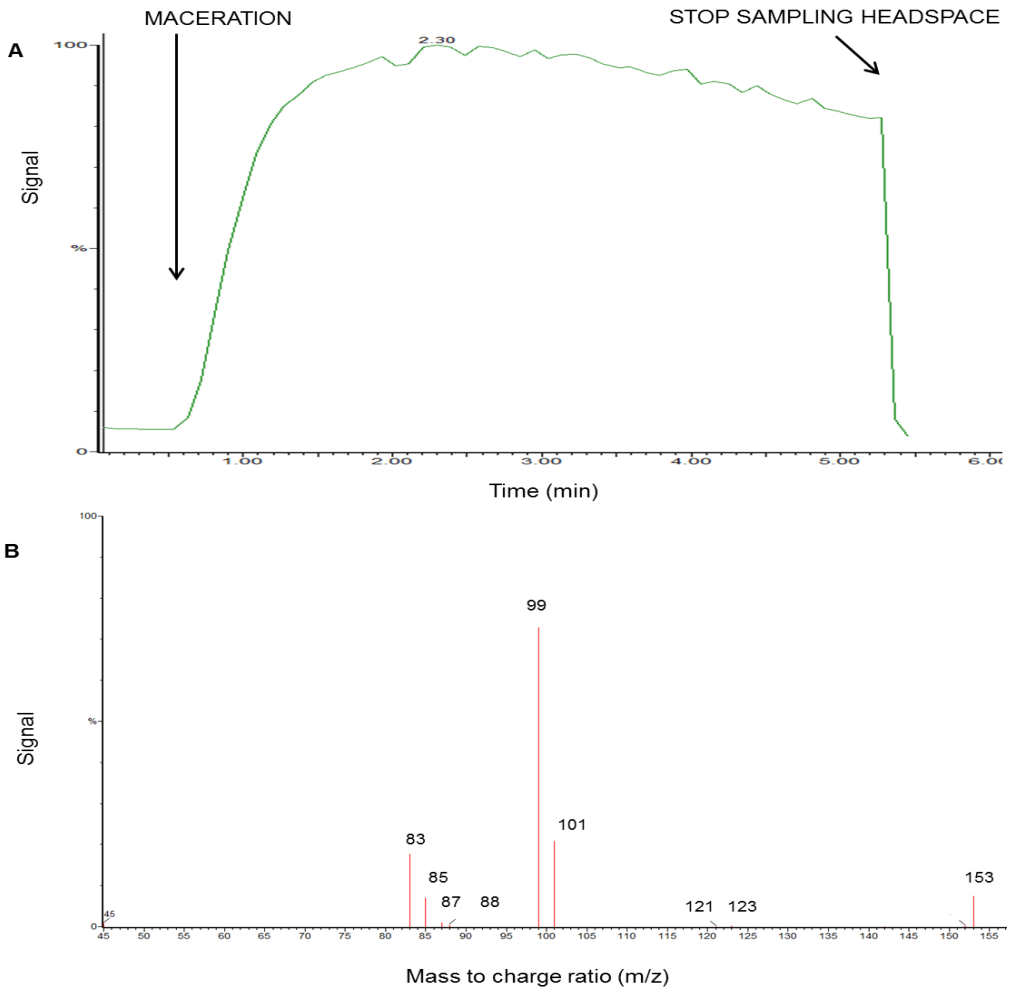


Figure 2.2 Chromatogram (A) for a tomato sample. After initial 30s baseline, tomato fruit is macerated and volatiles are released into a headspace followed by introduction into APCI-MS. Increase of signal corresponds to volatiles reaching vacuum region and registered as signal on MS. A typical spectrum (B) for tomato sample with corresponding m/z: 83 – hexenol, 85 – C5 unsaturated volatiles (1-penten-3-one, E-2-pentenal,), 87- methylbutanals, pentanal, 99 – hexenals, 101 – hexanal, 121 – 2-phenylethanol, 123 – 2 – phenylacetaldehyde, 152 – 1 - nitro phenyl ethane, 153 – citral/methyl salicylate.

2.2.4 Normalisation procedure

SIM analysis was undertaken via two channels covering 18 m/z each. The signal for each m/z was expressed as a percentage of the total ion signal for each channel.

2.2.5 Statistical analysis

Analysis of data was undertaken by application of Student's t-test available from MS Office Excel package (Microsoft, Redmond, U.S.A). The ILs were compared with a control M82D for S. pennellii or with E6203 for S. habrochaites. The level of significance was highlighted in graphs with stars corresponding to the relevant level of significance ($P<0.05$ (*), $P<0.01$ (**); $P<0.001$ (***)).

In case of this study, to examine the data and identify the correlations between ILs and volatiles, principal component analysis (PCA) was undertaken using GenStat 12 edition (VSN International, U.K.). PCA was used for determining the key variables in a multidimensional tomato volatile data to aid in explanation the differences in the observations, and to simplify the analysis and visualization of the data. PCA was undertaken to establish correlations between volatile compounds and relevant introgressions. Only selected volatiles were subject of the analysis due to possibility of complexity in the analysis. To visualise the data PCA biplots were used. Biplots are a type of exploratory graphs used in statistics. A biplot allows information on both samples and variables of a data matrix to be shown in a graphical form.

2.3 Results and Discussion

2.3.1 Overview of volatile metabolites detected by APCI-MS: results from the S. pennellii ILs

QTL maps for *S. pennellii* population based on data from 2007 (summer crop) and 2008 (winter crop) are shown in Figures 2.3a-b and 2.3c-d. 585 fruits were collected in 2007 and 865 were picked in 2008 this resulted in a much denser QTL map for *S. pennellii* in 2008. From this population the majority of detected volatiles are represented by C5, C6 and C7 aldehydes and alcohols such as 1-penten-3-one, E-2-pentenal, hexenal, hexenol, hexanal, heptanal, 2-heptenal. Citral and methyl salicylate were also present in this population. These volatiles are linked with introgressed wild species genome on several different chromosomes. The summary is presented in Table 2.3 and Fig. 2.3, where different volatiles are linked with several bins on different chromosomes. The majority of the volatiles were associated with regions on several chromosomes. However, a number of volatiles were identified to be present on one chromosome e.g. 6-methyl-5-hepten-2-one (Chr. 12 bin C, Fig. 2.3b).

Data obtained for *S. pennellii* IL's are consistent with other published data using GC-MS (Tieman *et al*., 2006; Table 2.3). These authors used hydrocarbon-trapping method supported by Super Q column volatile collection, followed by GC-MS analysis on DB-5 column. Tieman *et al.,* (2006) created a QTL map with volatiles. Several volatiles mapped to the same genomic regions in both studies. However, in some cases volatiles were associated with different bins.

Tieman *et al.,* (2006), linked methylbutanals with Chr. 2 (bin G), Chr. 5 (bin A), Chr. 7 (bin B), Chr. 9 (bin J), Chr. 10 (bin G).E-2-pentenal and 1-penten-3-one were found on Chr. 8 (bin A), Chr. 10 (bin B). 2-

phenylacetaldehyde was found also on Chr. 8 (bin D). Finally, the authors discovered the enhanced level of 6-methyl-5-hepten-2-one on chromosome 12 (bin D, in this research bin C). Therefore, our data is consistent with published information. In addition, a number of other bins have been identified in both this study and Tieman *et al.,* (2006) and details are presented in Table 2.2

Table 2.2 Summary table of volatile regions identified on chromosomes and comparison with published information (Tieman *et al.,* 2006). Numbers correspond to chromosomes and letters indicate relevant bins. In bold the same QTLs identified in both studies.

Volatile	**Two years of screen by APCI-MS, combined data**	**Data from Tieman *et al.,* 2006**
1-penten-3-one	1F, 2A, 2C, **2K** , 3A , 4G, 5A, 5H, 6E, 6G, 7A, 7B, 7E, 7F, **8A**, **10B**, 11A, 11B, 11D, 12B, 12E	1G, **2K**, 4I, 5D, **8A**, 9D, **10B**,
E-2-pentenal	1F, 2A, 2C, 2K , 3A , 4G, 5A, 5H, 6E, 6G, 7A, 7B, 7E, 7F, **8A**, **10B**, 11A, 11B, 11D, 12B, 12E	1G, 4I, 5D, **8A**, **10B**
Pentanal	1G, 2G, 3I, 5A, 5H, 6G, 7A, 7B, 7E, 9I, 9J, 10G, 11B	4I, 12E
Methylbutanals	1G, **2G**, 3I, **5A**, 5I, 6E, 6G, 7A, **7B**, 7E, 7F, **9J**, **10G**, 11B	**2G**, 2K, 3I, 4I, **5A**, 6E, **7B**, **9J**, 10B, **10G**, 11C
Hexenal	1J, 2A, 4E, 8H, 12A, 12I	5B, 9D, 10B
Hexanal	1I , 1J, 4A, 3G, 3I, 8E, 9B, 10G	Not identified
E-2-heptenal	1D, 3B, 4E, 8A, 8D, 8H, 9A, 9C, 9E, 9G, 9K, 10G, 12A, 12I	10B, 12E
Citral/Methyl salicylate	1A, 1I, 1J, 2A, 2B, 2D, 3A, 3B, 3C, 3D, 3I, 4A, 4C, 4H, 4I, 5A, 5I, 6E, 7E, 8A, 8D, 8F, 9A, 9B, 9D, 9I, 9J, 10A, 10B, 10E, 11B, 11C, 11G, 11H, 12A, 12B, 12C, 12D, 12G	Not identified
2-phenylacetaldehyde	1G, 1I, 1J, 2D, 2L, 3C, 3D, 3I, 4C, 4D, 4F, 5A, 5I, 6E, 6G, 7A, 7B, 7D, **8D**, 8E, 9G, 9I, 9J, 110A, 10B, 10E, 10G, 11A, 11B, 11G 12A, 12B, 12C, 12D, 12G,	5C, **8D**
2-phenylethanol	1I, 4C, 4I, 5C, 5E, 5I, 6E, 7E, 7F, 8A, 8F, 8H, 9A, 9E, 10A, 11H, 12A, 12B, 12C, 12D	8D
6-methyl-5-hepten-2-one	12C	3C,12D

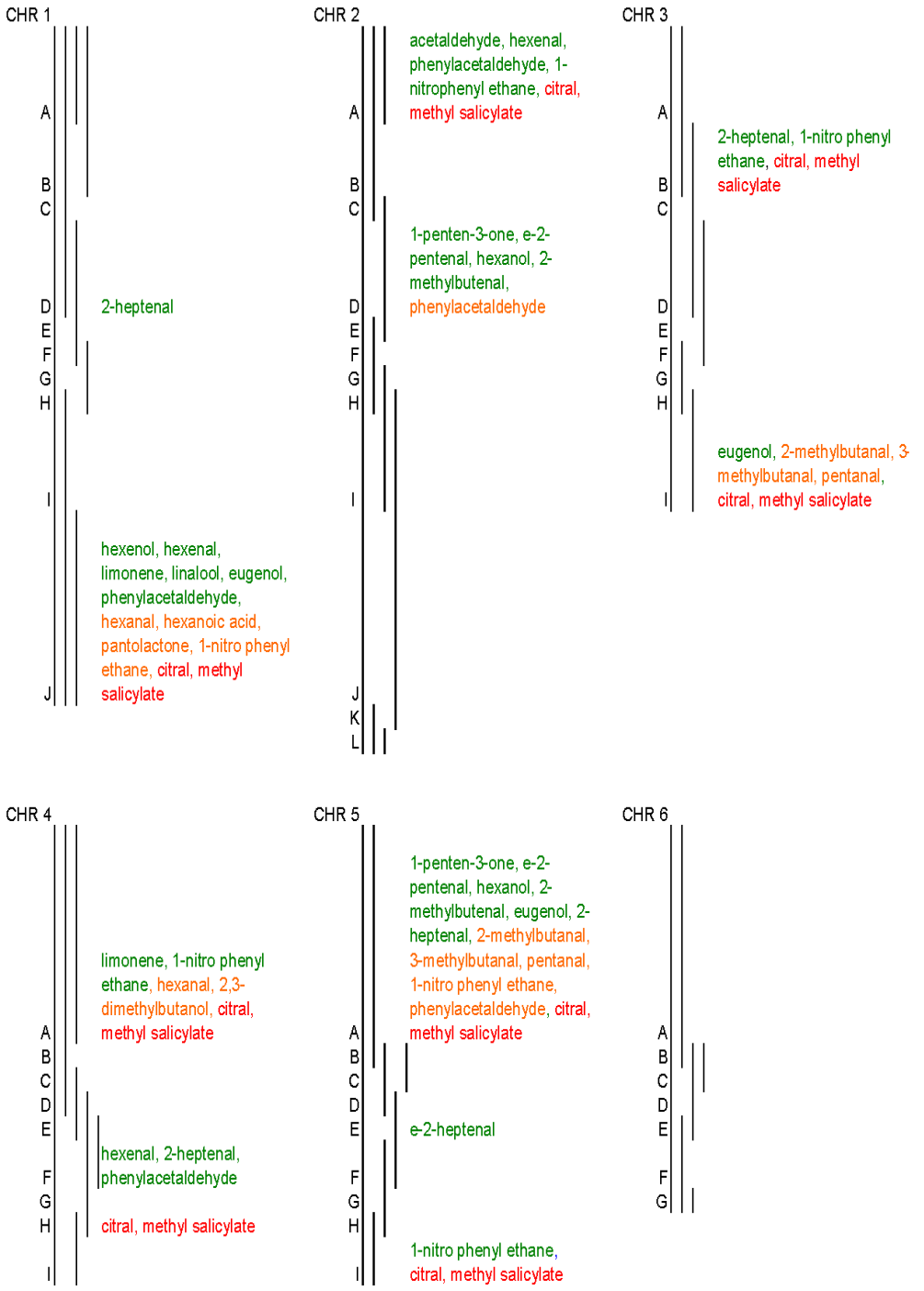


Figure 2.3a *S. pennellii* 2007 volatile data for chromosomes 1 to 6. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters describe the relevant bins.

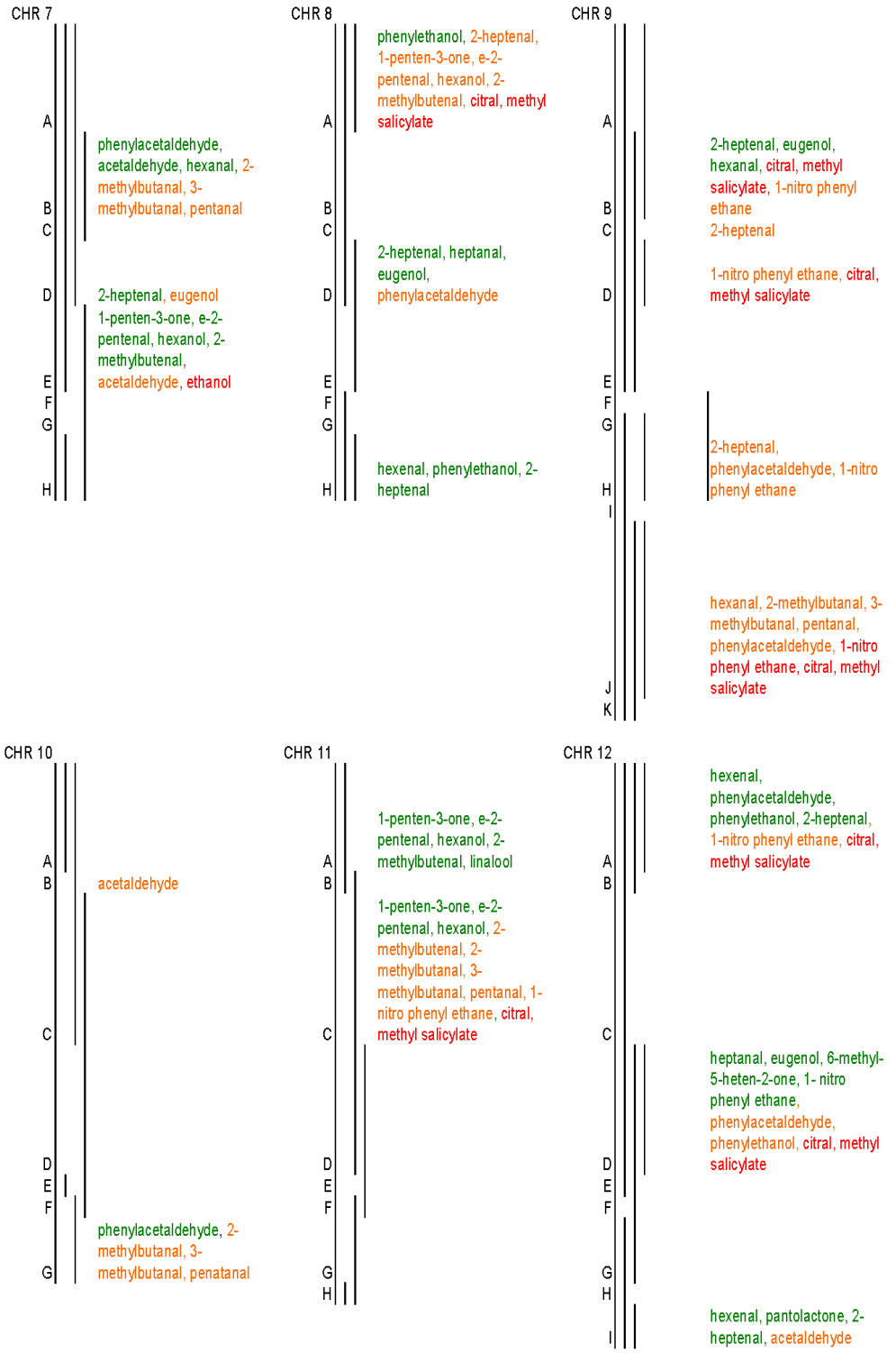


Figure 2.3b *S. pennellii* 2007 volatile data for chromosomes 7 to 12. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters describe the relevant bins.

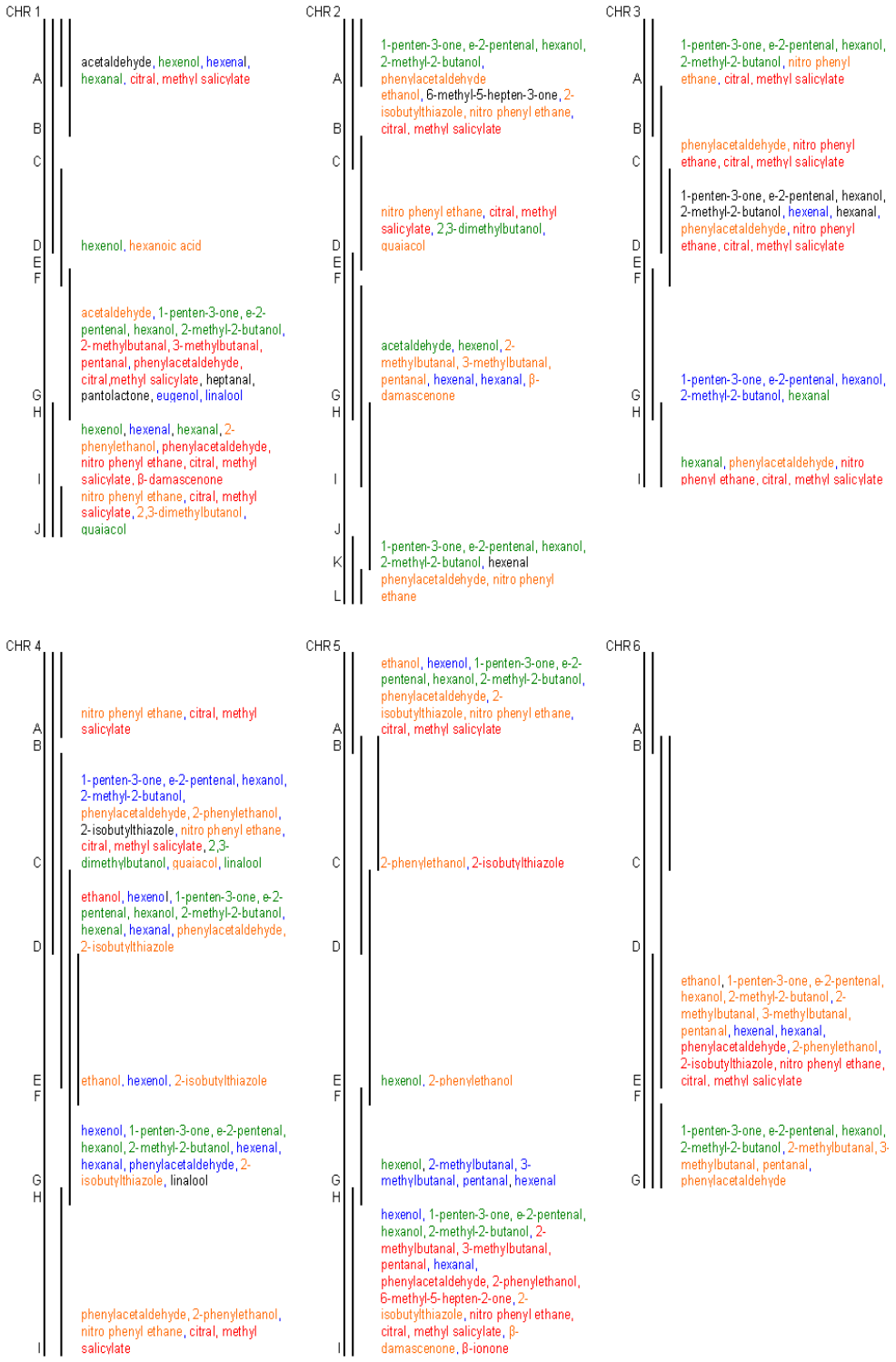


Figure 2.3c *S. pennellii* 2008 volatile data for chromosomes 1 to 6. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters describe the relevant bins.

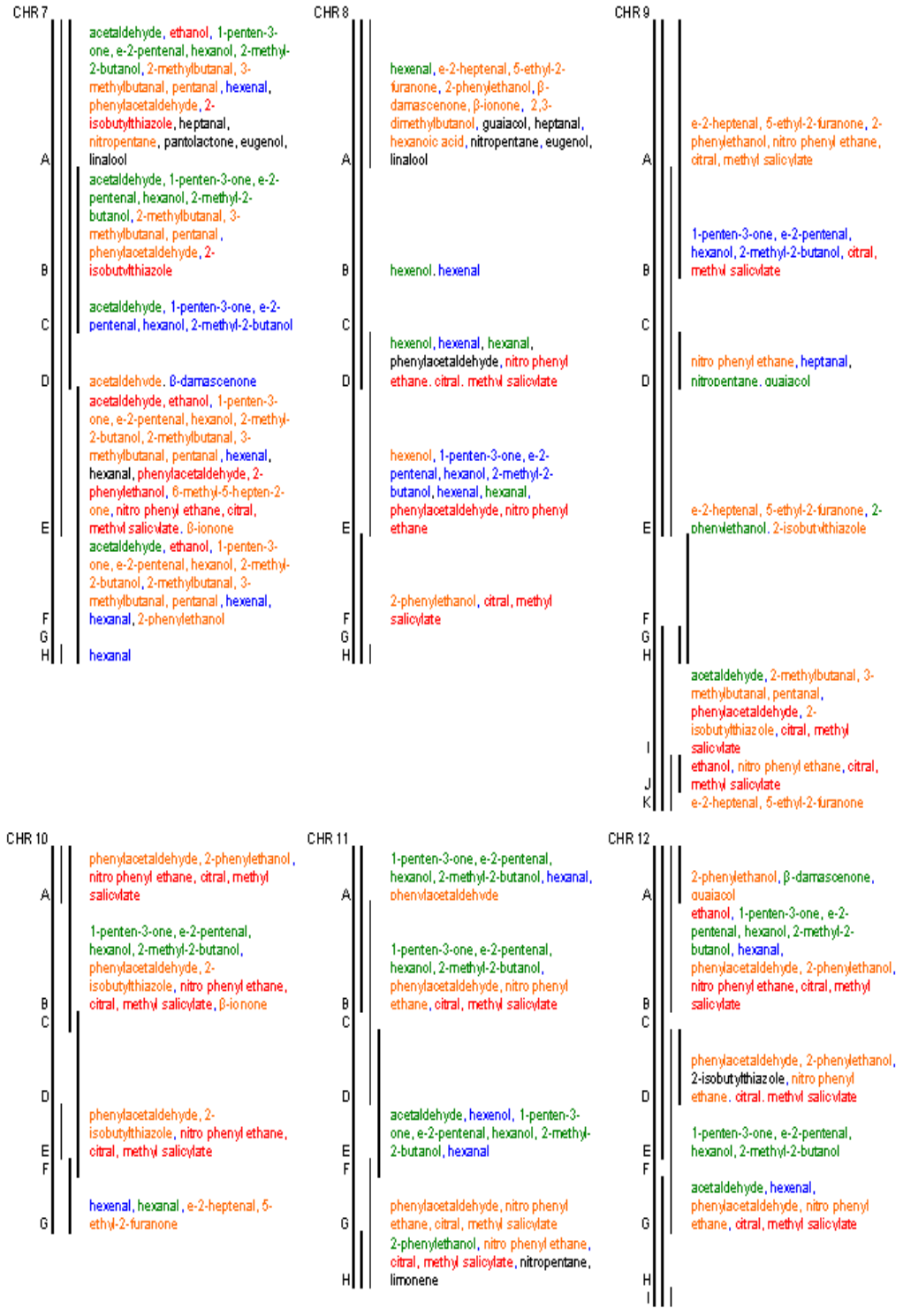


Figure 2.3d *S. pennellii* 2008 volatile data for chromosomes 7 to 12. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters describe the relevant bins.

Some volatiles presented similar effects in the same regions over the two years of study. C5 unsaturated volatiles include 1-penten-3-one and E-2-pentenal, were associated with chromosome 5 (bin A). Pentanal showed similar effects over two years on Chr. 7 (bin A). Amino acid derived methylbutanals were giving an identical response on Chr. 7 (bin A) and Chr. 9 (bin J). Citral and methyl salicylate were identified in several regions over the two years of work (Chr. 1, bin J; Chr. 4, bin A; Chr. 5, bins A and I; Chr. 9, bin J). 2-phenylacetaldehyde presented similar effects on chromosome 5 (bin A), Chr. 7 (bin A) and Chr. 8 (bin D). Finally, 2-phenylethanol gave comparable effects on Chr. 8 (bin A) and Chr. 12 (bin A).

A number of volatiles were inconsistent over two years of study and as a result, they were not identified on the same chromosomal regions in 2007 and 2008. The most noticeable volatile in this category was 6-methyl-5-hepten-2-one, linked with Chr.12 (bin C) during 2007 screen, but not present at higher levels in 2008. A group of C6 volatiles, to include hexenol, hexenals and hexanal showed inconsistent effects over the two seasons. This was later confirmed by comparison of t-test across two years of experiments, when some results were significant only during first or second year or were not significant at all.

The data for C5 unsaturated volatiles, C6 volatiles, methylbutanals, citral and methyl salicylate, will be further evaluated using PCA followed by graphical presentation of volatiles distribution.

2.3.1.1 Focusing in on principal effects

Figure 2.3e shows the outcome of principal component analysis. Each variable is represented as a vector, and the direction and the length of the vector indicates how each variable contributes to the principal components. For instance, Figure 2.3e shows that the first principal component has positive coefficient with only one variable (m/z 99) while the negative coefficient has six variables (m/z 85, m//z 87, m/z 121, m/z 123, m/z 101, m/z 153).

The second principal component has negative coefficient with three variables (m/z 101, m/z 123, m/z 153). Positive coefficient is represented by four variables (m/z 85, m/z 87, m/z 99, m/z 121).

IL 2-2 correlates with variable m/z 85 (Figure 2.4b for cross reference), IL 7-5-5 correlates with variable m/z 87 (Figure 2.4a for cross reference). There is also a weak correlation for IL 9-2-6 and 10-3 with variable m/z 99 (Figure 2.4c for cross reference). In addition, lines 1-4, 5-5, 8-2-1 and 9-1-2 correlate with variables m/z 101 (Figure 2.4d for cross reference) and m/z 153 (Figure 2.4e for cross reference).

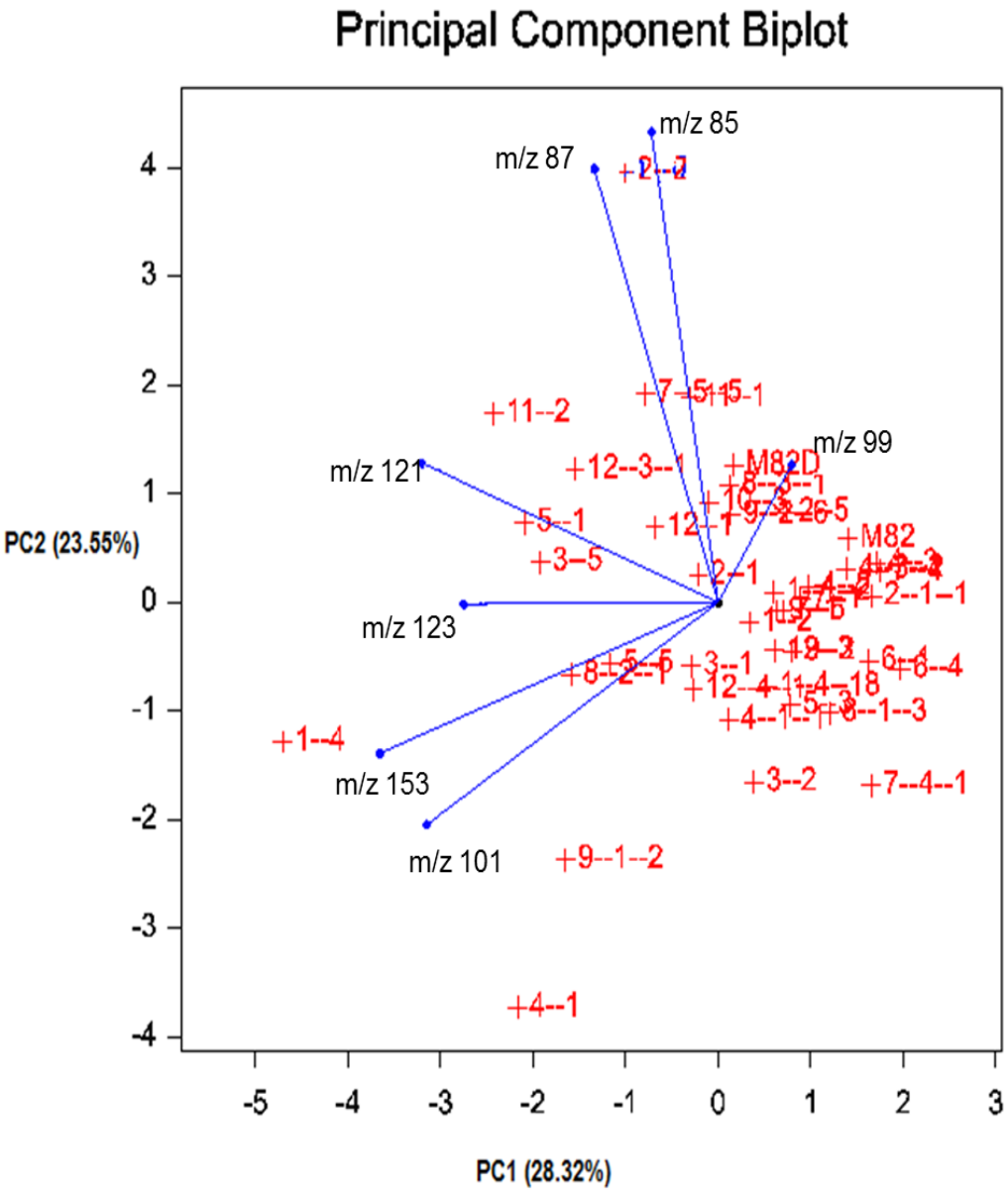


Figure 2.3e PCA biplot for volatile m/z vs. introgressions in the *S. pennellii* IL population in 2007. All ILs are presented, but only selected volatiles shown. First component explains 28% of variation; second component is responsible for 24% of variation. m/z 85 – C5 unsaturated volatiles, m/z 87 – methylbutanals, m/z 99 – hexenals, m/z 101 – hexanal, m/z 121 – 2-phenylethanol, m/z 123 – 2-phenylacetaldehyde, m/z 153 – citral/methyl salicylate. Red pluses indicate individual data points, blue lines represent coefficients.

PCA biplot presented on Figure 2.3f characterises correlations between volatiles and ILs during second year APCI screen. Figure 2.3f shows that the first principal component has positive coefficients with two variables (m/z 99, m/z 101), while the negative coefficient has five variables (m/z 85, m/z 87, m/z 121, m/z 123, and m/z 153). The second principal component has negative coefficient with four variables (m/z 101, m/z 121, m/z 123, and m/z 153). Positive coefficient is represented by three variables (m/z 85, m/z 87, and m/z 99). ILs 1-3 and 6-3 correlate with variable m/z 85 (Figure 2.4b for cross reference), ILs 1-3, 6-3 and 7-2 correlate with variable m/z 87 (Figure 2.4a for cross reference). There is also a good correlation between ILs 1-4 and 8-2-1 with variable m/z 101 (Figure 2.4d for cross reference), showing a consistent trend over two years.

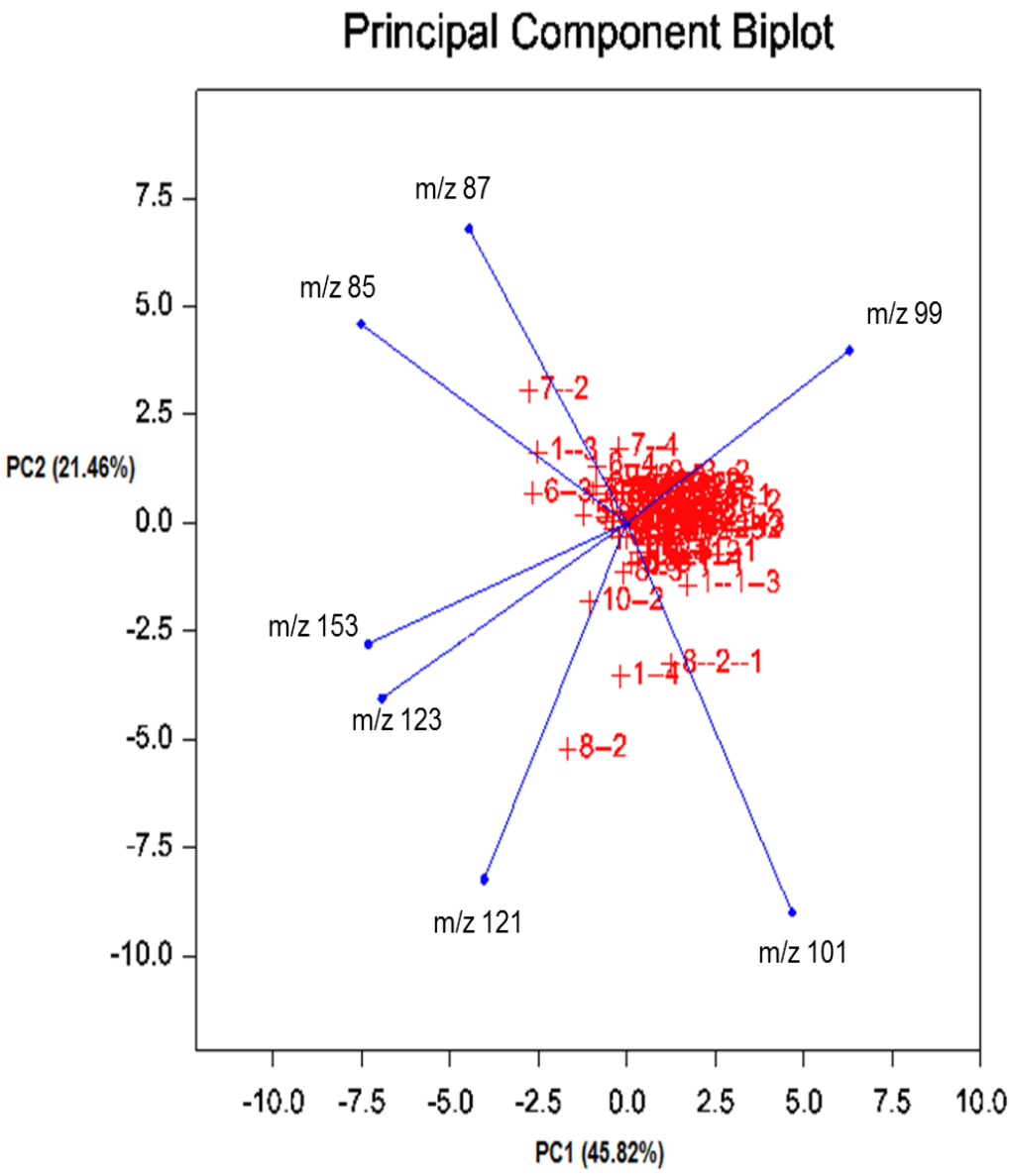


Figure 2.3f PCA biplot for volatile m/z vs. introgressions in the *S. pennellii* IL population in 2008. All ILs are presented, but only selected volatiles shown. First component explains 46% of variation; second component is responsible for 21% of variation. m/z 85 – C5 unsaturated volatiles, m/z 87 – methylbutanals, m/z 99 – hexenals, m/z 101 – hexanal, m/z 121 – 2-phenylethanol, m/z 123 – 2-phenylacetaldehyde, m/z 153 – citral/methyl salicylate. Red pluses indicate individual data points, blue lines represent coefficients.

2.3.2 Detailed analysis of the distribution of key volatiles as determined by APCI-MS

The key volatiles that were of interest are highlighted below and their distribution is shown in more detail in the Figures 2.4a-e. The data presented was evaluated using the Student t-test to identify significant ($P<0.05$ or $P<0.01$) ILs with respect to volatile production.

Figure 2.4a presents the data collected for *S. pennellii* ILs during 2007 and 2008 for methylbutanals. The methylbutanals were represented by two volatiles: 2-methylbutanal and 3-methylbutanal and their corresponding m/z was 87. The precursors of these compounds, are the amino acids, leucine (3-methylbutanal) and isoleucine (2-methylbutanal) (Tieman *et al.,* 2006; Goff and Klee 2006) and it is assumed, that synthesis of these compounds is initiated by enzymatic decarboxylation (van der Hijden and Bom, 1996). Krumbein and Auerswald (1998) have described 3-methylbutanal as unpleasant. In 2007, a series of lines were significantly lower in volatile concentration in comparison with control fruit M82D with P value of <0.05. The lowest line was IL4-1 ($P<0.001$) and the highest was IL7-5-5; however, it was not significant due to standard error of the mean for this particular IL. Lines 3-5 and 7-5-5 fall into bins (chromosome 3 bin I and chromosome 7 bin B) identified by Tieman and colleagues (Tieman *et al.,* 2006).

The number of lines tested in 2008 with APCI-MS was greater because of higher number of collected fruits. The lowest line was IL8-2-1 (not significant) and the highest was IL1-3 ($P<0.001$). IL7-5-5 confirms the trend observed in 2007 and agrees with Tieman work (Tieman *et al.,* 2006). It seems that a different number and set of lines shows high values for this particular volatile over the two years of study. However, three lines were identified to have generally higher values than control over the two years, but

the levels were not significant at P<0.05. These lines were IL9-2-6, IL10-3, and IL11-2.

Data for 2007 and 2008 C5 unsaturated volatiles are compared in Figure 2.4b, which presents the data, collected for *S. pennellii* ILs during 2007 and 2008. C5 unsaturated volatiles were represented by 1-penten-3-one, E-2-pentenal with corresponding m/z 85. They are a product of lipoxygenase pathway and their precursors are fatty acids (Goff and Klee 2006). 1-penten-3-one has been associated with green note (Krumbein and Auerswald, 1998). In 2007 the lowest lines was IL4-1 (P<0.05) the highest was IL2-2, not significant at P<0.05. However, in 2008 the lowest IL in comparison with control fruit M82D was IL8-3 (P<0.001) and 7-2 was significantly higher (P<0.01) then M82D control fruit.

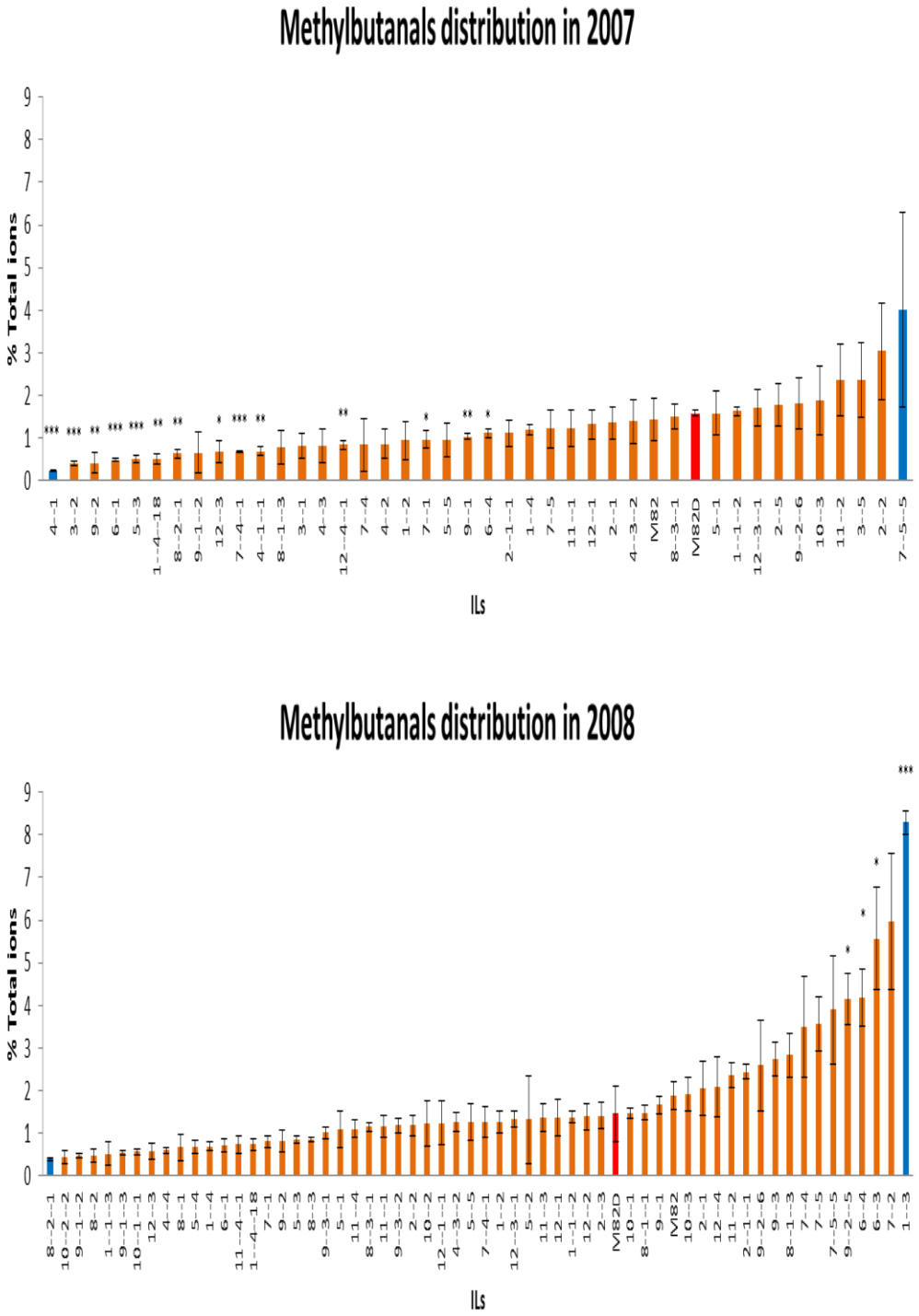


Figure 2.4a Methylbutanals distribution for *S. pennellii* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). M82D highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

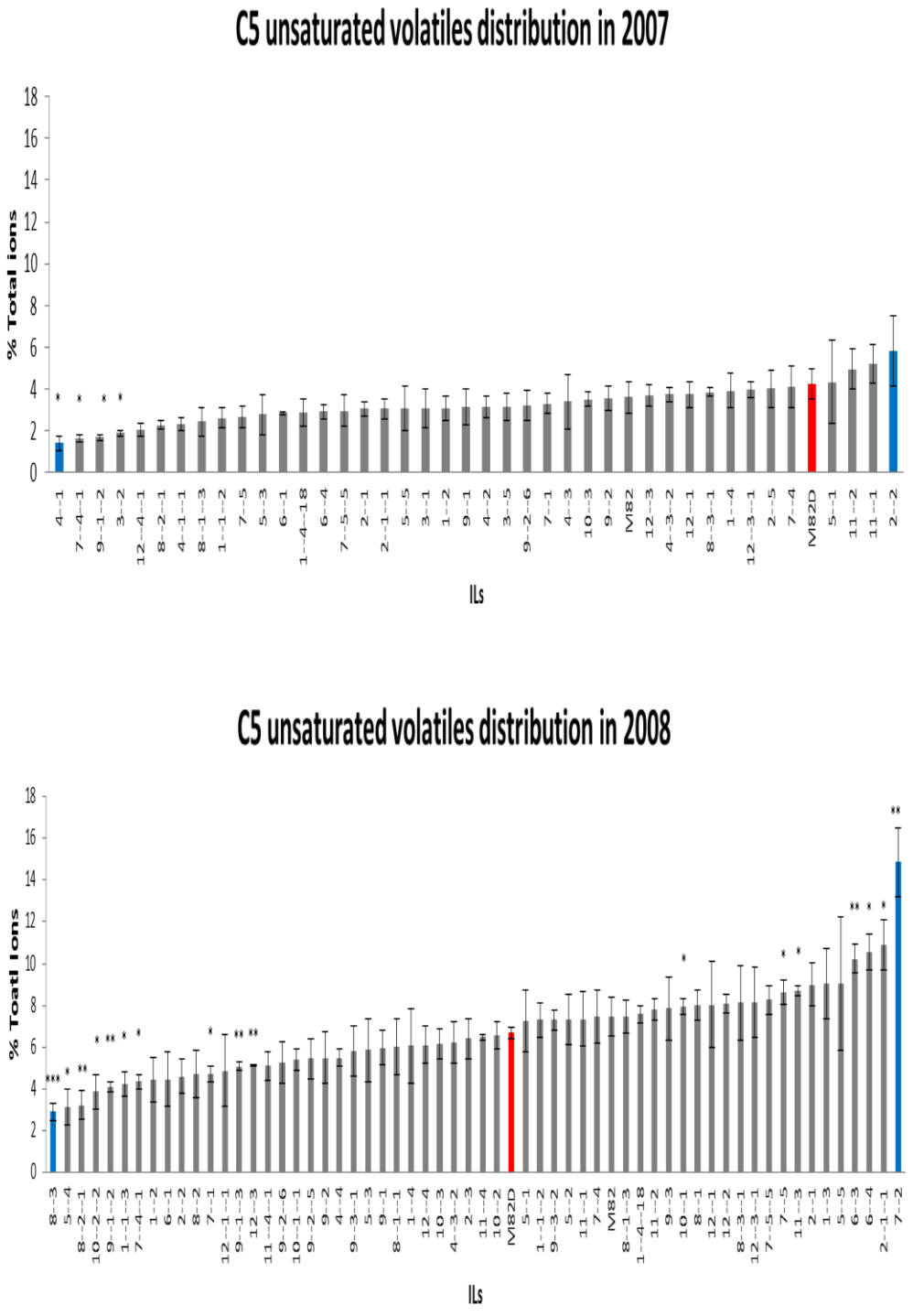


Figure 2.4b C5 unsaturated volatiles distribution for *S. pennellii* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). M82D highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

E-2-hexenal and Z-3-hexenal are volatiles associated with lipoxygenase pathway, their precursors are similarly to C5 unsaturated volatiles, fatty acids (Goff and Klee 2006), and their corresponding m/z is 99. Z-3-hexenal has been associated with fresh green tomato (Petro-Turza, 1987). For hexenals (Figure 2.4c), in 2007 none of the lines was significantly different from control M82D except IL4-3-2 ($P<0.01$).

In contrast with 2007 data, in 2008 a number of lines were significantly higher ($P<0.05$) in volatiles in comparison with control. The lowest was IL8-3 ($P<0.001$) and the highest was 9-3-1 ($P<0.01$). Hexanal has a corresponding m/z 101, and it is linked to lipoxygenase pathway similarly to hexenals. This volatile was also related to green note (Kazeniac and Hall, 1970; Petro-Turza, 1987). Hexanal was evaluated and characterised in ILs in 2007 (Figure 2.4d). The lowest volatile signal intensity was associated with IL4-3-2 (not significant), while IL 4-1 and IL1-4 were significantly higher ($P<0.01$) than control fruit M82D.

In 2008, a number of lines showed significantly ($P<0.05$) higher hexanal levels in comparison with control fruit M82D. As in 2007, the highest levels were present in introgression IL1-4 ($P<0.01$). A number of lines were higher in hexanal signal intensity than control over two years of experiments, and many of them were significant in 2008 in contrast to 2007. These lines were IL1-2 ($P<0.01$), IL1-1-3 ($P<0.01$), IL2-1 ($P<0.01$), IL5-2 ($P<0.05$), IL5-3 ($P<0.01$), IL7-4-1 ($P<0.001$) IL7-5 ($P<0.01$), IL7-5-5 ($P<0.001$), IL8-1 ($P<0.01$), IL8-2 ($P<0.01$), IL8-2-1 ($P<0.01$), IL8-3 ($P<0.05$), IL 8-3-1 ($P<0.01$), IL9-1-3, IL9-2-6 ($P<0.05$), IL10-1-1 ($P<0.01$), IL 10-2-2 ($P<0.01$), IL10-3 ($P<0.001$), IL11-1 ($P<0.01$) and IL12-1-1 ($P<0.01$). Line 1-4 was significant ($P<0.01$ in both years) during 2 years of APCI-MS experiments (Fig. 2.4d).

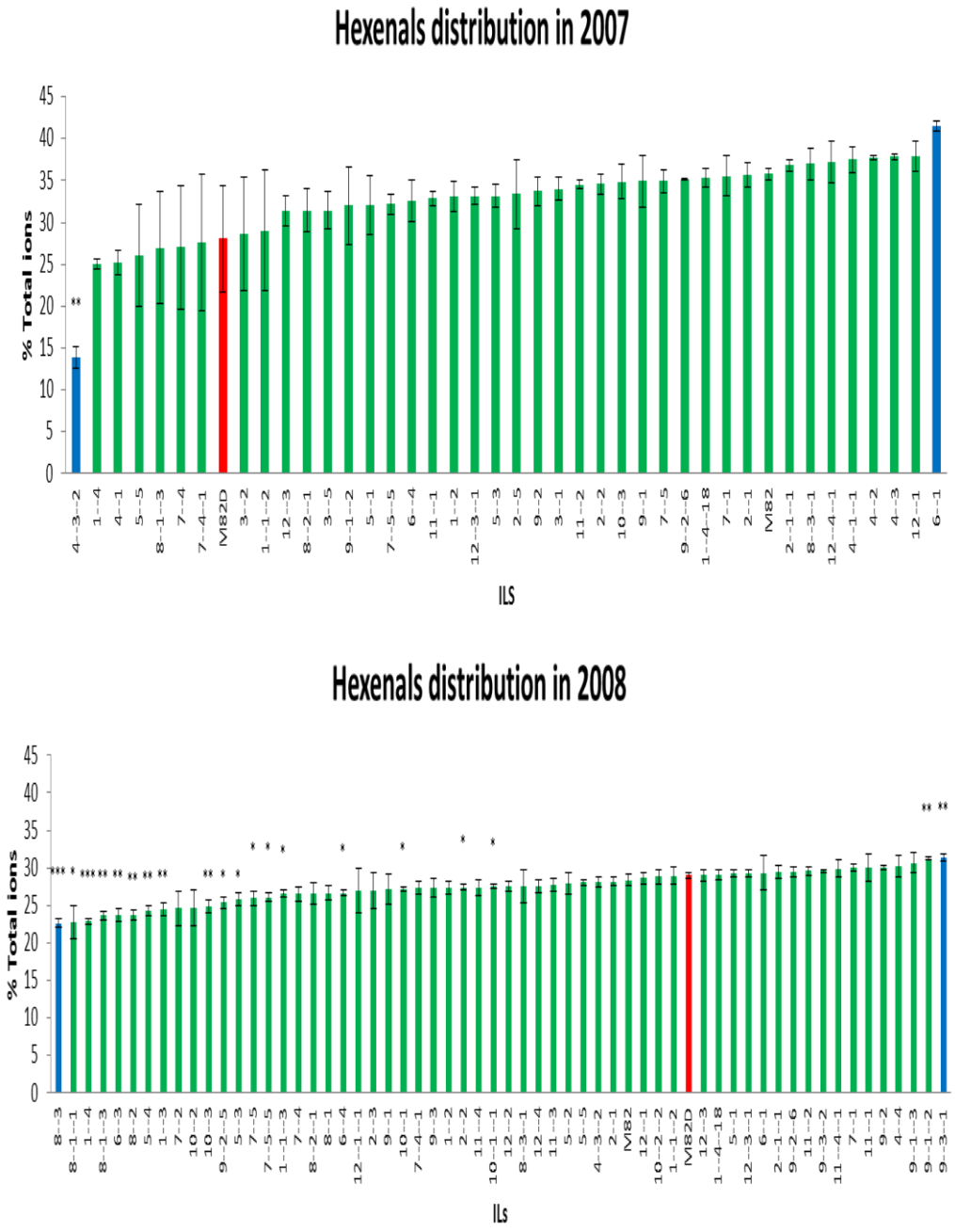


Figure 2.4c Hexenals distribution for *S. pennellii* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). M82D highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

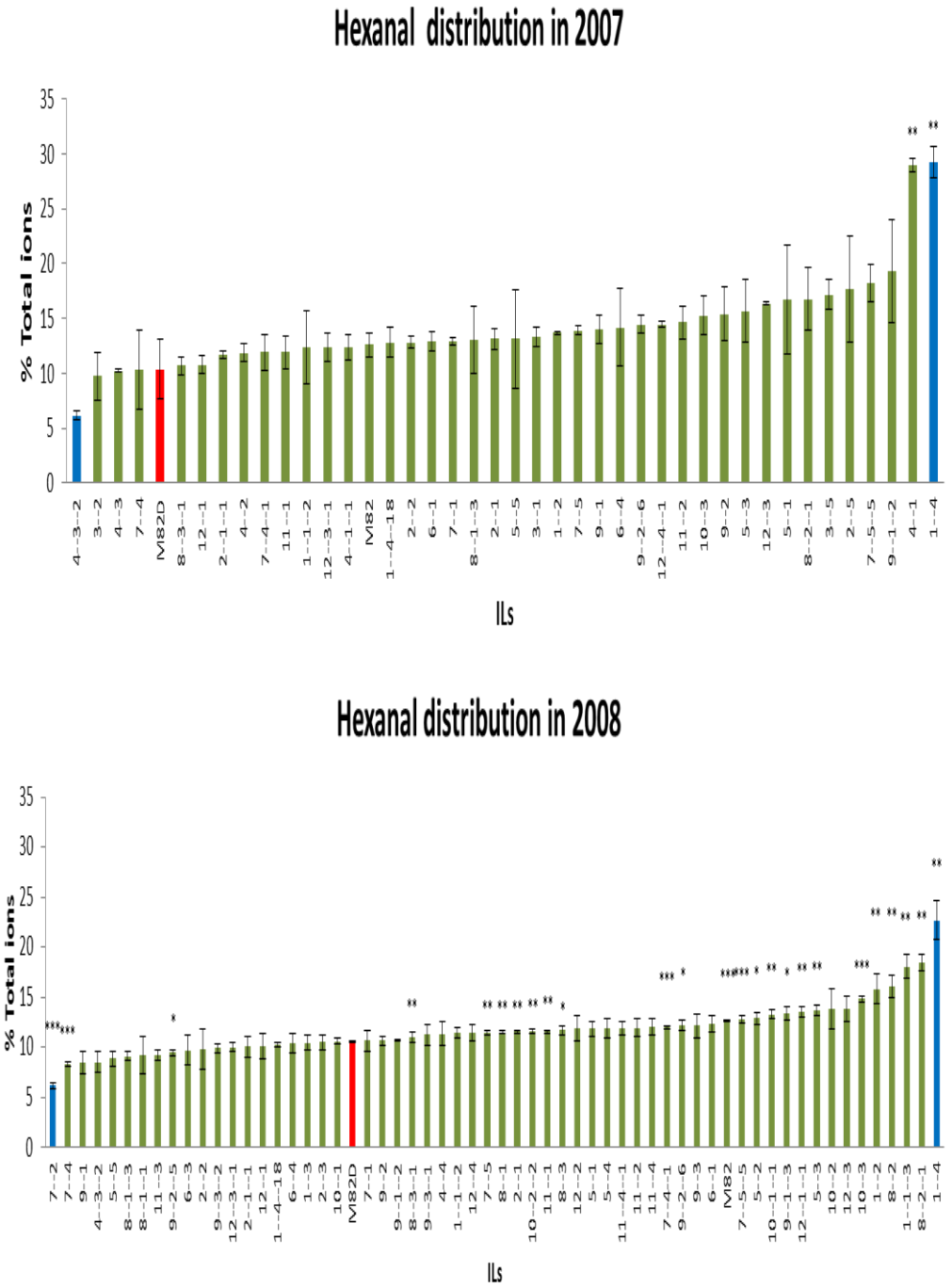


Figure 2.4d Hexanal distribution for *S. pennellii* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). M82D highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

The extent of volatile evolution related to citral and methyl salicylate was also analysed (Figure 2.4e). These volatiles have a corresponding m/z 153, citral is associated with carotenoid pathway (Lewinsohn *et al.,* 2005) and methyl salicylate is a product of shikimate pathway. These volatiles have been described as citrus (citral) and oily (methyl salicylate) by Krumbein and Auerswald (1998). Methyl salicylate (MeSA), or oil of wintergreen, has been identified as an important volatile in tomato flavour (Buttery and Ling, 1993).

2007 there were number of significant lines ($P<0.01$ and $P<0.001$, refer to Fig. 2.4e), the introgression with the lowest value was IL12-3, but not significant, IL4-3 was significantly low ($P<0.01$). High level of volatile signal was in IL1-4 ($P<0.01$).

In 2008, the trend is slightly different; the lowest introgression was IL5-2; however, it was not significant. The highest line in terms of volatile signal seemed to be IL8-2, but it was also not significant. During two years of experiments, a number of lines were higher in volatile signal. In comparison with control M82D; these lines were IL 1-4 (significant at $P<0.01$ in 2007 and $P<0.05$ in 2008), IL2-1 ($P<0.001$ in 2007 and $P<0.01$ in 2008), IL5-1 ($P<0.01$ in 2007 and $P<0.001$ in 2008), IL5-5 (<0.001 in 2007 and $P<0.01$ in 2008), IL9-1-2 ($P<0.01$ in 2007 and $P<0.05$ in 2008), IL12-1 ($P<0.001$ both years). Introgressions 1-4, 1-4-18, 5-1 and 5-5 support the data presented on the maps (Figures 2.3a and 2.3b) and can be associated with chromosome 1 (bin J) and chromosome 5 (bin A and I). With the APCI data it is not possible to identify if the trends are citral or methyl salicylate related. Similarly, to hexenals, the m/z 153 will be resolved in the next Chapter (Chapter 3, Fig.3.10).

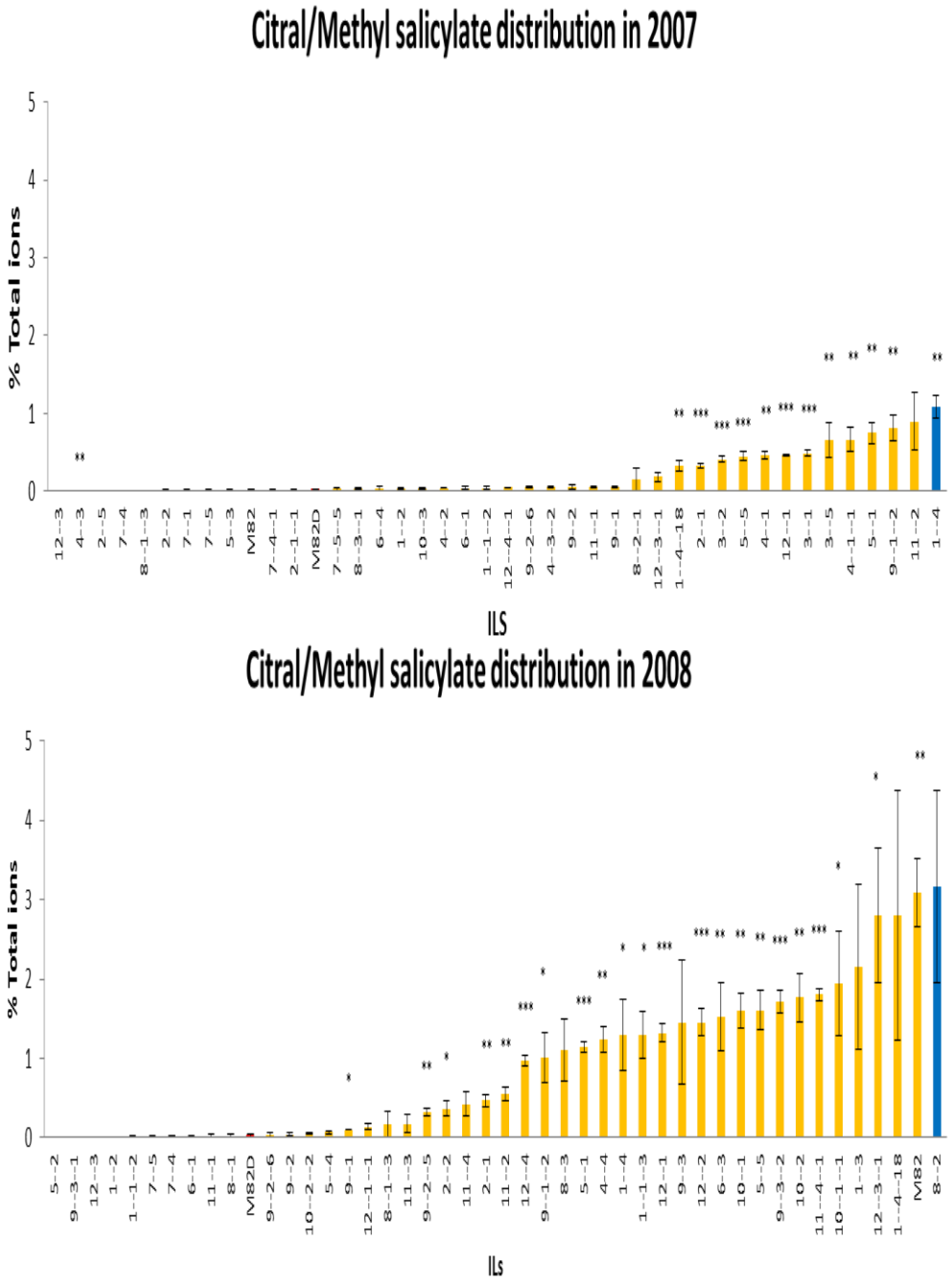


Figure 2.4e Citral/Methyl salicylate distribution for *S. pennellii* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). M82D highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

2.3.2.1 Lipoxygenase derived volatiles

C6 volatiles (hexenals and hexanal), a product of degradation of lipids, are key compounds responsible for fresh tomato flavour. Figure 2.5 presents a ratio of m/z 99 to m/z 101 (hexenals to hexanal) in order to see if there is a correlation and a difference in release of these compounds in tomato ILs, possibly due to different precursors (linolenic acid for hexenals and linoleic acid for hexanal).

The interesting correlation on Fig. 2.5 is shown for IL1-4. From previous data presented in this chapter (Fig 2.4c and 2.4d), the conclusion was that IL1-4 is low in volatile signal for hexenals, while for hexanal the result is opposite (high in volatile signal) and this reflects on a low ratio value. In contrast, compounds high in m/z 99 and low in m/z 101 have a greater ratio value (e.g. 2-1-1, 1-4-18, 7-1 and 12-1). However, because of the lack of identification of isomers in APCI, it is not possible to verify if the high hexenals trend is driven by E-2-hexenal or by Z-3-hexenal. This hypothesis will be examined in more detail in the next chapter, based on GC-MS data.

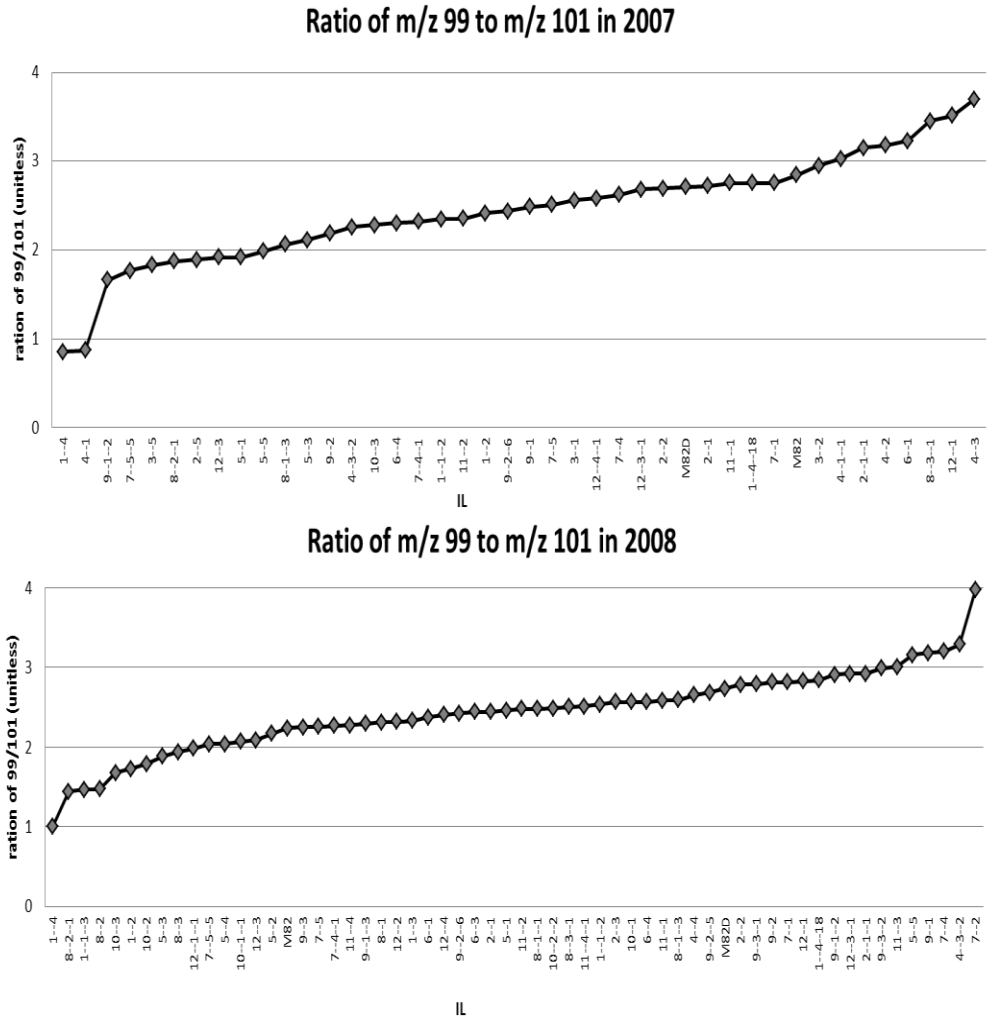


Figure 2.5 Ratio of hexenals (m/z 99) to hexanal (m/z 101) over two years of experiments. The consistent trend over two studies indicates IL 1-4 as low in m/z 99 and high in m/z 101.

2.3.2.2 The statistical analysis of presented data

The statistical analysis of multiple data points can lead to an error and wrong assumption of false positive or negative values. The t-test with a P value of 0.05 indicates that the measured value can happen randomly with a probability of 5% (5 samples out of 100 are significant by random). However, it is possible to overcome it with big data sets, if data are compared across two periods of treatment or two studies with the same sampling material. Table 2.3 shows significance tests results (P values) for selected ILs over two years of study. In some cases, the results were not significant in first year (2007), but were actually significant over the second year (2008).

From Table 2.3 it is possible to conclude that in case of m/z 99 none of the lines was significant over two year period, however, there was a number of significant lines during second year of screening. In case of m/z 101, IL1-4 was significant in the first and second year. In addition, a number of ILs was significant also during the second year. For m/z 153 five ILs showed significant levels during two years of screening, these were IL 1-4, IL2-1, IL5-1, IL 5-5, IL 12-1. A number of lines was significant in one of the screening years as well.

Table 2.3 The summary of significance tests for selected ILs and three m/z 99, 101, and 153. t-tests for two years are shown. In bold, significant values at P<0.05 and lower.

IL	2007 t-test (m/z 99)	2008 t-test (m/z 99)	2007 t-test (m/z 101)	2008 t-test (m/z 101)	2007 t-test (m/z 153)	2008 t-test (m/z 153)
1--1--2	0.923	0.964	0.666	0.191	0.420	0.515
1--2	0.492	0.130	0.285	**0.023**	0.637	0.346
1--4	0.663	**0.000**	**0.004**	**0.003**	**0.002**	**0.047**
1--4--18	0.327	0.930	0.462	0.209	**0.016**	0.152
2--1	0.309	0.345	0.387	**0.006**	**0.001**	**0.006**
2--1--1	0.245	0.702	0.650	0.640	0.830	0.224
2--2	0.371	**0.040**	0.416	0.726	0.366	**0.023**
4--3--2	0.096	0.302	0.199	0.108	0.059	0.224
5--1	0.613	0.711	0.327	0.153	**0.005**	**0.000**
5--3	0.477	**0.026**	0.246	**0.005**	0.493	0.224
5--5	0.832	0.173	0.622	0.077	**0.001**	**0.003**
6--1	0.104	0.887	0.425	0.109	0.285	0.529
6--4	0.540	**0.012**	0.441	0.834	0.905	0.224
7--1	0.330	0.154	0.400	0.955	0.252	0.224
7--4	0.920	0.081	0.994	**0.001**	0.117	0.558
7--4--1	0.968	0.146	0.651	**0.001**	0.561	0.224
7--5	0.353	**0.047**	0.262	**0.009**	0.479	0.474
7--5--5	0.562	**0.015**	0.070	**0.008**	0.993	0.224
8--1--3	0.914	**0.001**	0.539	**0.031**	0.085	0.453
8--2--1	0.652	0.199	0.177	**0.001**	0.416	0.224
8--3--1	0.251	0.565	0.901	0.464	0.860	0.224
9--1	0.388	0.410	0.285	0.141	0.070	**0.025**
9--1--2	0.643	**0.005**	0.175	0.217	**0.010**	0.033
9--2	0.439	0.116	0.235	0.866	0.342	0.598
9--2--6	0.327	0.618	0.221	**0.042**	0.204	0.912
10--3	0.365	**0.010**	0.203	**0.000**	0.296	0.224
11--1	0.498	0.622	0.635	**0.003**	0.056	0.745
11--2	0.370	0.403	0.237	0.196	0.080	**0.004**
12--1	0.214	0.729	0.884	0.730	**0.000**	**0.000**
12--3	0.645	0.943	0.091	0.059	**0.023**	0.329
12--3--1	0.474	0.684	0.534	0.294	0.078	0.031
12--4--1	0.249	0.224	0.206	0.367	0.066	**0.000**

Summary of *S. pennellii* screen

- A large number of different volatile compounds were detected; however, many of the volatile effects were not repeatable between the two years of experiments.
- The t-tests have shown that the results were in some cases not significant over two years of study. For some ILs the data was significant in one year or not at all (summary in Table 2.3).
- In some cases despite results not being significant, ILs showed similar trend over period of two years
- Robust effects were apparent with the following volatiles, with respect to C5 unsaturated volatiles only IL7-2 showed enhanced levels across each of the years. For hexenal, IL9-3-1 showed higher volatile signal intensity, while IL1-4 presented similar trend for hexanal.
- In case of citral and methyl salicylate, levels in IL1-4 were significantly higher over two years of study in comparison with M82D.

2.3.3 Overview of volatile metabolites detected by APCI-MS: results in the S. habrochaites ILs

QTL maps were also created for the *S. habrochaites* ILs (Fig. 2.6a-d). These maps contain information about chromosome markers in *S. habrochaites* population as well as show information about detected volatiles. Values are expressed as fold increase in comparison with control fruit E6203.

The maps suggest that there is no volatile activity linked specifically to chromosome 11. Limited volatile activity can be related to regions linked with genes on chromosomes 3, 6, and 8. The most interesting effects appear on chromosome 7. It might be potential QTL for tomato flavour with genes linked to introgression 3950 supported by the presence of citral/methyl salicylate. These volatiles are important contributors to tomato flavour, but citral was reported as a volatile giving a positive note (Lewinsohn *et al*., 2005) and methyl salicylate was giving a negative note (Kazeniac and Hall 1970).

S. habrochaites lines were subject of volatile analysis undertaken by Mathieu *et al.,* (2009). The differences between this study and Mathieu *et al.,* (2009) were in the grown plants and methodology used. Mathieu *et al.,* (2009) used mostly field tomatoes and analysed the volatiles using GC-MS, while this study used glasshouse tomatoes and direct mass spectrometry (APCI-MS).

In, summary of APCI screen for *S. habrochaites*, in comparison with Mathieu *et al.,* (2009) strong dissimilarities were observed. Despite several ILs falling into the same chromosome, they did not exactly match the same regions. In addition, some volatiles identified in this study, were not identified in Mathieu *et al.,* (2009). Only 2-phenylethanol was identified in both studies in line 3932.

Figures 2.6a-b and 2.6c-d show QTL maps for *S. habrochaites* population; based on data from 2007 and 2008 screen. Similarly, to data for *S. pennellii* 2008, more fruits were analysed and the map for *S. habrochaites* population based on data from 2008 is more detailed. Some chromosomal regions show similar volatile evolution in each of the two years of experiments: Chr. 1 (line 3913), Chr. 5 (line 3938), and Chr. 4 (line 3934/3936 for 2-isobutylthiazole only).

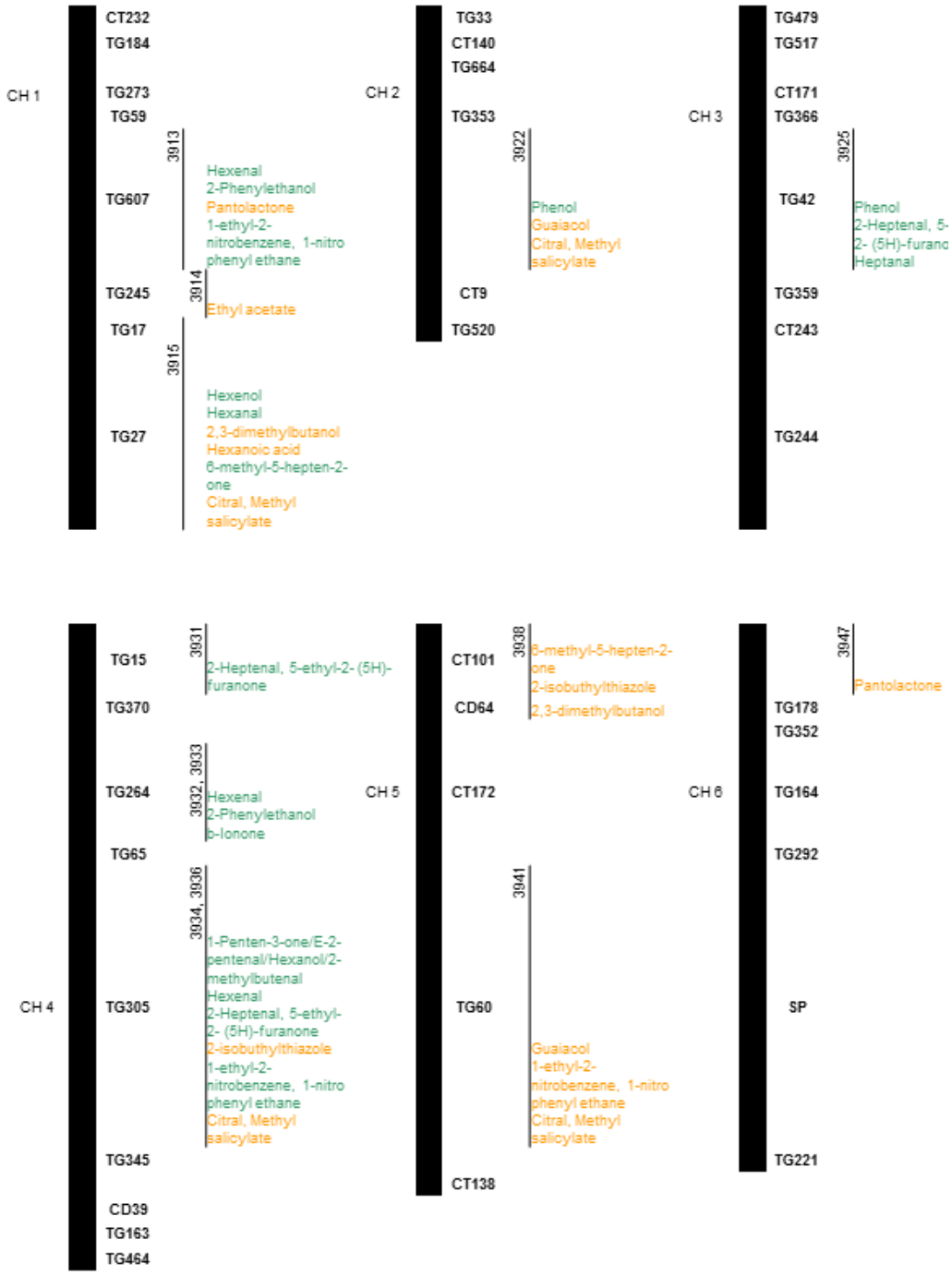


Figure 2.6a *S. habrochaites* 2007 volatile data for chromosomes 1 to 6. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters with numbers describe the relevant markers.

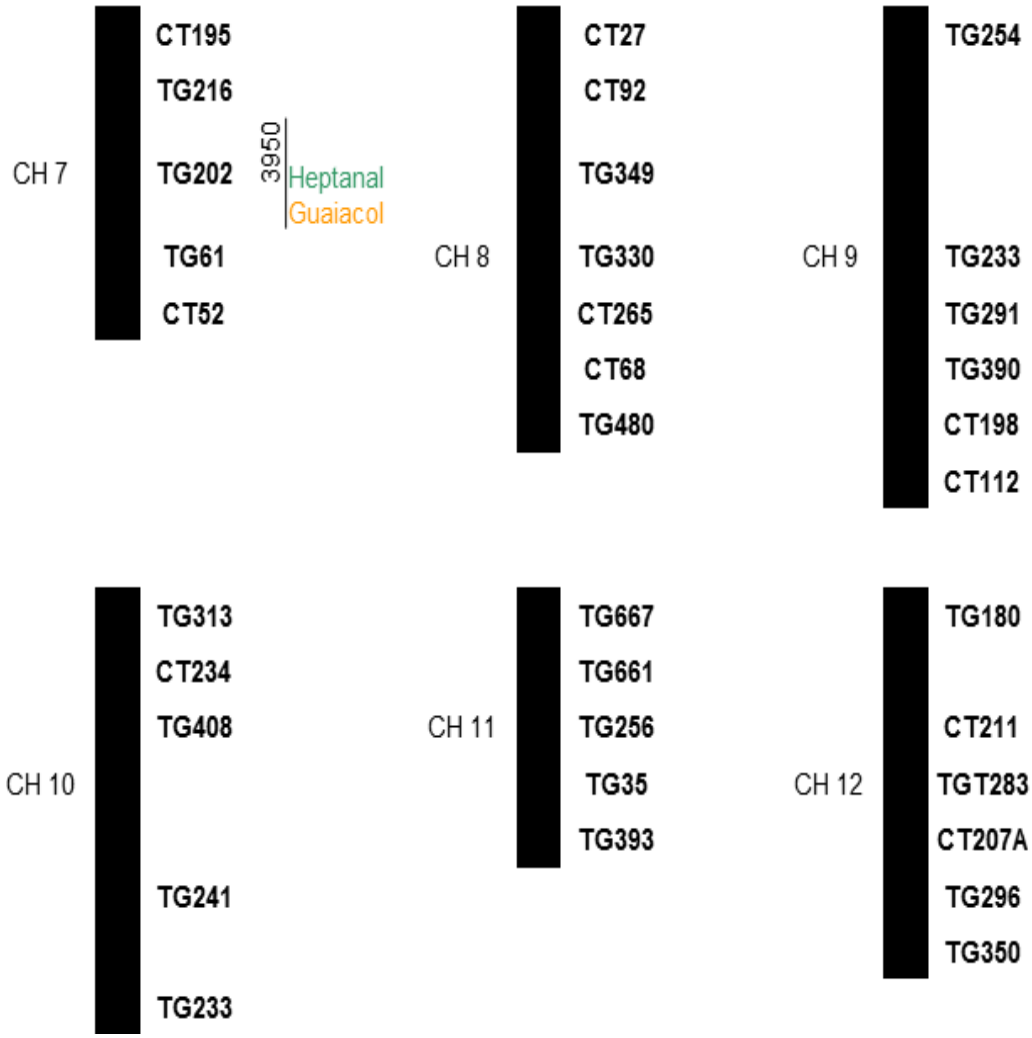


Figure 2.6b *S. habrochaites* 2007 volatile data for chromosomes 7 to 12. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters with numbers describe the relevant markers.

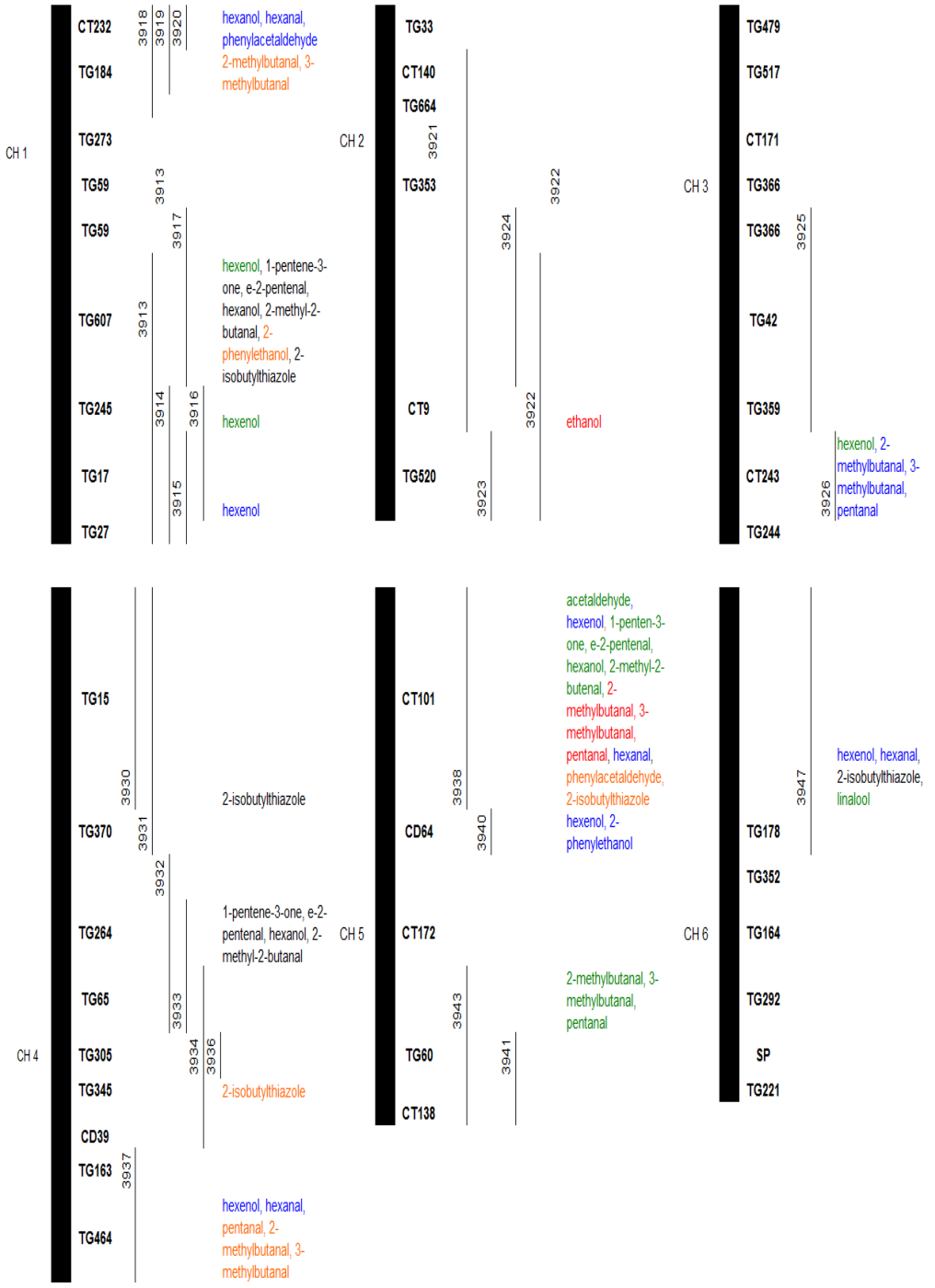


Figure 2.6c *S. habrochaites* 2008 volatile data for chromosomes 1 to 6. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters with numbers describe the relevant markers.

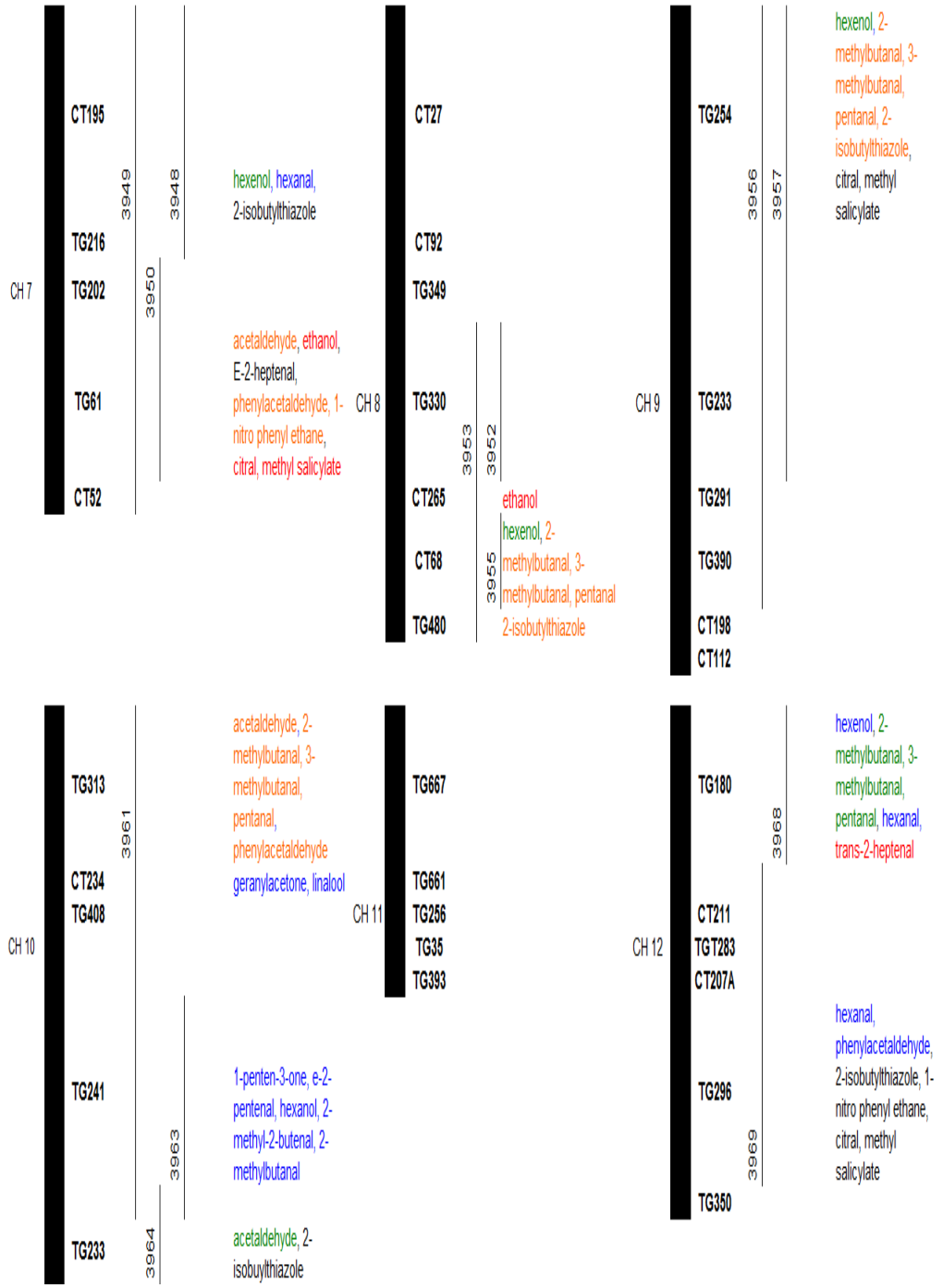


Figure 2.6d *S. habrochaites* 2008 volatile data for chromosomes 7 to 12. Map positions of flavour related volatiles or vQTL. Values are averages expressed as fold increase in comparison with M82D control fruit: Volatiles are annotated and fold changes are colour coded as 0.0-0.5 black, 0.5-1 blue, 1-2 green, 2-5 orange, 5 and more red. Each chromosome is marker defined. Student's t-test was performed and significant values are presented (at $P<0.05$). Vertical lines correspond to chromosomes and introgressions, while letters with numbers describe the relevant markers.

2.3.3.1 Focusing in on main effects

Principal component analysis was undertaken to identify main effects (Fig. 2.6e-f). Figure 2.6e shows both of the principal components in dimensional space where each variable is represented as a vector. The length and direction of the vector indicates how each variable contributes to the principal components.

For instance, Figure 2.6e shows that the first principal component has positive coefficient with four variables (m/z 85, m/z 87, m/z 99, and m/z 121), while the negative coefficient has three variables (m/z 101, m/z 123, m/z 153). The second principal component has negative coefficient with five variables (m/z 85, m/z 87, m/z 101, m/z 121, and m/z 153). Positive coefficient is represented by two variables (m/z 99, m/z 123).

Moreover, it is possible to conclude from the Figure 2.6e e that m/z 99 and m/z 101 are both in opposition based on the coefficients for the both principal components. Variables m/z 121 and m/z 152 are in opposition to m/z 123, based on second principal component. In addition, m/z 85 and m/z 87 cluster together.

The strong correlation between variable m/z 101 and line 3915 was observed (see Figure 2.7d). Moreover, a weak correlation between lines 3923, 3950, 3956 was detected for variable m/z 153 (compare with Figure 2.7e). In addition, a correlation between lines 3931, 3938, 3967and variable m/z 87 have been identified (Figure 2.7a for cross reference).

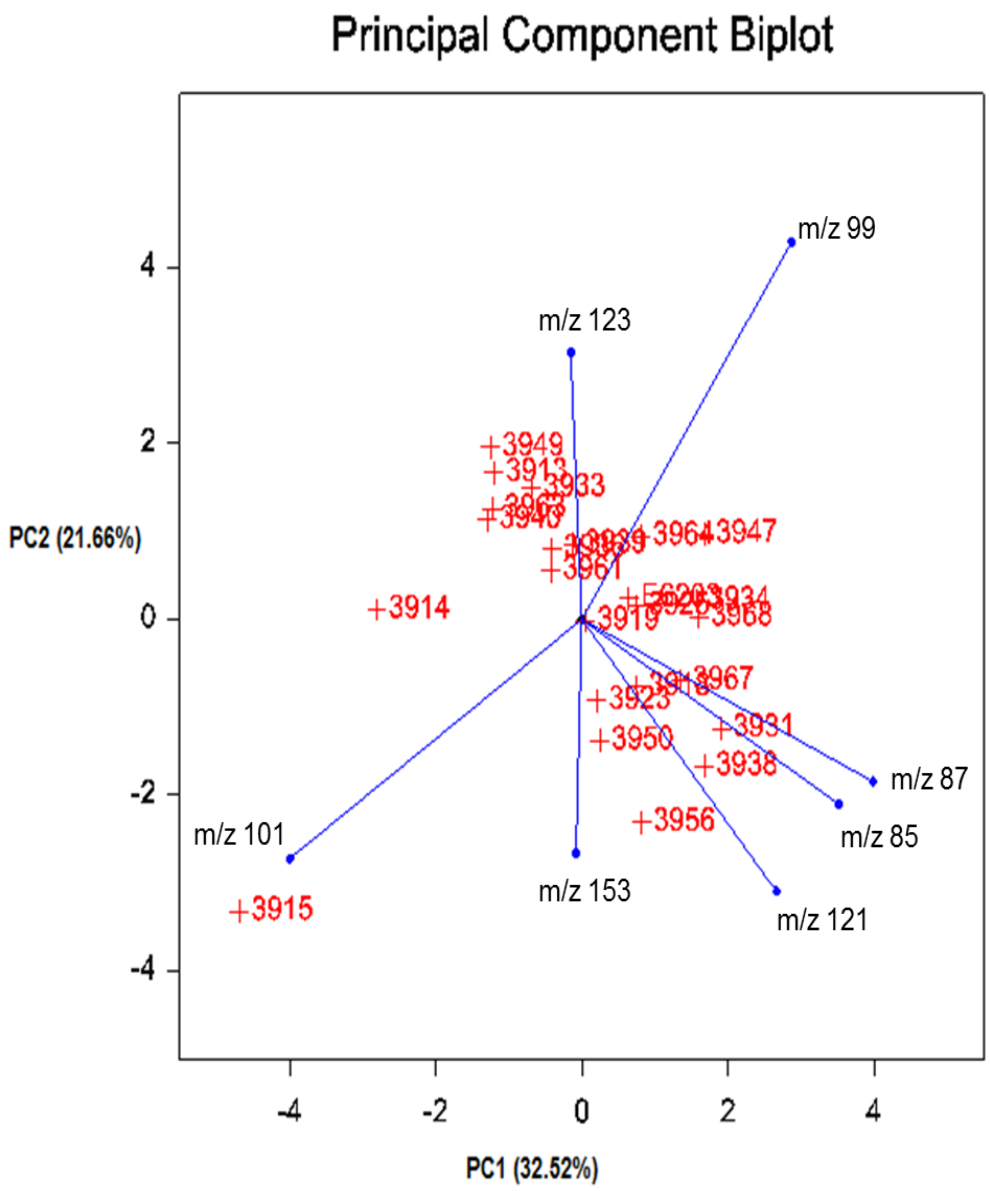


Figure 2.6e PCA biplot for *S. habrochaites* population in 2007. All ILs presented, but only selected volatiles shown. First component explains 32% of variation; second component is responsible for 21% of variation. m/z 85 – C5 unsaturated volatiles, m/z 87 – methylbutanals, m/z 99 – hexenals, m/z 101 – hexanal, m/z 121 – 2-phenylethanol, m/z 123 – 2-phenylacetaldehyde, m/z 153 – citral/methyl salicylate. Red pluses indicate individual data points, blue lines represent coefficients.

Assessment of ILs from 2008 screen was undertaken using PCA in support of QTL map generation and graphical presentation of volatiles distribution. Figure 2.6f shows both of the principal components in dimensional space. Each variable is represented as a vector. The direction and length of the vector specifies how each variable contributes to the principal components.

Figure 2.6f shows that the first principal component has positive coefficient with three variables (m/z 99, m/z 101, and m/z 123), while the negative coefficient has four variables (m/z 85, m/z 87, m/z 121, m/z 153). The second principal component has negative coefficient with four variables (m/z 101, m/z 121, m/z 123, and m/z 153). Positive coefficient is represented by three variables (m/z85, m/z 87 m/z 99). Moreover it was possible to conclude from the Figure 2.6f that m/z 99 and m/z 101 are both in opposition based on the coefficients second principal component. Variables m/z 121 and m/z 152 are in opposition to m/z 123, based on first principal component. In addition, m/z 85 and m/z 87 cluster together, similarly like during 2007 screen. Lines 3938 and 3957 moderately correlate with m/z 87 (see Figure 2.7a). A strong correlation was observed for lines 3955 and 3947 in regard of variable m/z 99 (Figure 2.7c for cross reference). The weak correlation was observed with line 3920 and variable m/z 101 (Figure 2.7d for cross reference).

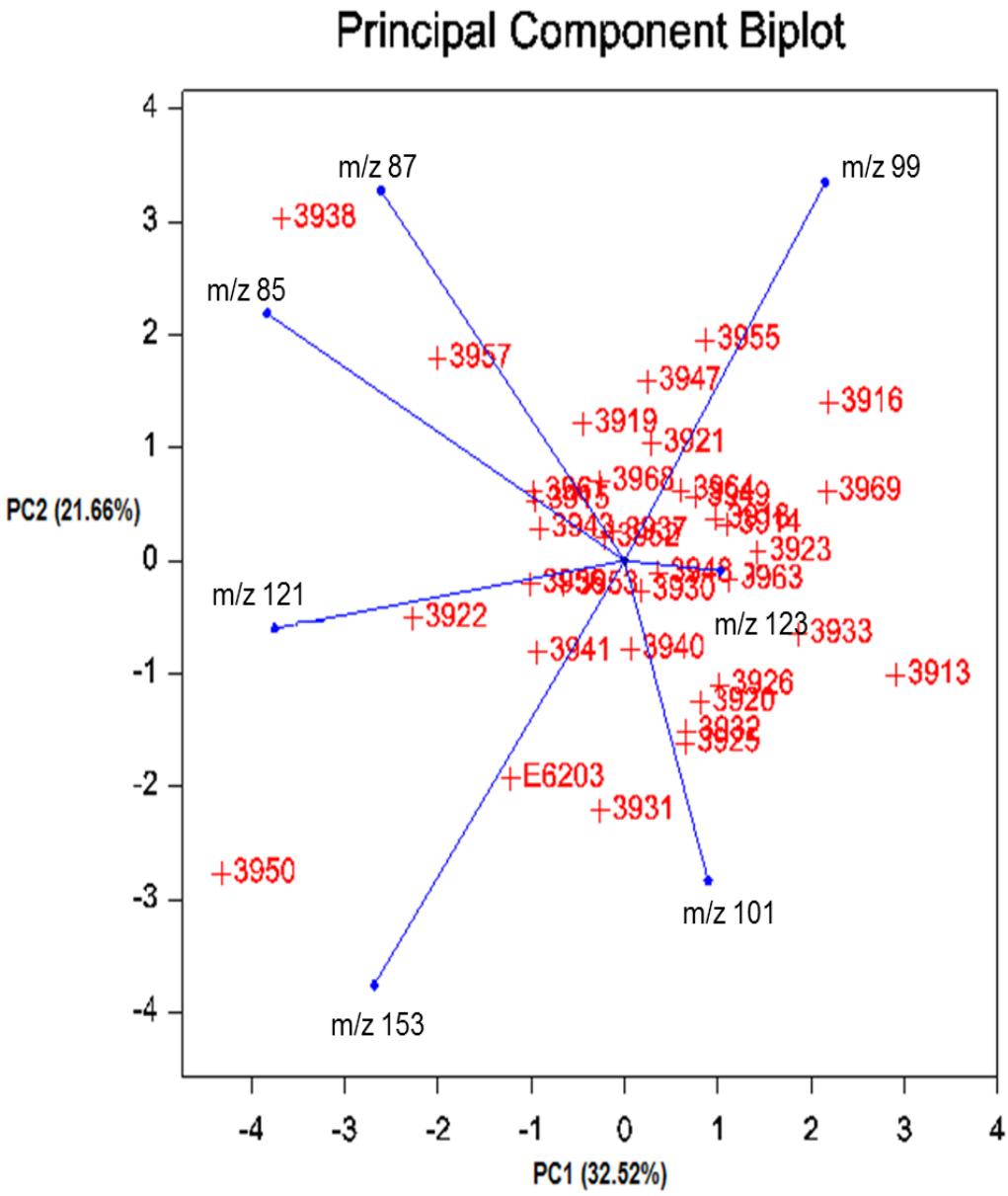


Figure 2.6f PCA biplot for *S. habrochaites* population in 2008. All ILs presented, but only selected volatiles shown. First component explains 32% of variation; second component is responsible for 21% of variation. m/z 85 – C5 unsaturated volatiles, m/z 87 – methylbutanals, m/z 99 – hexenals, m/z 101 – hexanal, m/z 121 – 2-phenylethanol, m/z 123 – 2-phenylacetaldehyde, m/z 153 – citral/methyl salicylate. Red pluses indicate individual data points, blue lines represent coefficients.

2.3.4 Detailed analysis of the distribution of key volatiles as determined by APCI-MS

Figure 2.7a presents the data collected for *S. habrochaites* ILs during 2007 and 2008 for methylbutanals (Fig 2.7a). In 2007, line 3913 was significantly lower ($P<0.01$) in volatile concentration in comparison with control fruit E6203. The highest line in terms of methylbutanals was 3931; however, this effect was not significant. Consequent analysis of plant material in 2008 revealed that line 3969 was significantly low ($P<0.05$), while 3938 was highest in volatile concentrations when compared with control E6203 in 2008 ($P<0.05$). Line 3913 was significantly lower during two years of experiments with APCI-MS. In 2008, lines 3941 ($P<0.01$), 3955 ($P<0.05$), 3961 ($P<0.05$), 3962 ($P<0.05$) and 3967($P<0.01$) were significantly higher than control E6203.

C5 unsaturated volatiles (1-penten-3-one and E-2-pentenal) were also evaluated and the results of two years of APCI experiments for these volatiles are assessed on Figure 2.7b. In 2007, lowest line in volatile concentration in comparison with control fruit E6203 (Figure 2.7b) was 3913 ($P<0.01$). In addition, line 3933 was also significantly low ($P<0.001$). In contrast, line 3925 was the highest; however, it was not significant, but lines 3967 ($P<0.001$) and 3934 ($P<0.05$) were significant. The following year analysis demonstrates that line 3913 was lowest and significant ($P<0.05$) in volatile signal in comparison with control fruit E6203. Line 3938 was significantly ($P<0.05$ in 2008) highest in comparison with control fruit E6203, other lines 3919, 3947, 3950, 3956, 3964, 3968 followed the trend over two years, but they were not significant. Lines 3913 ($P<0.01$ in 2007 and $P<0.05$ in 2008) and 3933 ($P<0.001$ in 2007 and $P<0.05$ in 2008) were both significantly lower during two years of experiments with APCI-MS.

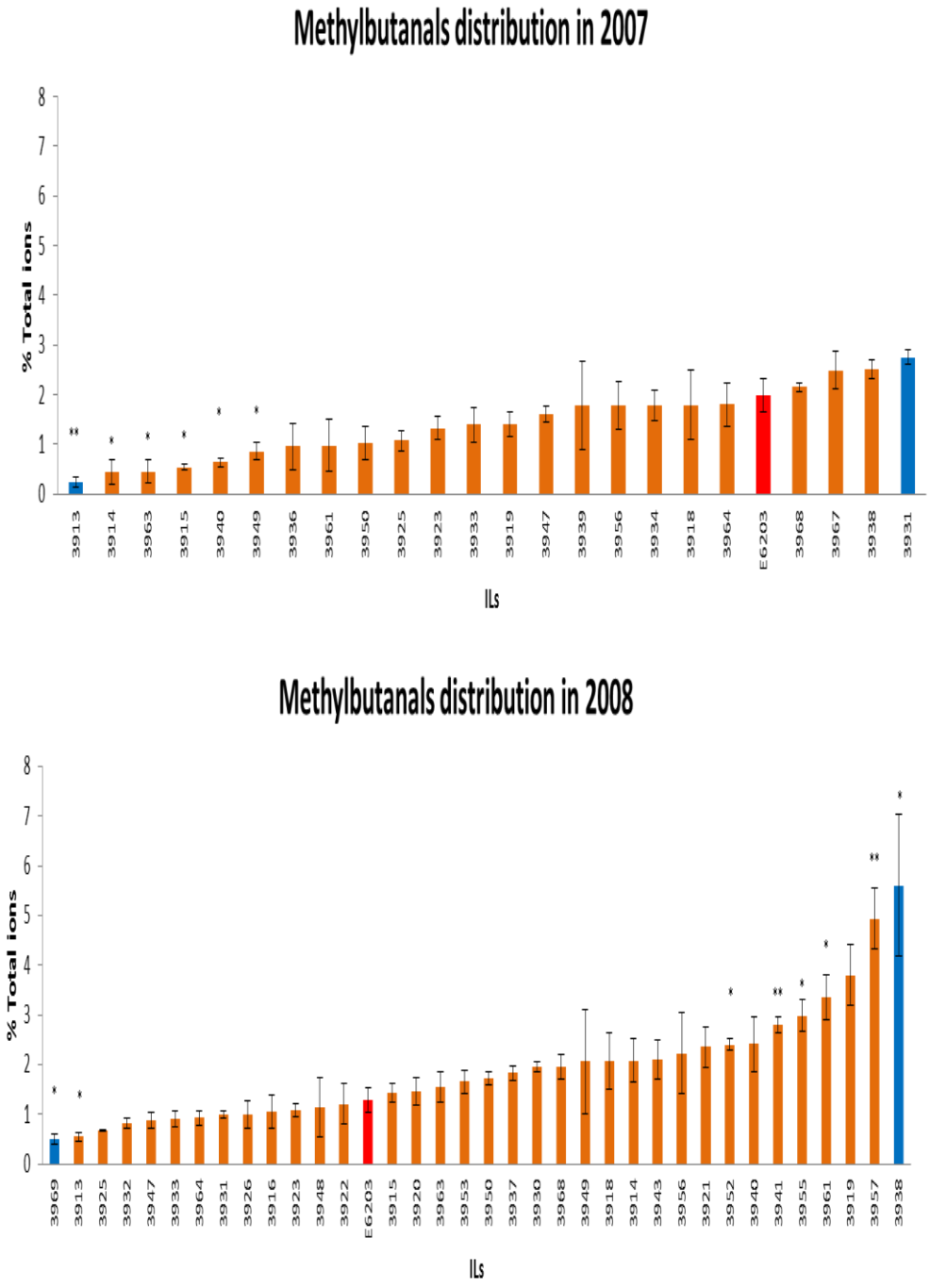


Figure 2.7a Methylbutanals distribution for *S. habrochaites* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). E6203 highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

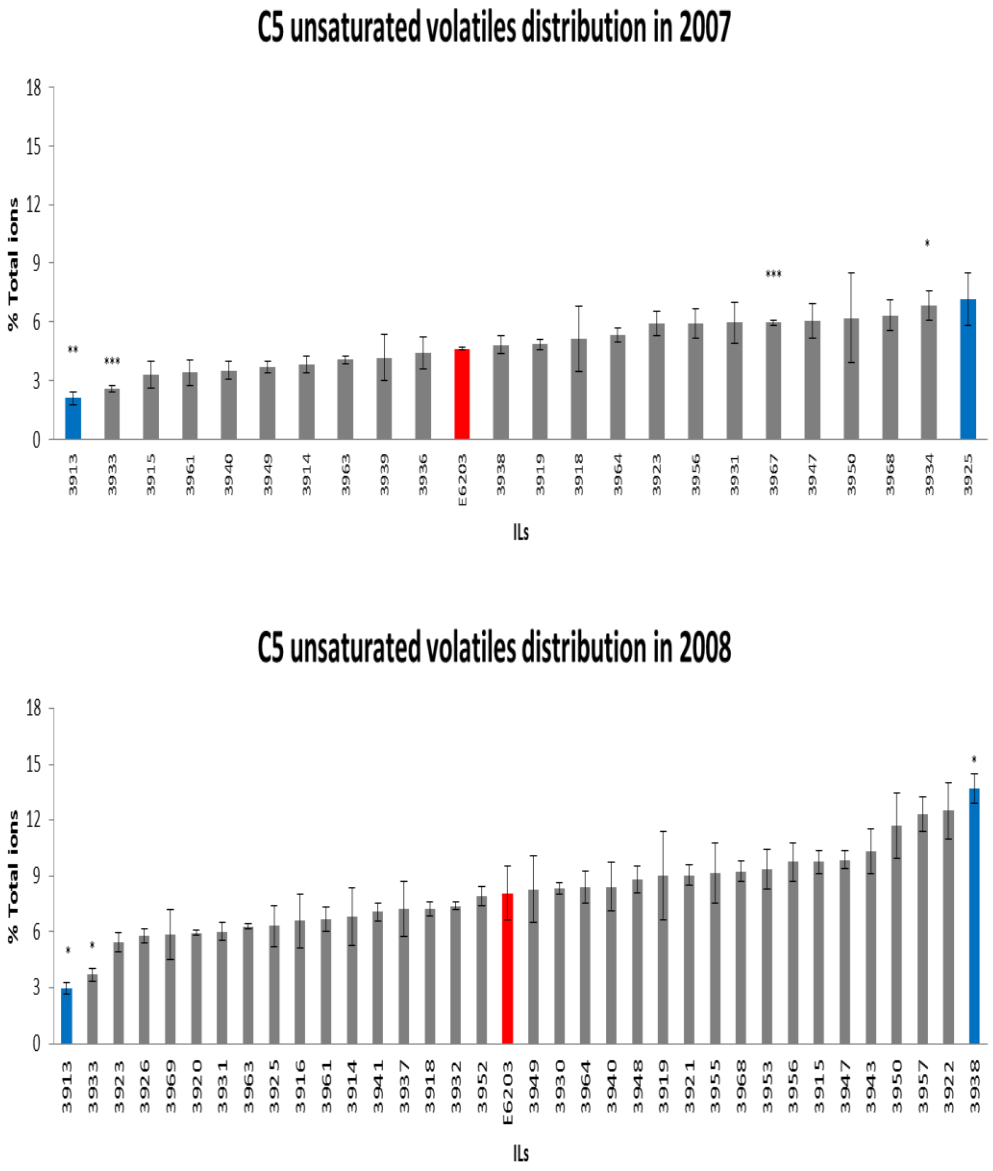


Figure 2.7b C5 unsaturated volatiles distribution for *S. habrochaites* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). E6203 highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

Analysis of hexenal contribution in the *S. habrochaites* ILs was attempted as well. Figure 2.7c presents the data collected for ILs during 2007 and 2008 for Hexenals. In 2007, line 3915 was significantly lowest (P<0.001) in volatile concentration in comparison with control fruit E6203 (Figure 2.7c)

while line 3934 was significantly highest ($P<0.01$) in volatile concentration in comparison with E6203 control. In contrast, data from 2008 indicates that the lowest line was 3950, but this line was not significant. The highest line in volatile concentration was 3916 also significant ($P<0.05$). Lines 3947, 3961, 3964 and 3968 were significantly higher during 2 years of experiments with APCI-MS (with $P<0.05$ for both years, only 3947 in 2008 had $P<0.001$). Interestingly line 3914 was significantly low ($P<0.05$) in 2007, but it was significantly higher ($P<0.05$) in 2008, when compared with control E6203.

Figure 2.7d presents the data collected for *S. habrochaites* ILs during 2007 and 2008 for hexanal. The screen of 2007 reveals a series of lines that were significantly lower ($P<0.05$) in volatile concentration in comparison with control fruit E6203. The lowest line was 3947 ($P<0.001$). Line 3915 was significantly the highest ($P<0.001$) in volatile concentration in comparison with E6203 control. Assessment of ILs in 2008 showed a number of lines that were significantly lower in volatile concentration in comparison with control fruit E6203. Line 3947 was significant ($P<0.05$) and was consistently the lowest. Line 3920 was significantly highest ($P<0.05$) in comparison with control fruit E6203. During the two years of experiments four lines demonstrated higher concentrations in comparison with E6203 control fruit, these lines were 3913, 3914, 3940 and 3961, however they were not significant (only 3914 significant at $P<0.001$ in 2007).

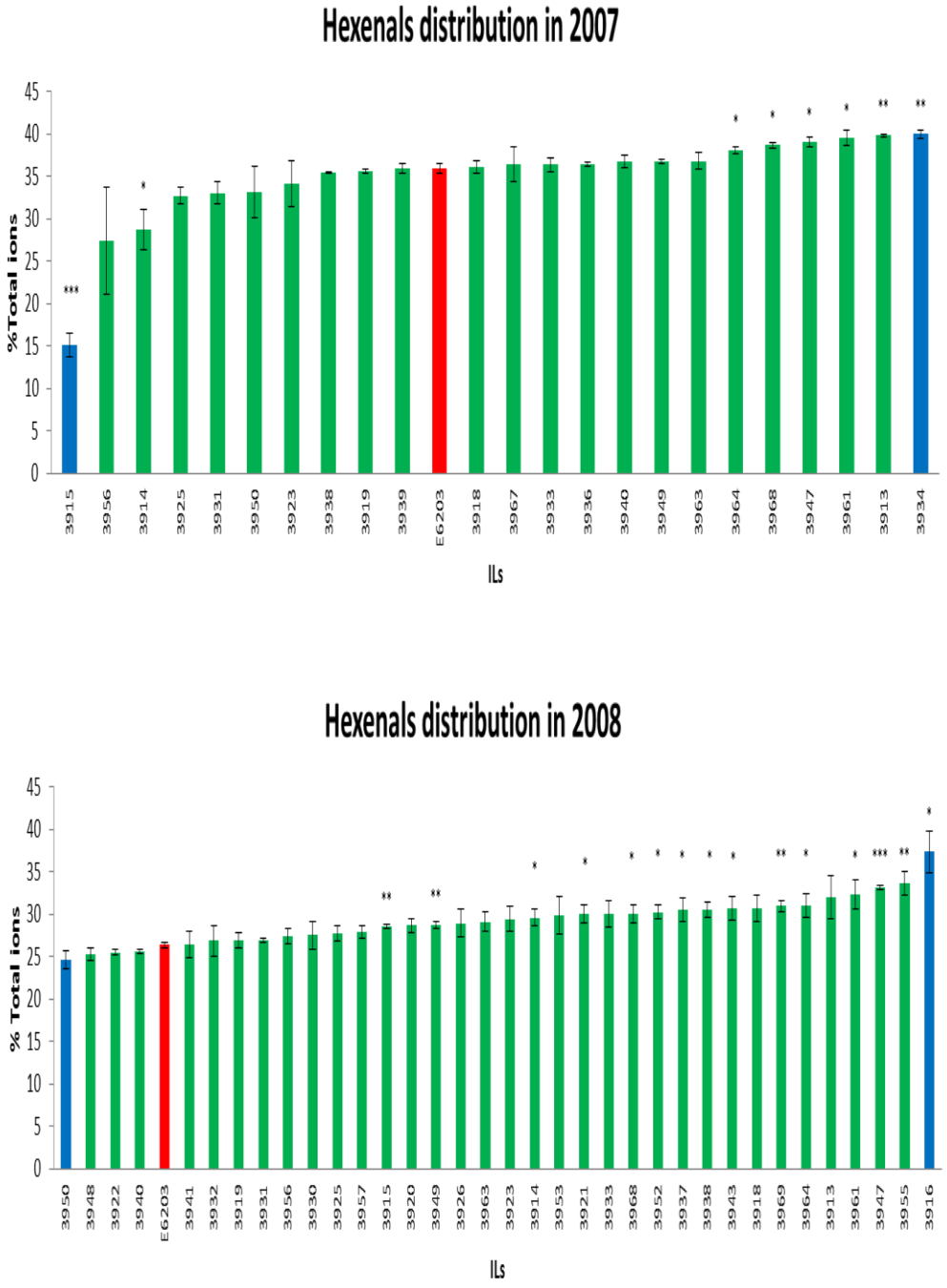


Figure 2.7c Hexenals distribution for *S. habrochaites* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). E6203 highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

Exploration of *S. habrochaites* ILs was performed as well for m/z 153 corresponding to citral and methyl salicylate. Figure 2.7e shows the data collected during 2007 and 2008. In 2007, the lowest line was 3933, but it was not significant. The highest line to report was 3950 and similarly like the low one, it was not significant. The 2008 screen indicated the same line 3957 as the lowest and at a significant level ($P<0.001$). During this year screen also line 3950 was the highest and significant ($P<0.05$). Lines 3913 ($P<0.05$ in 2007 and $P<0.001$ in 2008), 3915 ($P<0.05$ in 2007 and $P<0.01$ in 2008) and 3947 ($P<0.05$ in 2007 and $P<0.001$ in 2008) were consequently significant over two years of studies in comparison with control E6203 (Fig. 2.7e).

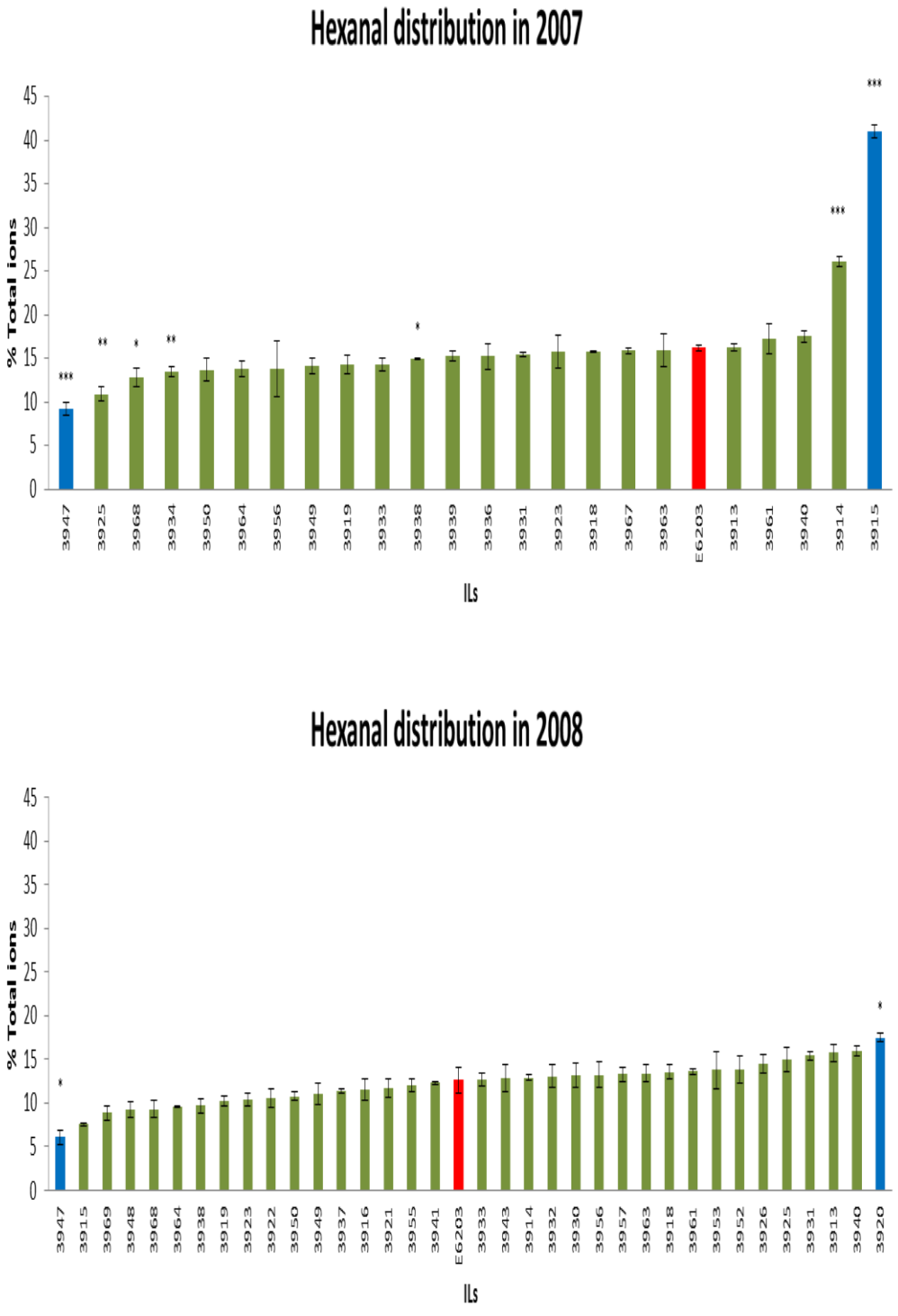


Figure 2.7d Hexanal distribution for *S. habrochaites* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). E6203 highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

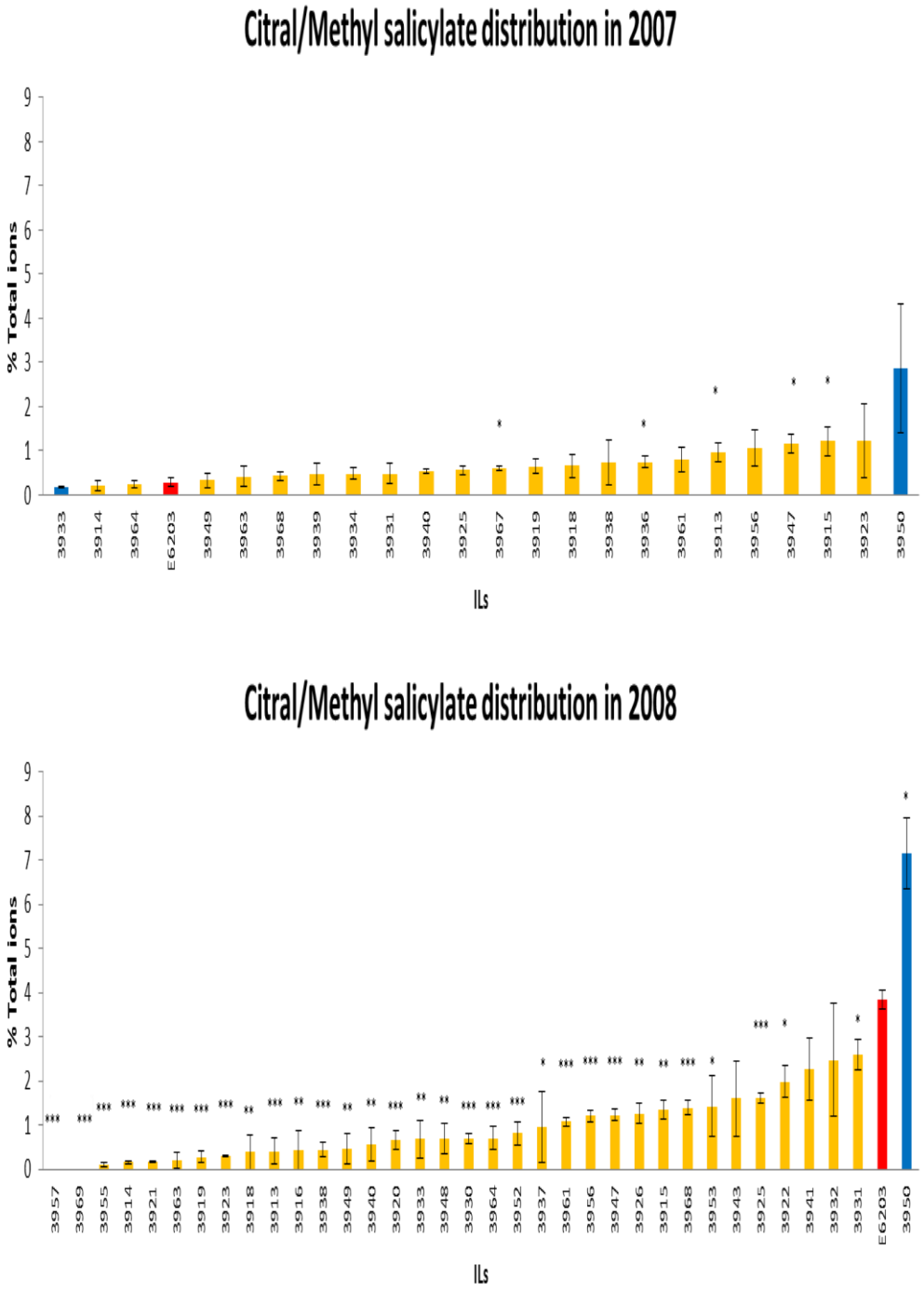


Figure 2.7e Citral/Methyl salicylate distribution for *S. habrochaites* ILs. 3 technical replicates were used for calculations. Error bars are standard error of mean (n=3). E6203 highlighted in red, while the highest and lowest IL highlighted in blue. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

Our data can be successfully compared with other authors published work. Tieman and colleagues (Tieman *et al.,* 2006) generated a QTL map with volatile effects, and their results can be associated with ours. The presented work for *S. habrochaites* ILs also leads to conclusion that there may exist a possible association. Methylbutanals were identified on Chr. 5, which agrees with the *S. pennellii* data, Tieman *et al.,* (2006) and work of Mathieu *et al.,* (2009). The data for *S. habrochaites* ILs can be further compared with Mathieu *et al.,* (2009) work. C5 unsaturated volatiles were identified on the same chromosomes, but in the different regions and ILs 7 (3947, 3950 in our study, 3950 in Mathieu *et al.,* 2009) and 12 (3968 in our study, but 4002 in Mathieu *et al.,* 2009) and hexenal was also identified on Chr. 12 (3968 in our study and 3995 in Mathieu *et al.,* 2009).

The difference in volatiles distribution between our study and published work, likely reflects the different analytical approaches. Tieman *et al.,* (2006) used a hydrocarbon trapping system followed by collection of volatiles on Super Q column and separation on GC-MS with DB-5 column attached. Mathieu *et al.,* (2009) followed this method for discriminative analysis of *S. habrochaites* population.

However, simplicity and reliability make this technology very beneficial and sufficient for large scale profiling. A number of different tomato compounds (acetaldehyde, ethanol, methylbutanol, hexenol, methylbutanal, hexenal, hexanal, 6-methyl-5-hepten-2-one, 2-isobutylthiazole) were successfully characterised in fresh tomato (Boukobza *et al.,* 2001) using APCI-MS.

2.4 Conclusions

- APCI-MS proved to be appropriate technique for volatile analysis in *S. pennellii* and *S. habrochaites* ILs.

- The biggest disadvantage of APCI-MS approach is lack of recognition between isomers (positional and stereo isomers) and compounds with the same molecular mass.

- The *S. habrochaites* lines did not present a lot of volatile activity in comparison with *S. pennellii* based on PCA and graphical analysis. Further experiments with these lines will not be continued. This approach indicated potential candidate introgressions.

- Out of 76 *S. pennellii* ILs only 18 were chosen based on data generated using APCI approach over two years of study. These were either significant over two years or in one year only or presented a trend. These promising lines were IL1-4, IL1-4-18, IL2-1-1, IL4-1-1, IL4-3, IL4-4, IL5-1, IL8-2, IL8-2-1, IL9-1, IL9-1-2, IL9-1-3, IL9-3, IL 9-3-1, IL9-3-2, IL11-2, IL12-1, and IL12-2.

- For *S. pennellii* several trends were identified based on m/z 99 and m/z 101 data and they will be further investigated in the next chapter.

CHAPTER 3:

Detailed analysis of selected ILs from *S. pennellii* population using Gas Chromatography-Mass Spectrometry with solid phase microextraction (GC-MS SPME).

3.1 Introduction

3.1.1 GC-MS SPME outline

Gas chromatography-mass spectrometry is commonly used analytical tool, to identify and quantify volatile and semi-volatile compounds present in complex mixtures. GC is capable of separation of compounds with high resolution, and MS provides detailed information on most compounds leading to their exact identification. GC-MS has been used successfully for analysis of volatiles in tomato. Some researchers used standard solvent based GC-MS, to evaluate volatile compounds in tomato-based products (Narain *et al.,* 2010).

Fundamental work undertaken by Buttery (Buttery et al., 1987; 1990; 2004) involved using a Tenax trapping system. Other authors (Linforth *et al.,* 1994; Brauss *et al.,* 1998, Krumbein and Auerswald 1998) also found this technology efficient. The sample in a Tenax system is concentrated under pure air or other gas. The released volatile compounds travel from sample tube into a purge head, where they are pushed via the flow of gas. The purge gas enters purge head and leaves via Tenax trap, leaving volatiles behind in the trap. The compounds from the trap are then solvent eluted and quantified on GC-MS.

At the present time, tomato volatile research involves analysis of compounds by GC-MS SPME (Marković *et al.,* 2007, Tikunov *et al.,* 2005). Solid phase microextraction; was first developed by Pawliszyn and his

colleagues (Arthur and Pawliszyn 1990; Pawliszyn 1997). This is a headspace analysis technique, which can be effectively used for analysis of volatile compounds. The aspects of GC-MS SPME, design and limitations are discussed in Materials and Methods section.

3.2 Materials and methods:

3.2.1 Gas chromatography mass spectrometry (GC-MS) – method development.

Gas chromatography-mass spectrometry is common analytical tool used widely to identify and quantify volatile and semi volatile compounds present in complex mixtures. Sample preparation can vary from simply dissolving some of the sample in a suitable solvent to extensive clean-up procedures using various forms of liquid chromatography. Data analysis can take another 1 to a couple of h depending on the level of detail necessary. Accuracy of 5-10% of a mean is typical for liquid based GC-MS. Headspace techniques are less precise with accuracy of 10-20% of a mean.

GC-MS is presented in Figure 3.1. Samples were injected through injector port into a GC column placed in GC oven. Analytes travel through column and were separated by their size (smaller compounds travel faster while bigger travel much slower). Later, they are transferred into mass spectrometer (quadrupole), where molecules enter a high vacuum region. There compounds are bombarded by high-energy electrons, resulting in fragmentation. Fragmentation patterns depend on the structure of the compound. Generated ions are than lead to the analyser where an electric field separates them based on their mass.

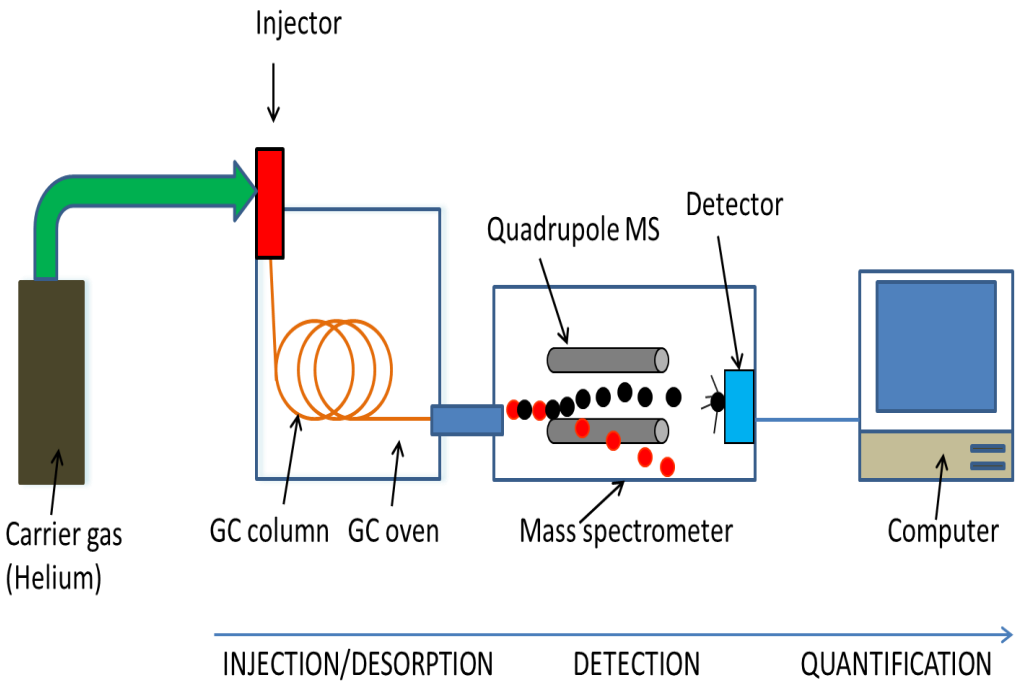


Figure 3.1 A schematic representation of GC-MS. Samples are delivered to gas chromatograph via injection port, kept hot to ensure that compounds will remain in the gas phase. In GC column thermal desorption occurs and compounds separated by their mass are transformed to mass spectrometer (quadrupole, but only 2 out of 4 rods are shown), where compounds with right m/z are detected. Quantification is undertaken using computer software.

Typical chromatogram for tomato sample is shown on Figure 3.2. Major compounds arrive within 3 to 18 min time slot, based on their mass (smaller earlier, heavier arrive later). A typical, ion profile for hexanal is presented in Figure 3.3, with characteristic ions of 44, 56, 82.

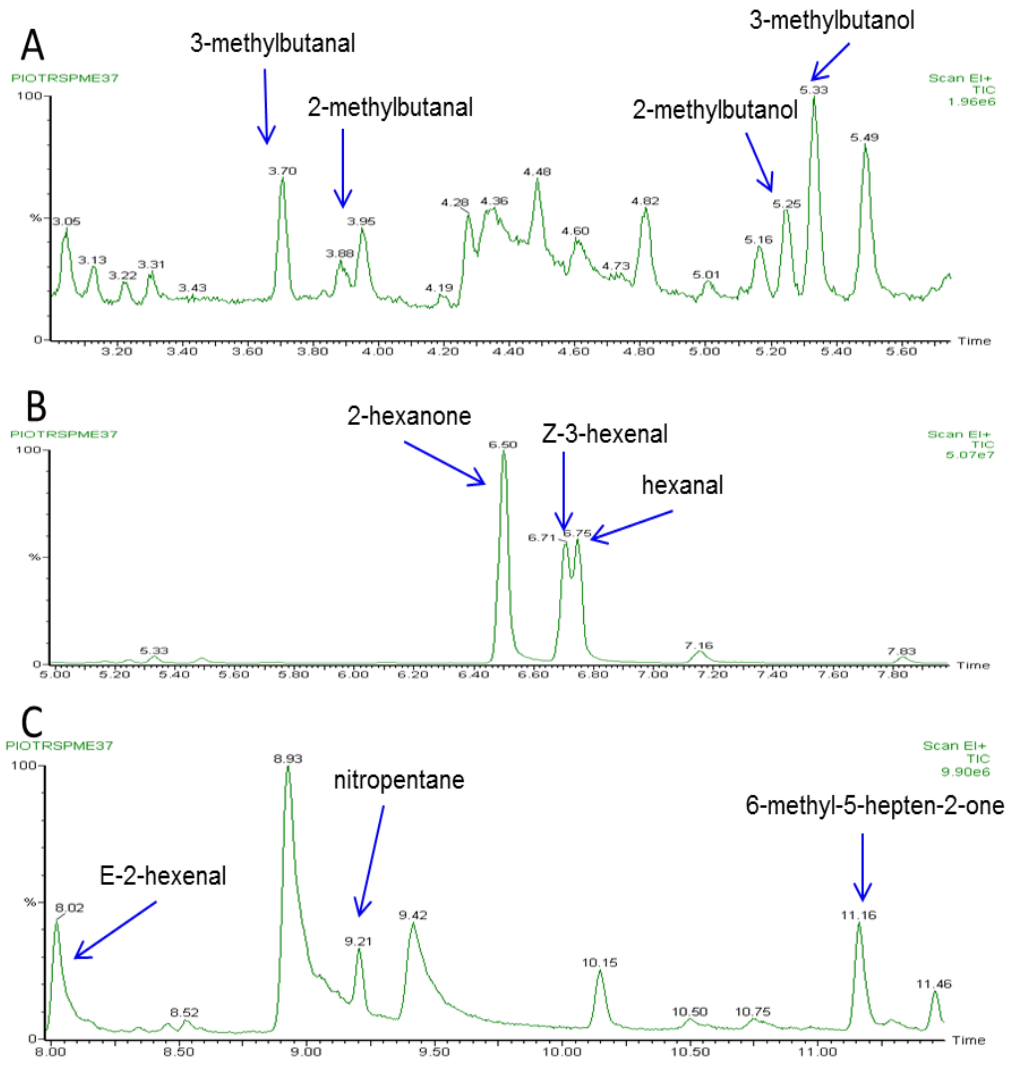


Figure 3.2 Full scan chromatographic profile for tomato fruit sample 3.00 to 5.70 min of GC run (A). Peaks: 3.70, 3-methylbutanal; 3.88, 2-methylbutanal; 5.25, 2-methylbutanol; 5.33, 3-methylbutanol. Full scan chromatographic profile; for tomato fruit sample 5.00 to 7.90 of GC run (B). Peaks: 6.50, 2-hexanone (Internal standard); 6.71, Z-3-hexenal; 6.75, hexanal. Full scan chromatographic profiles for tomato fruit sample 8.00 to 11.50 of GC run (C). Peaks: 8.02, E-2-hexenal; 9.21, nitropentane; 11.16, 6-methyl-5-hepten-2-one.

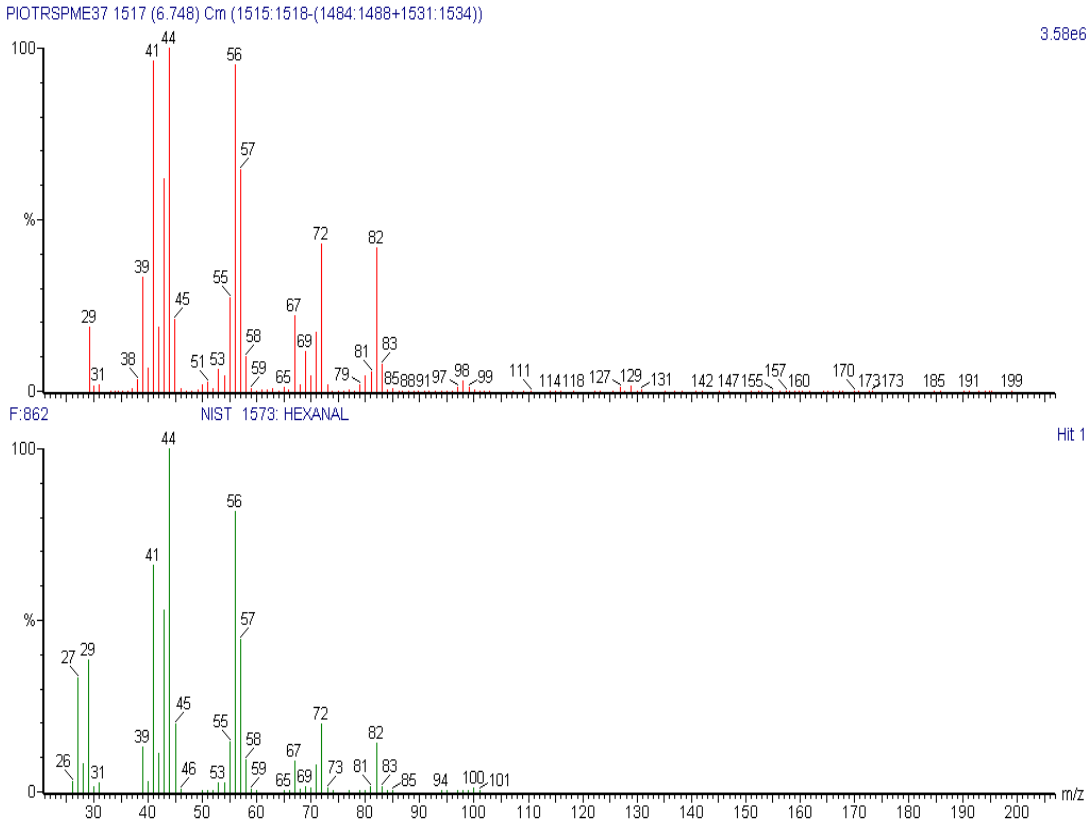


Figure 3.3 Mass spectra for hexanal. Top – spectrum for the sample, bottom - library hit. Characteristic ions are 44, 56, 82.

3.2.1.1 Initial experiments with manual GC-MS SPME

An alternative way for non-solvent volatile extraction was proposed. It is called solid phase microextraction (SPME), and involves the use of a fibre, that can be simply regarded as inverted chromatography column with adsorptive phase being outside. Initial SPME experiments were performed with use of manual system. To perform manual SPME an injector liner and septum must be changed. A special SPME holder is used for extracting and injecting analytes.

Preliminary experiments with use of manual SPME allowed answering some key questions in regard of stability of tomato extracts as well as fibre exposure time (extraction time) and as a result optimize the methodology. Tomato extracts were left for the period of 20, 187.5, and 355 min and after

these times the volatile content was measured. The second experiment was undertaken to establish the best extraction time, where SPME fibre was exposed into headspace for 5, 10, and 20 min.

Data presented on Figure 3.4 suggests that there is no significant difference between sample holding times that would affect volatile concentration. Short sample holding time was 20 min, medium sample holding time was 187.5 min and long sample holding time was 355 min. It suggests that even after 6 h the samples are stable. The highest differences were observed for E-2-hexenal.

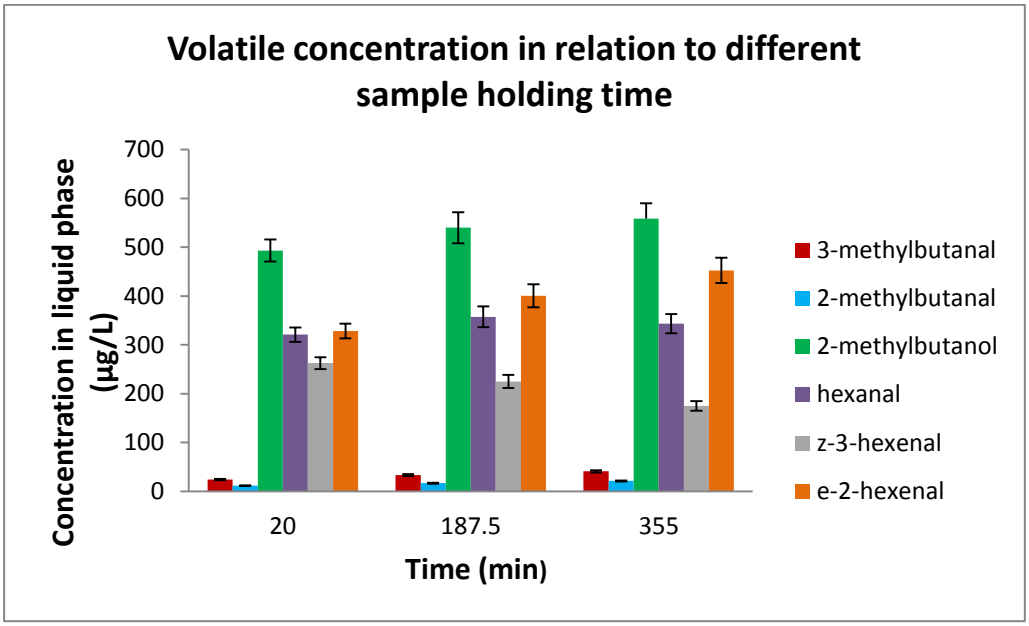


Figure 3.4 Concentration of volatiles in relation to holding time. Holding time was a time for a sample to be left before the analysis. Error bars are standard errors of the mean (n=3). Three technical replicates were used per holding time.

Figure 3.5 shows concentrations of four volatiles in relation to fibre exposure (extraction time). Extraction time was as below. Short trapping time: 5 min, medium trapping time: 10 min, long trapping time 20 min. Results suggest that, extended, long trapping time, increases volatile concentrations. It

is true for 2 out of 4 volatiles. The only exceptions are 3-methylbutanal and 2-methylbutanal. Possible reasons for this difference might be explained by the fact that C6 volatiles are released upon maceration and pre-longed trapping time is beneficial for their extraction. In addition, some competition effects between volatiles and fibre may occur and ability to transfer between headspace-fibre-injector may also vary between compounds.

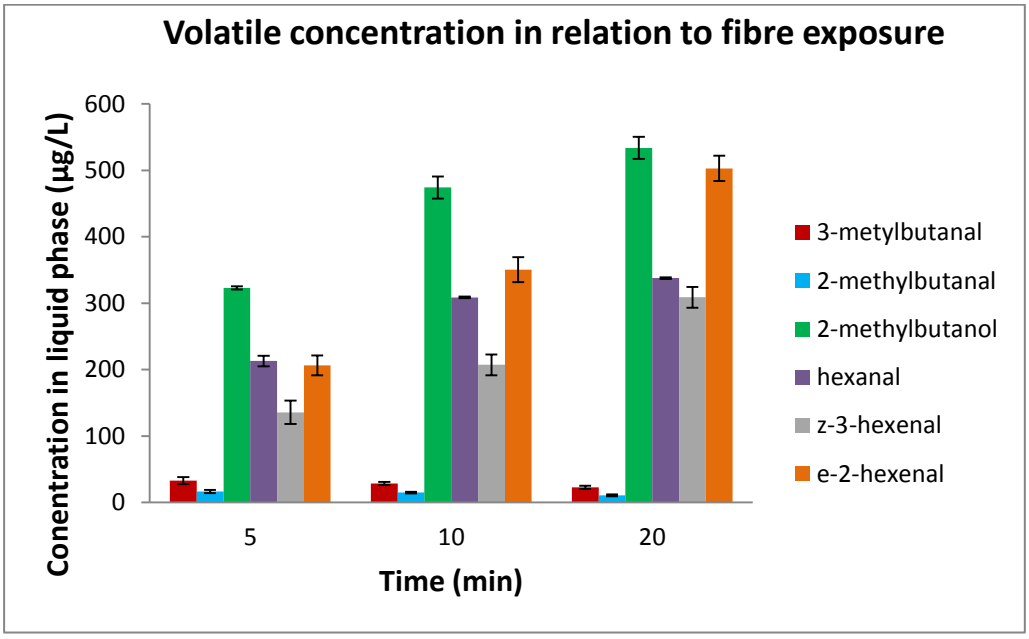


Figure 3.5 Concentration of volatiles in relation to trapping time. Trapping time was a time of sample extraction into exposed fibre. Error bars are standard errors of the mean (n=4). Four technical replicates were used per trapping time.

3.2.1.2 Automated GC-MS SPME

There is a slight difference in use of manual and automated GC-MS SPME. Running bigger amount of samples is facilitated with use of automated SPME, while in contrast achieving this task with manual SPME might be difficult. Extraction procedure, desorption and injection are performed constantly using standard autosampler. In addition, automated sample

handling procedures not only shorten the total analysis time, but also provide better accuracy and precision relative to manual techniques (Pawliszyn 2002).

An automated SPME method was developed. This method involves an increase in sampling temperature and addition of an agitation step. These two conditions allow better performance than in manual SMPE.

The method for automated SPME is presented below. Whole tomato fruit was blended for 2 min in a small blender, followed by addition of 2M calcium chloride (w/v; added to stop enzyme activity) and blending for 1 min. 50 μL of 3-heptanone (0.5 mg/L) was added as an internal standard into 100 mL of the blended tomato sample. Butylated hydroxytoluene (BHT) was added to stop free radical oxidation at 25 mg per sample, followed by 1 min blending. Blended samples were transferred into SPME vials (20 ml capacity, flat bottom, 23 mm x 75 mm; Nr 27199, Supelco, Sigma-Aldrich, Gillingham, U.K.) and sealed with a SPME seal (SU 860053, Supelco, Sigma-Aldrich, Gillingham, U.K.)

For GC-MS, the sample was extracted for 10 min using SPME fibre (3 phase: divinylbenzene/carboxen/polydimethyloxilane, needle length 1 cm, 57329-U, Supelco, Sigma-Aldrich, Gillingham, U.K.) with constant agitation at 500 rpm with temperature set to 35^{o}C. Sample was injected into mechanical SPME injector (Merlin Instrument Company, Newark, U.S.A) in splitless mode (split closed for 1 min) using a COMBI PAL autosampler (CTC Analytics, Zwingen, Switzerland). Desorption time was 2 min followed by 10 min fibre conditioning. The injector of the Trace GC 2000 (Thermo Fisher Scientific, Loughborough, U.K.) gas chromatograph was maintained at 250^{o}C, with an initial oven temperature of 40^{o}C, which was increased to 200^{o}C at 8^{o}C/min. After 20 min, temperature was increased to 260^{o}C and MS was switched off

for the last 3 min. Helium (18psi) was used as the carrier gas to elute the volatiles from the ZB-5 column (30 m, 0.25 mm ID, 1 μm thickness; Phenomenex, Cheshire, U.K.). The DSQ mass spectrometer (Thermo Fisher Scientific, Loughborough, U.K.) was initially operated in full scan mode with a scan range from 40-200 Da. However, selected ion mode was later introduced to increase sensitivity for specific compounds.

Volatile compounds with their retention times and key ions for quantification are presented in Table 3.1 and Table 3.2 presents the selected ions with relevant retention time.

Table 3.1 Volatile compounds, retention times, and ions for quantification in tomato fruit sample.

Volatile compound	Retention time (min)	Ion for Quantification	Alternative ions
3-methylbutanal	3.70	58	43, 71
2-methylbutanal	3.88	57	41, 86
3-methylbutanol	5.25	55	42, 70
2-methylbutanol	5.33	56	41, 70
Z-3-hexenal	6.71	98	55, 69
Hexanal	6.75	82	44, 56
E-2-hexenal	8.02	83	41, 69
6-methyl-5-hepten-2-one	11.16	108	43, 69
2-isobutylthiazole	12.41	99	58, 126
2-phenylethanol	14.35	122	65, 91
Methyl salicylate	16.04	120	92, 152
Citral B (neral, Z-citral)	16.79	109	41, 69
Citral A (geranial, E-citral)	17.29	152	69, 109

Table 3.2 Retention time and ions monitored in selected ion mode in GC-MS SPME.

Retention time (min)	Ions monitored
0-4:10	57, 58, 61, 71
4:10-6:00	27, 41, 79, 84
6:00-7:50	43, 56, 58, 69, 82,
7:50-8:00	67, 69, 82, 83
8:00-9:00	57, 85, 114
9:00-11:00	41, 55, 83, 96
11:00-12:00	43, 108, 111
12:00-15:50	68, 91, 93, 99, 122
15:50-17:00	69, 109, 120, 152
17:00-18:00	69,109
18:00-22:30	41, 69, 121, 177

3.2.1.3 Authentic standard preparation and sample concentration calculation

GC-MS experiments involve the use of an internal standard. In this research 2-hexanone (C6 ketone) or 3-heptanone (C7 ketone) were used. However, to obtain useful information about real concentrations of volatiles in the samples, we also used a set of authentic standards. These were the real volatile compounds diluted in distilled water and treated as a separate SPME sample. The concentrations of volatiles in authentic standard matched the concentrations in tomato fruits and lead to easy calculation of volatile compounds in the tomato fruit samples, but the method is quantitative, because it calculates the values based on authentic standards compounds.

Concentrations of the samples were calculated from the equation below:

Csample = (Psample/Pstd)*Cl*1000

Where:

Csample-concentration of the sample in the liquid phase (µg/L=ppb)

Psample-peak area of the sample

Pstd-peak area of the standard

Cl-concentration of standard in liquid phase (mg/L=ppm)

1000-mulitiplication factor to convert ppm to ppb

Standards and samples GC-MS procedure

10 ml of the tomato sample was put in a 20 mL vial. A standard of volatiles at a known concentration was prepared in water and 10 mL was transferred into a 20 mL vial. These were run on a GC-MS. After analysis, peak areas were compared and the concentrations of the compounds were accordingly calculated because if peak areas for standard and tomato sample were the same, the concentrations were the same as well. Table 3.3 presents the compounds and their partition coefficients and the details of required dilutions are presented in Table 3.4.

Table 3.3 Selected volatiles, their CAS number (unique numerical identifier), NIST library entries and relevant partition coefficients* (*from Henrywin v1.90 software, EPA, U.S.A).

Volatile	CAS No	NIST library entry	Partition coefficient 1* (unitless)	Partition coefficient 2* (unitless)	Mean
β-ionone	79-77-6	20824	7.11E-03	incomplete	N/A
hexanal	66-25-1	1573	8.61E-03	9.77E-03	9.19E-03
1-penten-3-one	1629-58-9	566	1.42E-03	7.94E-04	1.11E-03
2-methylbutanal	96-17-3	714	6.49E-03	1.07E-02	8.60E-03
3-methylbutanal	590-86-3	688	6.49E-03	8.32E-03	7.41E-03
E-2-hexenal	6728-26-3	1255	4.04E-03	1.86E-03	2.95E-03
2-isobutylthiazole	18640-74-9	7680	4.04E-03	incomplete	N/A
E-2-heptenal	18829-55-5	2632	5.36E-03	2.63E-03	4.00E-03
2-phenylacetaldehyde	122-78-1	3755	2.24E-04	incomplete	N/A
Z-3-hexenol	928-96-1	1567	6.33E-04	2.09E-04	4.21E-04
2-phenylethanol	60-12-8	3960	1.18E-05	4.17E-06	7.99E-06
3-methylbutanol	123-51-3	857	5.42E-04	6.76E-04	6.09E-04
methyl salicylate	119-36-8	10159	1.86E-04	9.12E-08	1.86E-04
E-2-pentenal	1576-87-0	567	3.04E-03	1.32E-03	2.18E-03
Citral	5392-40-5	10402	1.54E-02	1.78E-03	8.59E-03

Table 3.4 Partition coefficients, liquid phase concentrations for volatiles. The information about volume needed (μL in L of liquid) for each volatile and required dilution to achieve liquid phase concentration is provided. The volatiles need to be diluted because it was not viable to pipette quantities below 50 μl. The four-step dilution (three steps for compounds requiring 100x dilution) was essential to achieve this task.

Volatile	Partition coefficient (mean where applicable)	Liquid phase concentration *(mg/L=ppm)	Volume of volatile needed (μL in 1L of water)	Required dilution
β-ionone	7.11E-03	0.23	115	500x
hexanal	9.19E-03	0.17	87	500x
1-penten-3-one	1.11E-03	1.45	145	100x
2-methylbutanal	8.60E-03	0.19	93	500x
3-methylbutanal	7.41E-03	0.22	108	500x
E-2-hexenal	2.95E-03	0.54	271	500x
2-isobutylthiazole	4.04E-03	0.40	198	500x
E-2-heptenal	4.00E-03	0.40	200	500x
2-phenylacetaldehyde	2.24E-04	7.14	714	100x
Z-3-hexenol	4.21E-04	3.80	380	100x
2-phenylethanol	7.99E-06	125.24	N/A	N/A
3-methylbutanol	6.09E-04	2.63	263	100x
methyl salicylate	1.86E-04	8.60	860	100x
E-2-pentenal	2.18E-03	0.73	73	100x
Citral	8.59E-03	0.19	93	500x

The dilution process is described below for compounds requiring 500x dilution. First step dilution was omitted for compounds requiring 100x dilution (volatiles added straight to Solution 1).

Stock solution: volatile (μL in L of deionised water, Table 3.3)

200 mL of stock solution in 1L of deionised water (dilution 1 in 5, **Solution 1**)

500 mL of Solution 1 in 1L of deionised water (dilution 1 in 2, **Solution 2**)

100 mL of Solution 2 in 500 mL of deionised water (dilution 1 in 5, **Solution 3**)

100 mL of Solution 3 in 1L of deionised water (dilution 1 in 10, **Final solution**)

10 mL of Final solution was used in a 20 mL vial as a standard.

All chemicals used were of analytical grade (> 95% purity; Fisher Scientific, Loughborough, U.K. and Sigma-Aldrich, Gillingham, U. K.).

Below on the graph (Figure 3.6) data from GC-MS SPME for tomato juice samples is presented. The data gives a brief overview of repeatability of samples analysed. Three tomato juices were bought, shaken before opening and three representative samples were prepared per tomato juice. These were treated as single analytical samples therefore, there were nine analytical samples. 3-methylbutanal and hexanal were within one standard error for all three juices based on their mean values. Only E-2-pentenal showed a slightly different trend having higher standard error, especially for juice three, but it is still acceptable.

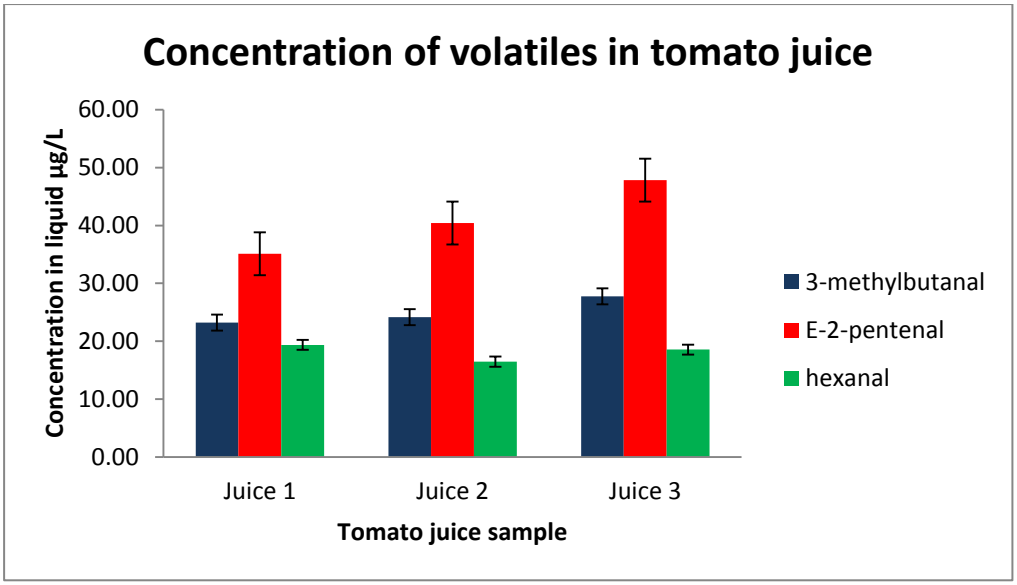


Figure 3.6 Graph representing three different tomato volatiles from tomato juice samples. Error bars are standard error of the mean (n=3). Three technical replicates were used per juice.

3.2.2 Statistical analysis

The data was a subject of t-test using Microsoft Office package (Microsoft, Redmont, U.S.A). The ILs were compared with a control M82D. The level of significance was highlighted in graphs with stars corresponding to the relevant level of significance ($P<0.05$(*); $P<0.01$(**)).

3.3 Results

3.3.1 Analysis of selected ILs form S. pennellii population

APCI-MS indicated the tomato ILs showing significant volatile evolution across two years, however not always constant. GC-MS was used to validate these data and obtain quantitative information. The number of replicate fruits was initially chosen to be three, but due to plant-to-plant variation, some of the lines only had two replicate fruits.

The distribution of methylbutanals and C5 unsaturated volatiles are illustrated in Figure 3.7. For 2-methylbutanal, it was observed that a number of lines were lower in concentration than a control, but only IL9-1-3 was significantly low ($P<0.05$). IL that was the highest in terms of 2-methylbutanal was IL4-3. In addition, similar trend can be observed with 3-methylbutanal where IL9-1-3 is also significantly low ($P<0.05$); however, some other ILs were significantly higher to include IL9-1 ($P<0.01$) and IL4-3 ($P<0.05$) while IL9-3 was the highest, but not significant.

In case of the data for 1-penten-3-one, IL9-3 was the lowest line ($P<0.01$), the biggest concentration of this C5 unsaturated volatile was observed in IL11-2. In addition IL 4-4 and IL 8-2-1 were also significantly low ($P<0.01$ for both ILs), while IL12-2 and IL1-4-18 were significantly high ($P<0.05$ for both ILs). In case of E-2-pentenal, only a small number of introgressions were identified, those were significantly low ($P<0.05$). The most striking was IL9-3 ($P<0.01$) followed by IL 4-4 ($P<0.05$), IL9-1-3 ($P<0.05$) and IL 8-2-1 ($P<0.05$), while IL11-2 was the highest; however, not significant. The impact of C6 volatiles and phenyl volatiles is summarised on Figure 3.8.

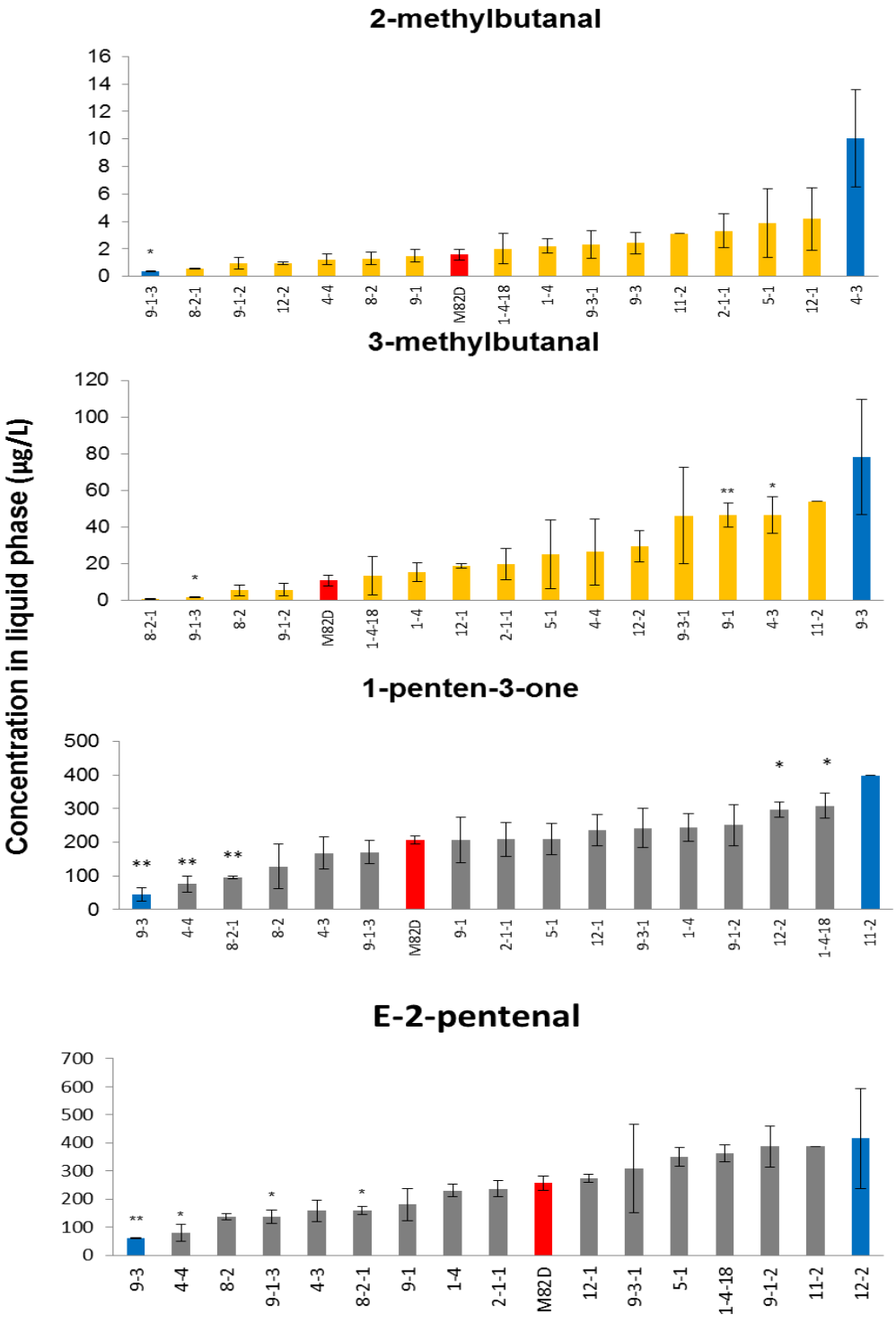


Figure 3.7 Distribution of methylbutanals and C5 unsaturated volatiles across selected ILs and control. In blue colour lowest and highest ILs. In red, M82D control. Fruits were collected at B+7. Error bars are standard errors of the mean (n = 3, unless otherwise stated). Majority of the ILs have 3 replicates (three fruits), but IL1-4-18, IL4-4, IL8-2, IL8-2-1, IL9-3, IL12-1 and IL12-2 had two replicate fruits. The only exception among all lines was IL11-2 with only one replicate. Level of significance labelled with stars; P<0.05(*), P<0.01(**).

Analysis of selected ILs, lead also to comparison ILs in terms of hexanal (Figure 3.8) E-2-hexenal, Z-3-hexenal, 2-phenylacetaldehyde and 2-phenylethanol. The data for hexanal; reveals no significant lines. The top one was IL1-4, while the lowest one was IL9-3.

In contrast, volatile information for E-2-hexenal indicates IL9-3 as significantly low ($P<0.05$). IL9 -1-2 was the most prominent in terms of e-2-hexenal, but still the results were not significant (at $P<0.05$). However, the data for Z-3-hexenal reveals significantly low IL 9-3 ($P<0.001$) and IL 4-4 ($P<0.001$). In addition, significantly low are also IL 9-1-3 ($P<0.05$) and IL 8-2-1 ($P<0.01$). The highest, but non-significant line was IL 9-1-2. Figure 3.8 also summarises distribution of 2-phenylacetaldehyde and 2-phenylethanol. In case of 2-phenylacetaldehyde a number of ILs were identified to have significantly higher values than the control. These were IL12-2 ($P<0.05$), L8-2-1 ($P<0.01$) and IL8-2 ($P<0.01$). Similar situation was observed for 2-phenylethanol data. IL8-2-1($P<0.01$) and IL8-2 ($P<0.01$) were the most striking introgressions, but IL 12-2, IL9-1-2, IL2-1-1, IL4-4, IL12-1 were also significant ($P<0.05$). The lowest line for both of these volatiles was IL9-1-3.

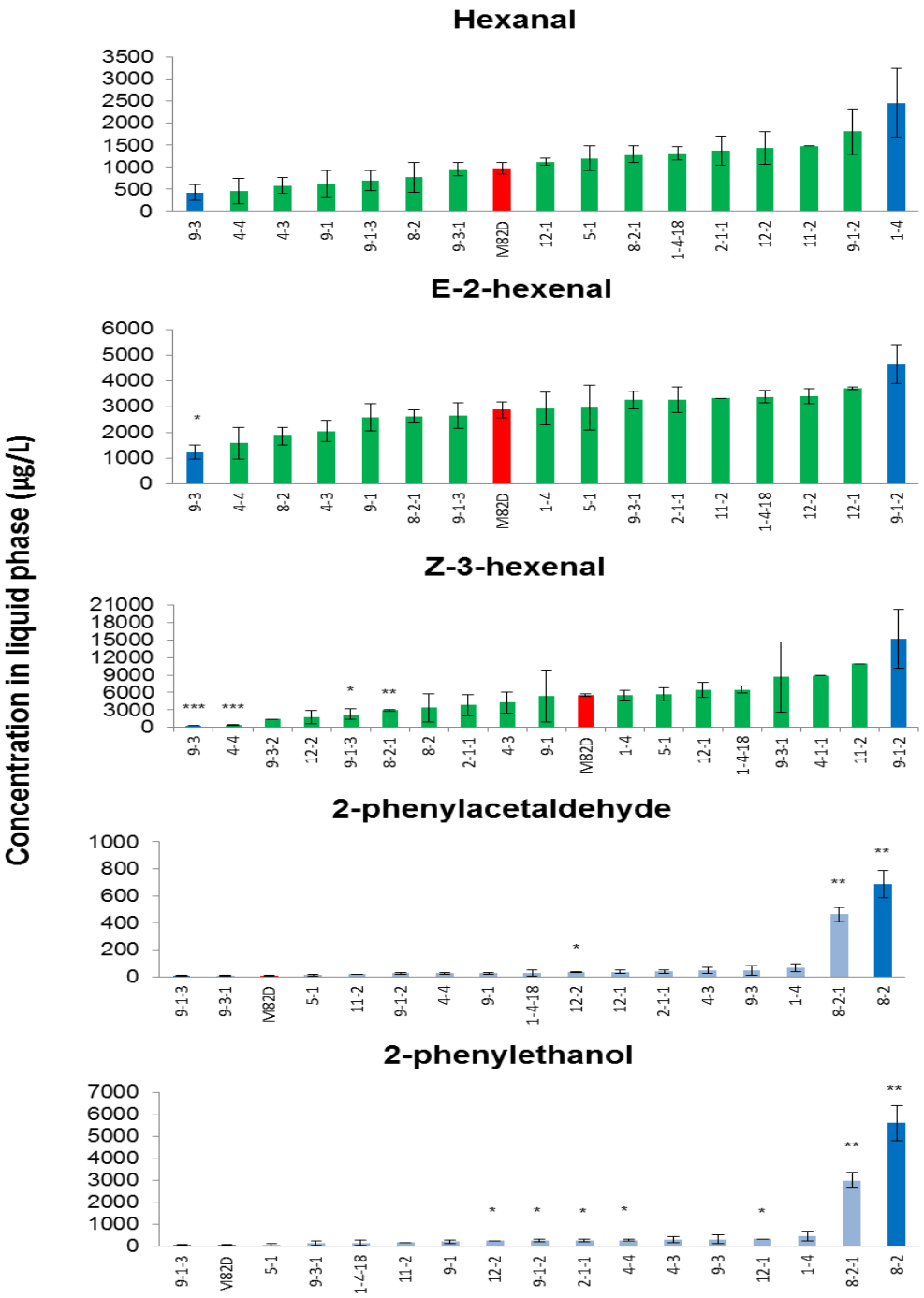


Figure 3.8 Distribution of C6 volatiles, 2-phenylacetaldehyde, and 2-phenylethanol across selected ILs and control. In blue colour lowest and highest ILs. In red, M82D control. Fruits were collected at B+7. Error bars are standard errors of the mean (n = 3, unless otherwise stated). Majority of the ILs have 3 replicates (three fruits), but IL1-4-18, IL4-4, IL8-2, IL8-2-1, IL9-3, IL12-1 and IL12-2 had two replicate fruits. The only exception among all lines was IL11-2 with only one replicate. Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

In Chapter 2 ratios of % ion for hexanal and hexenals were presented, however, it was not possible to identify which of these two volatiles is responsible for a trend. The hypothesis was to discover if hexanal abundance would affect hexenals. In other terms, was hexanal increasing or hexenals decreasing. Data in Figure 3.8 clearly shows that effect was due to hexanal increasing rather than changes in hexenals. IL 1-4 was the most interesting in Chapter 2 and this trend also appears in Figure 3.8, with strong response indicated by IL1-4.

Distribution of volatiles like methyl salicylate (Figure 3.9) and citrals (two isoforms) was also measured. For methyl salicylate, there was a number of significant introgressions in comparison with control M82D, but the most noticeable was IL1-4 ($P<0.05$), but IL1-4-18 ($P<0.05$), IL9-1-2 ($P<0.01$), IL8-2 ($P<0.05$), IL12-1 ($P<0.05$) and IL12-2 ($P<0.05$) were also significant. The lowest IL in comparison with M82D control for methyl salicylate was IL8-2-1 and the same trend was seen for citrals. In contrast for citral data, there is only one significantly low IL9-1-3 ($P<0.01$) that is characteristic for both isoforms. Other introgressions in case of citral, although they are higher than M82D control, they are not significant. The most striking was IL12-1, showing the highest concentration; however, the results were not significant at $P<0.05$.

APCI data presented in Chapter 2 indicated several trends related to m/z 153 corresponding to citrals and methyl salicylate. However, it was not possible to identify which compound was responsible for this occurrence. The GC-MS however has a power to resolve this interesting issue. Several introgressions were significantly high over two years of study with APCI ($P<0.05$-0.001, see Fig. 2.4e). These ILs were IL1-4, IL2-1, IL5-1, IL5-5, IL9-1-2, IL11-2 and IL12-1. Comparison with GC-MS data (Fig. 3.9) clearly indicates that this trend was due to methyl salicylate and not citral, with examples represented by IL1-4 ($P<0.05$) and IL9-1-2 ($P<0.01$). Interestingly, IL12-1 is significant ($P<0.05$) for methyl salicylate and is present at high concentrations as indicated by citral data, however it is not significant, which means that confirmation a trend from APCI may be difficult, without further examination.

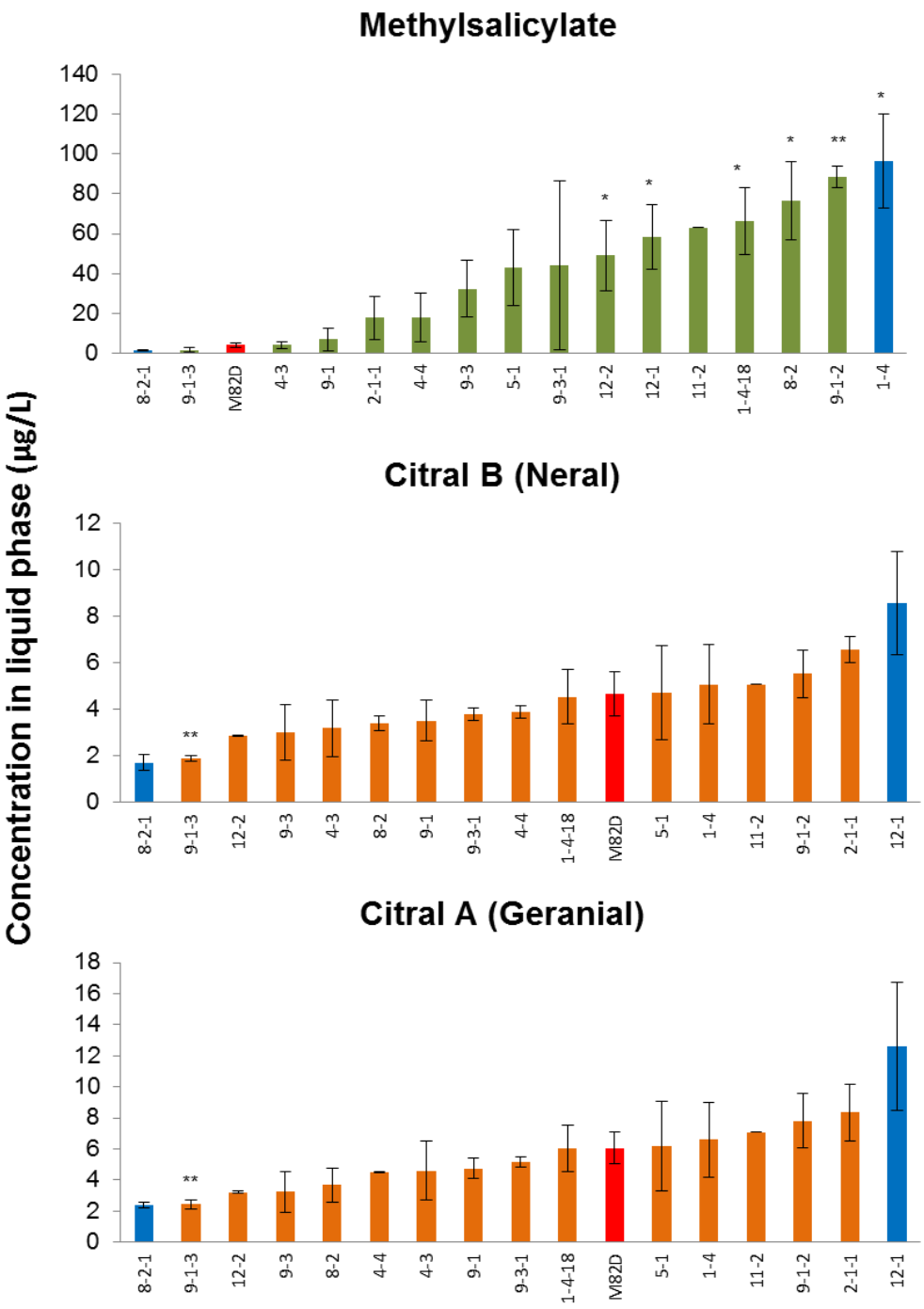


Figure 3.9 Distribution of methyl salicylate and citrals across selected ILs and control. In blue colour lowest and highest ILs. In red, M82D control. Fruits were collected at B+7. Error bars are standard errors of the mean (n = 3, unless otherwise stated). Majority of the ILs have 3 replicates (three fruits), but IL1-4-18, IL4-4, IL8-2, IL8-2-1, IL9-3, IL12-1 and IL12-2 had two replicate fruits. The only exception among all lines was IL11-2 with only one replicate. Level of significance labelled with stars; P<0.05(*), P<0.01(**).

3.3.2 Evaluation and impact of developmental stage on volatile release focusing on ILs 1-4, 9-1-2, 11-2 and 12-1.

3.3.2.1 Effect of developmental stage on ILs

The GC-MS data indicate that a few volatiles can be identified as showing robust differences in comparison with M82D control. We chose four of the volatiles for comparison; these were E-2-pentenal, hexanal, methyl salicylate and citral (both isoforms). The argument for that is that these particular volatiles are important and were reported as characteristic for tomato flavour (Buttery *et al.,* 1990) and also represent a selection of compounds from different biochemical pathways (lipoxygenase: E-2-pentenal, hexanal; shikimate: methyl salicylate; carotenoid: citral) We first investigated the changes in these volatiles during tomato fruit development. Figures 3.10 and 3.11 present these volatiles distribution across four developmental stages to include mature green, breaker, breaker+3 and breaker+7.

For E-2-pentenal all ILs showed increasing levels with fruit development. During MG stage, the volatile levels are quite low and they steadily increase with the time. IL 12-1 has the highest level of E-2-pentenal when compared with control M82D and other ILs. During B+3 the level of this C5 unsaturated volatile is similar for the control and all three other ILs, while IL11-2 shows a significant ($P<0.05$) increase. Finally at B+7, there is a dramatic increase of E-2-pentenal in control M82D fruit as well as these from IL11-2, while other ILs show a lower increase, IL 1-4 and IL 12-1 are significantly low ($P<0.05$). For hexanal, at the MG stage the levels of volatile are similar across all ILs and steadily increase with fruit development (Figure 3.10B). During B+7 IL1-4, IL11-2 and IL12-1 show similar concentration of this volatile while M82D is slightly higher and in 9-1-2 the highest found, however high standard error (M82D) and limited replication (1 fruit for IL9-1-2) do not make a strong conclusion.

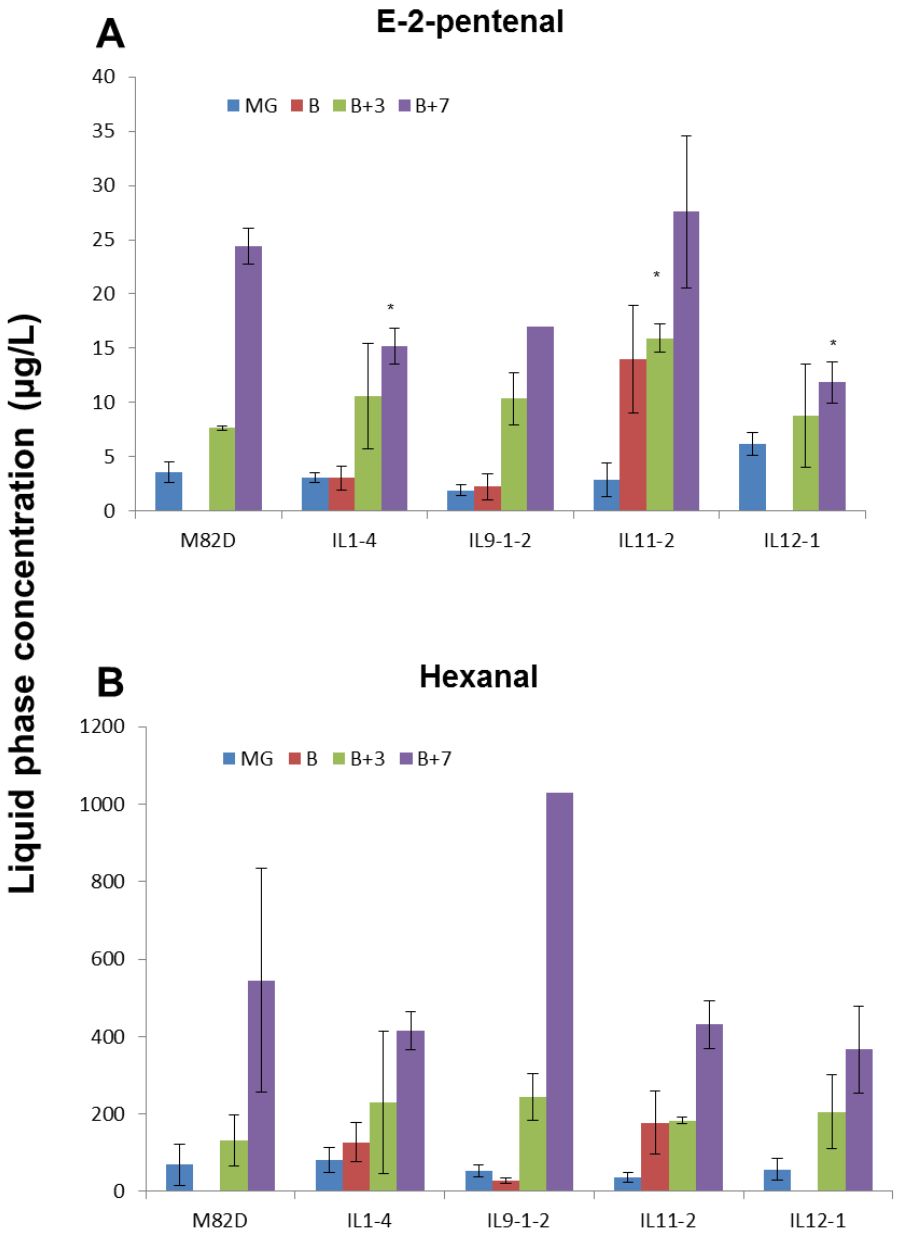


Figure 3.10 Distribution of E-2-pentenal (A) and hexanal (B) during fruit ripening. Error bars are standard errors of the mean (n = 3, unless otherwise stated). For mature green (MG) three fruits were collected for all ILs and control except IL11-2 were only two fruits were picked and analysed. For breaker (B) three fruits were collected and analysed for all three lines presented. For breaker +3 (B+3) two fruits were collected for all ILs and control. For breaker+7 (B+7) three fruits were collected and analysed for all three lines presented and control except IL9-1-2 were only one fruit was collected and therefore no standard error was calculated for this line. Level of significance labelled with stars; $P<0.05$(*).

In contrast to the other volatiles that were examined, methyl salicylate showed the highest levels of this volatile at MG stage, and the lowest values at B+7, with some variation between ILs (Fig. 3.11), but the data was not significant. During B+7 stage IL1-4 and IL11-2 were both significantly higher than control ($P<0.05$).

For citral A, most ILs showed a steadily increase from MG. However, IL11-2 had exceptionally high citral A during B stage. In addition, IL11-2 was significantly high ($P<0.05$) during B+3 stage in comparison with control M82D. During B+7, IL1-4 had the highest level of this volatile, however not significant.

The other isoform, citral B, was at high levels during MG stage in IL1-4 and M82D, but significantly ($P<0.05$) low in IL9-1-2. In all lines this compound slowly increased in concentration with developmental stage, with exception for IL1-4 where it' was significantly ($P<0.05$) high during B+3. The levels of this compound during B+7 were quite similar for all lines with exception to M82D, where control was the lowest in terms of this volatile concentration, however not significant.

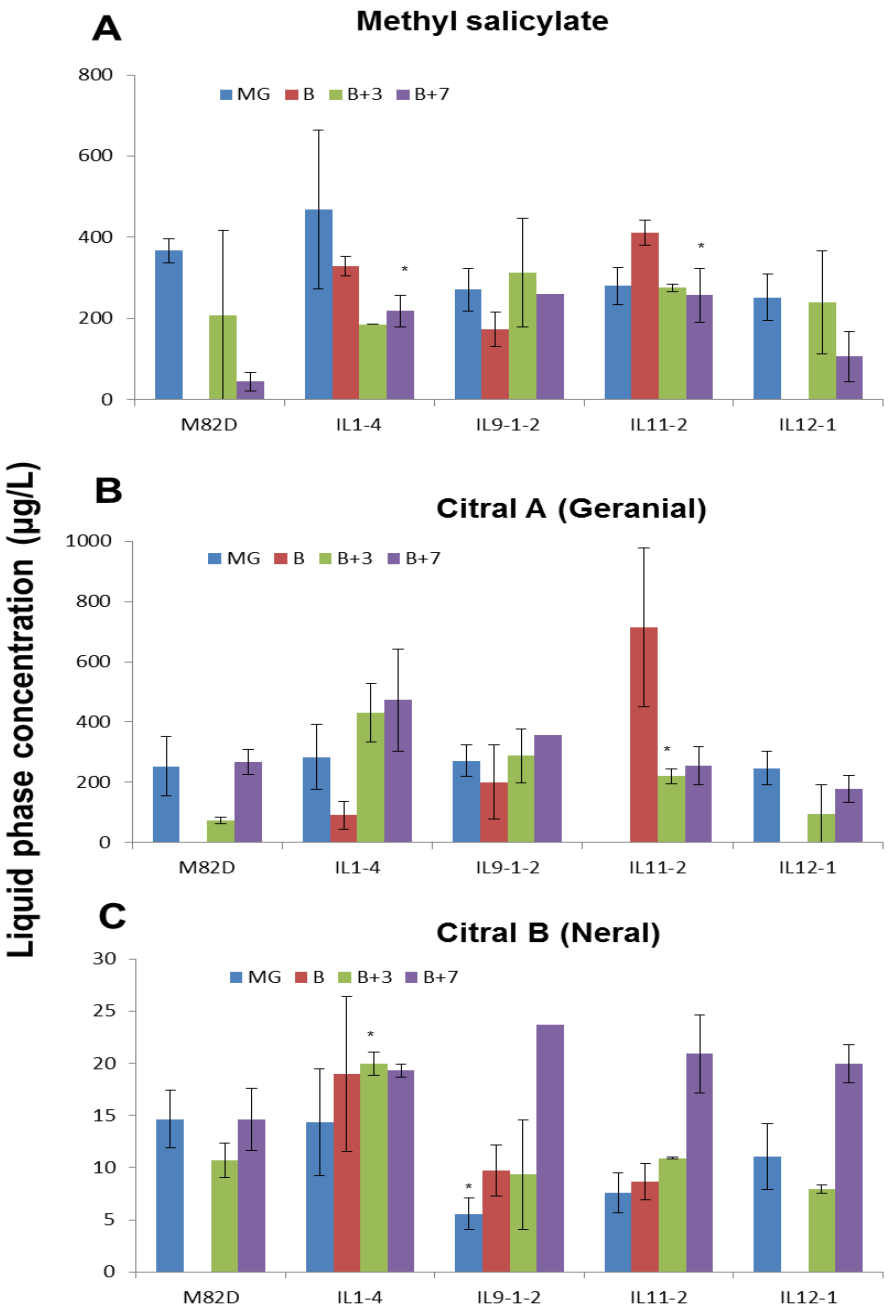


Figure 3.11 Distribution of methyl salicylate (A) and citrals (B and C) during fruit ripening. Error bars are standard errors of the mean (n = 3, unless otherwise stated). For mature green (MG) three fruits were collected for all ILs and control except IL11-2 were only two fruits were picked and analysed. For breaker (B) three fruits were collected and analysed for all three presented lines. For breaker +3 (B+3) two fruits were collected for all ILs and control. For breaker+7 (B+7) three fruits were collected and analysed for all three presented lines and control except IL9-1-2 were only one fruit was collected and therefore no standard error was calculated for this line as well. Level of significance labelled with stars; P<0.05(*).

3.4 Discussion

GC-MS SPME is an analytical approach that can be successfully used for analysis of volatiles because of its robustness and sensitivity (Yang and Peppard, 1994; Matich *et al.,* 1996; Verhoeven *et al.,* 1997; Song *et al.,* 1997, Augusto *et al.,* 2000; Verdonk *et al.,* 2003). Application of this technique, lead to an in depth analysis of selected ILs, as well as selected volatiles. From initial 76 ILs only 18 were chosen for further study based on APCI and after early evaluation with GC-MS, only four lines were selected. These four lines were IL1-4, IL9-1-2, IL11-2, and IL12-1 and they predominantly had one or a mixture of the major volatiles to include E-2-pentenal (C5), hexanal and E-2-hexenal (C6), methyl salicylate and citrals. In addition, IL 1-4 showed strong correlation with presence of hexanal both in APCI and GC-MS studies. Majority of the volatiles presented in this chapter are the core volatiles that characterise tomato flavour, identified and described by fundamental work of Petro-Turza (Petro-Turza 1987) and later by other colleagues (Buttery *et al.,* 1990, Yilmaz 2001). Methyl salicylate is also among top ten tomato flavour volatiles that could duplicate the tomato flavour (Buttery *et al.,* 1993; Marković *et al.,* 2007). Citral with its citrus aroma character is also an important tomato compound (Baldwin *et al.,* 2004).

The initial GC-MS SPME screen also included 2-phenylacetaldehyde and 2-phenylethanol. There was a clear indication that these volatiles may be present in high concentration on Chr. 8 (Tadmor *et al.,* 2002; Tieman *et al.,* 2006), specifically in IL8-2 and IL8-2-1. We confirmed the presence of these volatiles both via APCI and in initial GC-MS screen. These two volatiles have fruity/floral properties and may be considered desirable, but in elevated levels (like in IL8-2 and IL8-2-1) can be associated with undesirable flavour in tomato (Tadmor *et al.,* 2002).

3.5 Conclusions

- The application of GC-MS SPME allowed an extensive analysis of the selected tomato fruit material. The technique that was used proved to be effective and sufficient. Our data was in agreement with published work (Tadmor *et al.,* 2002, Tieman *et al.,* 2006).

- The main volatiles, which became the focus of the work were E-2-pentenal (C5), hexanal (C6), E-2-hexenal (C6), methyl salicylate, citrals (citral A and citral B) and the ILs showing robust effects were IL1-4, IL9-1-2, IL11-2, IL12-1 based on in depth analysis of 18 ILs via GC-MS SPME.

- Analysis of volatile distribution during developmental stages indicates a steady increase in terms of volatile levels with fruit ripening with exception of methyl salicylate which showed the opposite trend.

- The resolution power of GC-MS allowed identification of isomerising compounds (hexenals) and revealed the trend for m/z 153 (citrals and methyl salicylate).

- IL1-4 and IL11-2 were chosen for further study based on the data for C5 and C6 volatiles, methyl salicylate, and citral. IL1-4 showed the strongest trend in APCI data and GC-MS data.

CHAPTER 4:

Sensory evaluation of selected tomato volatiles in tomato juice and tomato purée.

4.1 Introduction

The sensorial approach was used for better understanding of volatiles interactions and their perception from a consumer point of view (represented by the panelists). In addition, the analytical data was compared with outcome from sensory data and answered some key questions in terms of possible volatile concentrations in tomato fruit required for a better flavour.

The sensory analysis focused on the key volatiles that had been measured from tomato ILs as described earlier in the thesis. This chapter describes two sets of sensory experiments. Initial tests were performed using tomato juice as a base. A further set of trials then involved using of tomato purée as a base.

The sensory evaluation of spiked tomato juice was conducted in order to better understand volatile-flavour interactions. In the first part of our study, we use a tomato juice as a base because water is not suitable simply as it cannot be associated with tomato either by content or by colour. Macerate of fresh tomato has a high variance in batches of tomato; chemical changes occur with time and potential interactions caused by enzymes, that they all make this system difficult to work with.

Tomato juice was spiked with selected volatiles. The four volatiles were chosen based on their flavour characteristics. Methyl salicylate has a strong pharmaceutical, medicinal note (Krumbein and Auerswald, 1998); citral

has a lemon-like note. E-2-hexenal is green, fruity (Buttery and Ling 1993; Krumbein and Auerswald 1998; Yilmaz 2001) and E-2-pentenal can be described as fruity, floral, viney (Tandon *et al*., 2000). Furthermore, Baldwin and colleagues (Baldwin *et al.,* 2008) evaluated selected volatiles in partially deodorised tomato purée. Among the compounds tested, there were C5 and C6 volatiles and reported that these volatiles positively contributed to tomato aftertaste and slightly positively affected sweetness; however, not at significant level. Three sensorial analyses were undertaken: threshold detection, hedonic tests, and flash profiling.

Threshold detection - is the lowest stimulus which can produce a sensation. The threshold of added desirable substances may be used as a research tool in the formulation of foods, beverages etc. A low detection threshold, for a given compound corresponds to a high sensitivity for the flavour in question. Thresholds can vary between people, so in sensorial experiments it is important to have an adequate number of participants with minimum number usually between 20 to 30.

Hedonic test - this is an acceptance measurement. The panelists have to rate their liking for the product on scale usually from 0-10. The hedonic scale assumes that consumer preferences exist on a continuum and that the preference can be categorized by response based on likes or dislikes (Lawless, 1998).

Flash profiling - In order to characterize more fully spiked tomato juices, a flash profile approach was also used. This is a sensory descriptive method (Sieffermann, 2000; Dairou and Sieffermann, 2002) derived from Free Choice Profiling where each subject chooses and uses his/her own words to evaluate and compare the products that are tested. It’s quicker and more economic in

comparison in terms of required budget and consumed time in comparison with conventional profile (Dairou and Siefferman, 2002).

4.2 Material and Methods

4.2.1 Tomato juice and volatile compounds

The tomato juice used during the entire project was from the brand Libby's. It had added salt, vitamin C and E. Before being given to panelists, the cups with juice were shaken, because the pulp of tomato juice can decant. The volatiles studied were: methyl salicylate (Sigma Aldrich, 99%), citral (Sigma Aldrich, 95%), E-2-hexenal (Sigma Aldrich, 95%) and E-2-pentenal (Sigma Aldrich, 95%). we decided to use commercial tomato juice as a matrix, although it is known that processed, concentrated tomato juice is different in composition from fresh tomatoes and this might be a potential limitation. To minimize the effect of juice-to-juice variation, we ordered tomato juice from the same supplier, the same manufacturer and we used a juice from the same batch.

4.2.1.1 Sensory panel

<u>The internal panel</u>

The internal panel was recruited on the campus, in order to participate in the determination of taste detection thresholds, triangle tests and hedonic test. Panelists were recruited among university students, and were within 18-30 years of age, approximately 50% were men and 50% were women. Information was provided to panelists in order to inform them about the composition of the product to test as well as to warn them about potential allergic reactions. Panelists were recruited to assess the four sessions of sensorial tests, one volatile each session and only one concentration at a time. The concentrations of volatiles were then adapted based on panelists

response and a new triangle tests took place. To perform hedonic test, we have recruited 100 people. This test was a hedonic evaluation of interactions between specific volatiles within selected range of concentrations. Panelists had to rate their liking for the product on a specific scale.

The external panel

The external panel was used for the flash profile. This method requires trained judges. These panelists are rigorously screened for their sensory acuity, discrimination and descriptive ability. We have chosen ten judges among those working for the University of Nottingham. Within this group were nine women and one man. They were chosen based on their preference for tomato juice.

4.2.1.2 Sensory methods for tomato juice studies

Detection threshold determination

Preliminary test was conducted with 5 judges. The goal of these preliminary tests was to find a balanced in the range of concentrations, not too weak and not too strong.

Modified British standard method (modified from: BS 5929-7:1992, ISO 3972:1991 Method of investigating sensitivity of taste)

Initially we used modified British standard method. This method is used to evaluate tastants in water. In our approach we used general principles of BS method but as a base we used tomato juice and we have spiked it with volatiles (Table 4.1).

Preparation of volatiles

The aromas were diluted in propylene glycol before the mix with tomato juice. Propylene glycol is a clear, odourless chemical that has been used as an additive in foods and beverages for years. 200µL of each volatile

was diluted in 100 mL of propylene glycol. 400 µL of this mixture was diluted in 100 mL of tomato juice in order to obtain a final stock solution of 8 ppm.

Table 4.1 Panelists sessions, volatiles and their corresponding concentrations used in modified British standard method. Four different volatiles were tested by a group of 30 people (untrained panelists) during four panel sessions. Each volatile was evaluated at 8 different concentrations, specific for each volatile, based on literature data, odour thresholds and preliminary experiments.

Session	Volatile	Concentrations (ppm)							
		1	2	3	4	5	6	7	8
1	Methyl salicylate	0.08	0.1	0.114	0.13	0.2	0.267	0.4	0.8
2	Citral	0.08	0.085	0.093	0.1	0.114	0.13	0.16	0.2
3	E-2-hexenal	0.08	0.1	0.13	0.16	0.2	0.3	0.4	0.8
4	E-2-pentenal	0.032	0.08	0.107	0.13	0.2	0.4	0.8	1.6

The panelists had to compare each sample with a reference tomato juice (without aroma) served in a glass of 125 mL at the beginning of the session and filled again when needed. Between each comparison, they had to cleanse their palate with water and a cracker. If they perceived no difference between the reference and the sample tasted, they wrote a zero in the box corresponding to the tasted sample. If they perceived a difference, they wrote a cross in the box corresponding to the tasted sample. With increased perception of the samples panelist added more crosses. They did this until the end of the session, except if they felt that the concentration of aroma was too strong. When they perceived a difference, they had to describe it as well as possible in the corresponding box.

Modified method based on American Society for Testing and Materials (ASTM) rapid method (E679)

To validate our results we used modified three-alternative forced choice (3-AFC). It is a method of sample evaluation in which three samples are presented: two are controls and one contains substances under test. The ASTM's rapid method (E679) aims at determining a practical value close to the threshold, based on minimum testing effort. It creates the best estimate determination of each panelist's threshold (Meilgaard *et al.,* 2007).

The best estimate threshold (BET) for each subject is the geometric mean of the highest concentration missed and the next higher concentration (Meilgaard, 1993). The group BET is the geometric mean of the individuals BETs. We used this way of analysis, which is normally used for the 3-AFC method (Meilgaard *et al.,* 2007); to analyze the data from modified British standard method.

Hedonic test

In order to see the interactions between the four volatiles an experiment was created with use of Design Expert 7.0 (Stat-Ease, Minneapolis, U.S.A). 40 solutions were prepared, following this design. 100 panelists had to taste 10 solutions plus the reference. Panelists were split into 4 blocks; they were 25 panelists in each block. The sample presentation was randomized, but was not balanced. The samples were left from the fridge at least 2 h before the evaluation, so they were served at room temperature. For the first part a cup of 20 mL was served, for the second part, a glass of 50 mL was served. The panelists had to rate their overall liking. A 9 point hedonic scale was used (see Figure 4.1). The anchors, were the terms "dislike extremely" to "like extremely", with "neither like nor dislike" in the middle. Between the samples judges had to clean their palate with water and cracker.

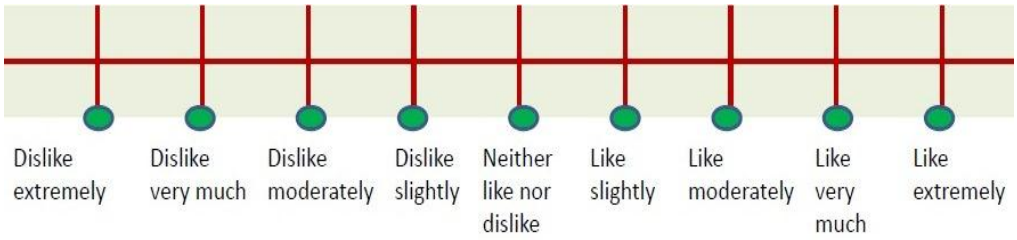


Figure 4.1 A 9-point hedonic scale for Hedonic test

Flash profile

The flash profile is a combination of free choice of terms and number of terms by the panelist, which means that each member of the panel group describes sample in their own words and is not limited by the number of words to use; and comparative presentation of the overall products to describe. With this method, we obtain a ranking of samples. This method suits to the research project about conception and formulation of new products (Siefferman, 2002).

Analysis method

For analysis of hedonic data Design Expert 6.0 was used (Stat-Ease, Minneapolis, U.S.A). Response surface model category was used, with D-Optimal subcategory. This type of model can measure up to 30 factors. The model was split into 4 blocks in each block there were 10 samples so whole design covered 40 runs. Within this model four categorical factors were measured (volatiles: methyl salicylate, E-2-hexenal, E-2-pentenal, citral) and two numerical factors were monitored (likeness and relative likeness).

To analyze flash profile data, XLSTAT 2009 (Addinsoft, New York, U.S.A) was used in complement to EXCEL, (Microsoft, Redmont, U.S.A). The Spearman test was used to test the repeatability of the assessors for each attributes. The discriminant ability of each attribute per judge was tested using a one way analysis of variance (ANOVA) on the rank data. Rank data is non-

parametric and should be analyzed using a Friedman's test, but this test cannot handle replications, so ANOVA was performed. Generalized Procrustes Analysis (GPA) was applied to the data, from the flash profiling to assess the consensus between judges' sensory map. GPA is a method of statistical analysis that can be used to compare the outcome of surveys, interviews, panels. It was developed for analyzing the results of free-choice profiling, a survey technique which allows respondents (such as sensory panelists) to describe a range of products in their own words or language. GPA is the only way to make sense of free-choice profiling data (Meullenet *et al.,* 2007). The GPA calculates a consensus from data matrices of a sensory profiling experiment. In this experiment, a data matrix corresponds to each judge. This consensus should reflect the true underlying data structure and indicate which products are similar and which ones differ strongly from each other on the GPA plot.

4.2.2 Sensory methods for tomato purée studies

4.2.2.1 Tomato purée and volatile compounds

The tomato fruits used during the entire project were from Tesco. Country of origin was Morocco, class II, medium size.

The volatiles studied were citral, E-2-hexenal and E-2-pentenal. All used volatiles were food grade from Aromco (Nuthampstead, UK).

4.2.2.2 Sensory panel

<u>The external panel</u>

The external panel was used for the flash profile. This method requires trained judges. These panelists are rigorously screened for their sensory acuity, discrimination, and descriptive ability. We have chosen ten

judges among those working for the University of Nottingham. Within this group were eight women and two men. They were chosen based on their preference for tomatoes.

4.2.2.3 Difference from control test

In these experiments, panelists were asked to judge six samples presented in randomised order and use a 9-point scale to characterise their result. Figure 4.2 shows a schematic of 9-point scale used in the study.

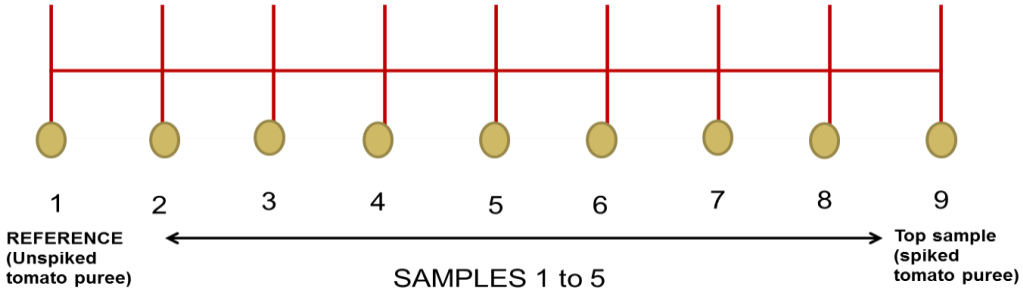


Figure 4.2 A 9-point scale used in difference from control test. Number 1 corresponds to reference and number nine to the spiked tomato purée at top concentration, which is very different from the reference. Other samples (1 to 5) should be within a range 2 to 9 on a scale based on individual choice of panelists.

4.2.2.4 Preparation of tomato purée

For each session 9-10 kg of tomatoes were used. The fruits were carefully washed with water. To minimise the risk of creating additional volatile effects resulting from C6 volatiles release upon cutting and maceration, tomatoes were blanched by microwaving (Full power, 800W, for 10 min, per 1kg of tomatoes). After microwaving, they were macerated in a blender (Kenwood BL 440, for 3 min, per 1kg). The macerates were then combined, in 5L beakers and stirred to ensure that they are well mixed. After maceration, it was left too cool for 30 min. Some of the sample was saved as

reference (unspiked tomato purée) and the rest was used to prepare dilutions for volatiles.

4.2.2.5 Preparation of stock solutions for volatiles

Volatiles (200 µL) were diluted in 100 mL of propylene glycol to give a stock solution (Solution 1) with a concentration of 2000 ppm. The working solution was then prepared by taking 500 µL of Solution 1 and diluting with 100 mL of tomato purée (if more was needed the proportion was still the same, for example 3 mL of solution 1 and 600 mL of tomato purée). The working solution then had a concentration of 10 ppm.

4.2.2.6 Sample preparation for volatiles

Samples for the test were prepared a day before analysis and kept overnight in a cold room. In total 1000 mL, spiked tomato purée was prepared per sample. A summary of the sample preparation is presented in Tables 4.2-4.4.

Table 4.2 Sample preparation for E-2-hexenal. Initial concentration corresponds to working solution, final concentration is the concentration of volatile in tomato purée served to the panelist. Dilution ratio is the ratio between final and initial concentrations.

Sample	1	2	3	4	5	6
Initial concentration (ppm)	10	10	10	10	10	10
Final concentration (ppm)	**1.2**	**1.9**	**2.8**	**4.2**	**6.3**	**9.5**
Volume of working solution (mL)	125	187	281	421	632	949
Volume of tomato purée (mL)	875	812	718	578	367	51
Total volume (L)	1	1	1	1	1	1

Table 4.3 Sample preparation for E-2-pentenal. Initial concentration corresponds to working solution, final concentration is the concentration of volatile in tomato purée served to the panelist. Dilution ratio is the ratio between final and initial concentrations.

Sample	1	2	3	4	5	6
Initial concentration (ppm)	10	10	10	10	10	10
Final concentration (ppm)	**1.2**	**1.8**	**2.7**	**4.1**	**6.1**	**9.1**
Volume of working solution (mL)	120	180	270	405	607	911
Volume of tomato purée (mL)	880	820	730	595	392	88
Total volume (L)	1	1	1	1	1	1

Table 4.4 Sample preparation for citral. Initial concentration corresponds to working solution, final concentration is the concentration of volatile in tomato purée served to the panelist. Dilution ratio is the ratio between final and initial concentrations.

Sample	1	2	3	4	5	6
Initial concentration (ppm)	10	10	10	10	10	10
Final concentration (ppm)	**1.0**	**1.5**	**2.2**	**3.4**	**5.1**	**7.6**
Volume of working solution (mL)	100	150	225	337	506	759
Volume of tomato purée (mL)	900	850	775	662	493	240
Total volume (L)	1	1	1	1	1	1

4.2.2.7 Sensory tests

Each sensory session consisted of two parts and in each; a panelist was given 30 mL of tomato purée per sample, with 6 x 2 samples in whole session. For each of the panelists samples were individually randomised to avoid any bias and statistical error.

4.3 Results and discussion

4.3.1 Determination of taste detection thresholds in tomato juice for specific volatiles

4.3.1.1 Modified British standard method

The panelists had to compare each sample with a reference. The samples were presented in an increasing concentration (see Table 4.5). Perception of difference was scored as 1; lack of difference was scored as 0. Using the results from all panelists, we have calculated best estimate threshold (BET's) for each panelist and for each volatile. The group BET is found based on results from each individual judge.

Table 4.5 An example of how Best Estimate Threshold is calculated. The BET for each subject is the geometric mean of the highest concentration missed and the next higher concentration. For the panelist # 1, BET is the geometric mean between 0.8 and 1.6 (the logical following concentration): BET1=√(0.8*1.6) = 1.13 ppm. For calculating the group BET, The geometric mean of all the BETs is used. Zero corresponds to panelist not identifying a sample, 1 corresponds to positive identification of the sample by the panelist.

Panelist	**Concentration of volatile (ppm)**								BET
	0.08	0.1	0.114	0.13	0.2	0.267	0.4	0.8	(ppm)
1	0	0	0	0	0	0	0	0	1.13
2	0	0	0	0	0	0	0	1	0.57
3	0	1	1	1	1	1	1	1	0.09

Detection threshold distribution varies for each volatile. We have noticed that none of the four tested volatiles has a normal distribution (Fig. 4.3-4.6). The most interesting volatile is citral, which shows very different distribution. The data suggest that we have two categories of people in the panel: those who perceived citral easily, and those who needed higher levels of the compound to be present. Methyl salicylate and E-2-hexenal have a slightly different profile than citral, where distribution of panelist's detection is more balanced, with sample distribution through the whole concentration scale (Fig. 4.5 and Fig. 4.6). E-2-pentenal has got profile that is different from other 3 volatiles (Fig. 4.7). Modified British standard method allowed identifying taste detection thresholds and also creation of volatile detection distribution profiles. These distribution profiles clearly show that there is no linear response upon volatile stimulus, and that the detection of the same volatile compound within the same matrix differs between panelists.

Figure 4.3 suggests that in terms of citral, panelists are either very sensitive or insensitive to this particular volatile stimulus. Nine panelists detected citral at a concentration of 0.224 ppm, five at concentration of 0.082 ppm, while the most sensitive were able to detect this compound at concentration of 0.063 ppm.

The distribution pattern for methyl salicylate (Figure 4.4) is different from citral (Figure 4.3). Panelists were capable of detecting this particular volatile either at a low concentration of 0.063-0.09 ppm or at higher levels of 0.23-0.33 ppm.

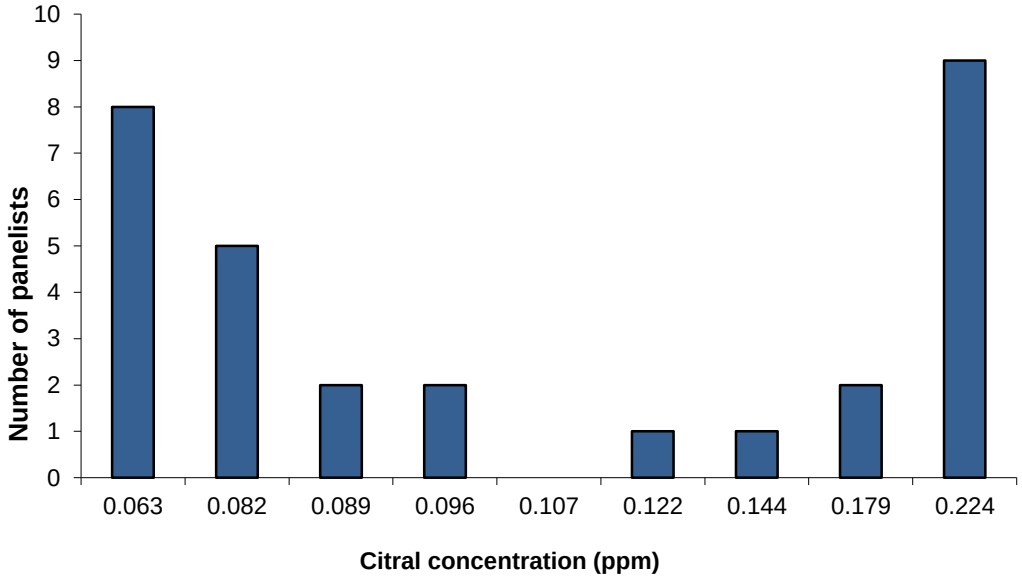


Figure 4.3 Distribution of the panelists according to the BET's for citral. Blue bars correspond to number of panelists positively identifying volatile at a specific concentration.

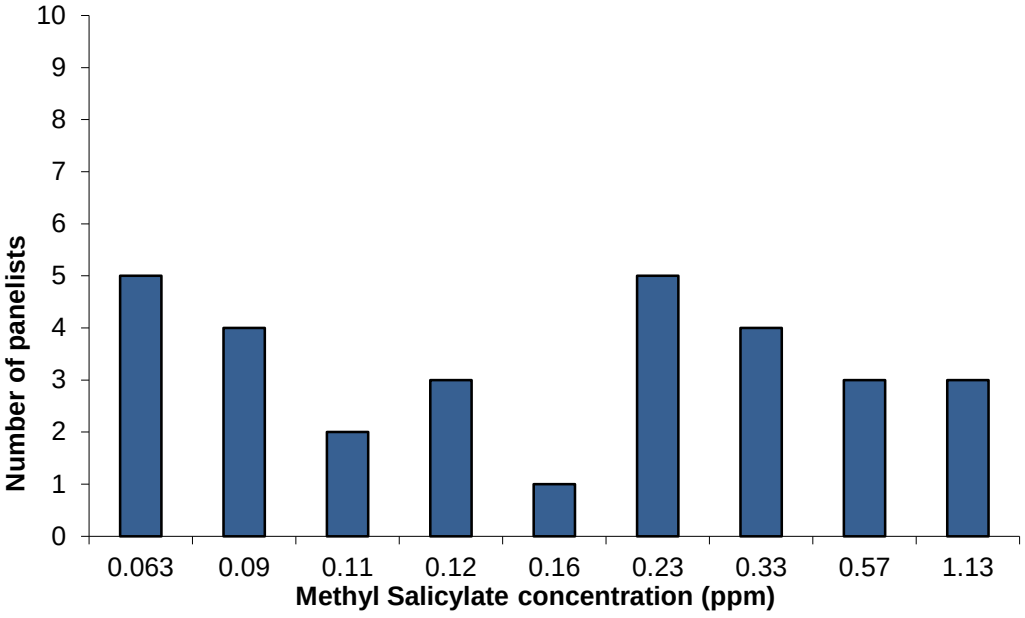


Figure 4.4 Distribution of the panelists according to the BET's for methyl salicylate. Blue bars correspond to number of panelists positively identifying

volatile at a specific concentration.

For E-2-hexenal (Figure 4.5), the data is similar to citral because majority of panelists could detect E-2-hexenal at low concentrations of 0.063-0.089 ppm, while others were more insensitive and detected only higher concentrations of 0.245-0.566 ppm.

In contrast with the other volatiles, data presented for E-2-pentenal (Fig. 4.6) indicates that the panelist distribution for this volatile is totally different from other volatiles. Panelists either detect at very low levels of 0.03 ppm or at higher concentrations of 0.118 ppm or finally at top concentrations of 2.263 ppm.

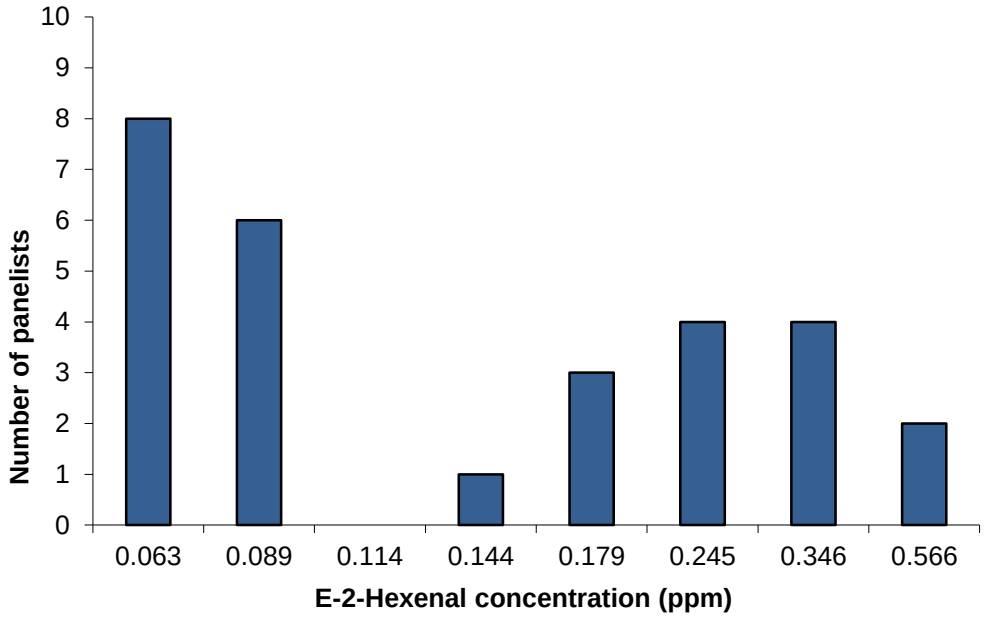


Figure 4.5 Distribution of the panelists according to the BET's for E-2-hexenal. Blue bars correspond to number of panelists positively identifying volatile at a specific concentration.

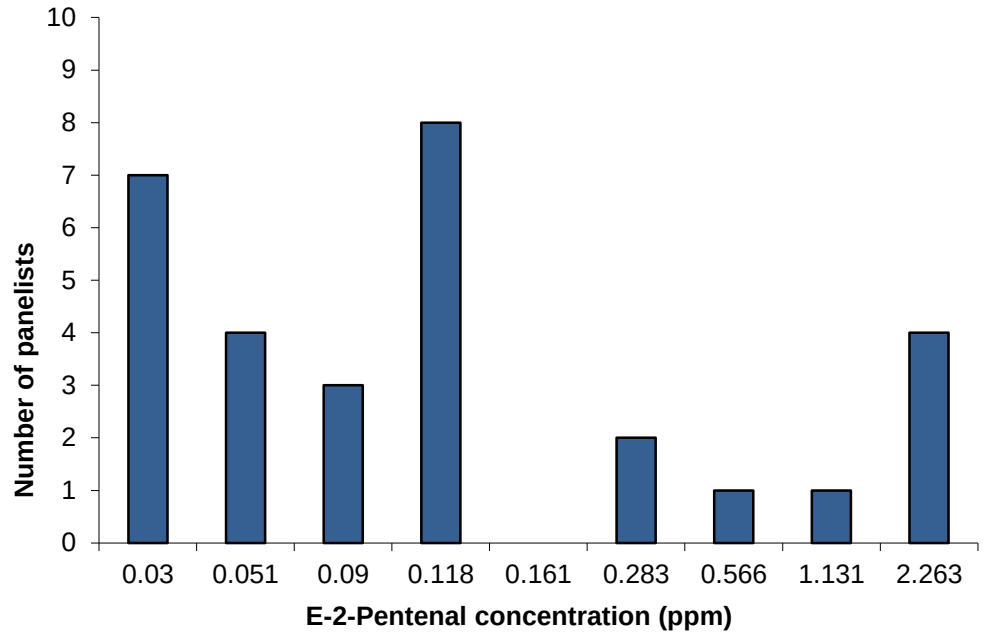


Figure 4.6 Distribution of the panelists according to the BET's for E-2-pentenal. Blue bars correspond to number of panelists positively identifying volatile at a specific concentration.

4.3.1.2 Modified American Society for Testing Materials (ASTM) method

The detection threshold results were verified using triangle tests. In a triangle test, three coded samples are presented to the panelists. Two are identical and one is different. The samples were tomato juice, spiked and non-spiked with a single volatile. The judge had to taste the product in order given, from left to right and select an odd sample. Because the results from the triangle test were not significant, meaning that not enough panelists could correctly identify the odd sample, a new approach had to be used. Modified American Society for Testing Materials method (ASTM's rapid method E679) is a three-alternative-forced-choice and is similar to triangle test in a sense that three samples are presented: two are controls and one is the substance under test. The samples were tomato juice, spiked (test sample) and non-spiked (controls) with a single volatile. The summary of modified BS method are presented in Table 4.6 and results of ASTM method are presented in Table 4.7.

Table 4.6 Review of the threshold detection determination (Modified British standard method BS 5929-7:1992, ISO 3972:1991). The BET for each panelist (see example in Table 4.5) was used to calculate the GEOMEAN BET. GEOMEAN BET is a geometric mean of all judges' responses and represents the concentrations of volatiles from the detection threshold test.

	Methyl salicylate	Citral	E-2-hexenal	E-2-pentenal
GEOMEAN BET (ppm)	0.195	0.114	0.136	0.133

Table 4.7 Concentrations of the 4 tested volatiles in tomato juice and their corresponding BET's using ASTM method. Three tests were undertaken for methyl salicylate, two tests for other volatiles. A significance level of ($P<0.05$) was chosen. One test involved samples with lower concentrations, in the other test samples concentrations were higher. Concentrations and level of significance correspond to ASTM method. NS - not significant.

Volatile	Test	Concentration (ppm)	Answers taken	Answers right	Significance
Methyl salicylate	1	0.5	N/A	N/A	NS
	2	0.8	17	10	0.02
	3	0.6	17	11	0.01
Citral	1	0.6	17	10	0.02
	2	0.45	17	6	NS
E-2-hexenal	1	0.8	17	12	0.01
	2	0.55	17	6	NS
E-2-pentenal	1	2	17	10	NS
	2	1.6	17	7	NS

Using a combination of methods of determining thresholds (BS 5929-7:1992, ISO 3972:1991 and ASTM rapid method E679) modified to investigate the volatiles in tomato juice gave the following results: Methyl salicylate – BET=**0.55 ppm**, Citral – BET=**0.52 ppm**, E-2-hexenal - BET=**0.66 ppm** and E-2-pentenal - BET=**1.79 ppm**.

Both modified British Standard Method and modified rapid ASTM method permitted to obtain an estimate of the taste thresholds in tomato juice. This task was very challenging because tomato juice is a complex mixture, much more difficult to perform sensory studies than for other matrices, for example water.

The olfactory perception can occur via two pathways: odours reach the olfactory epithelium through the nostrils (orthonasal olfaction) and through nasopharynx (retronasal olfaction). Retronasally perceived samples were usually delivered in a liquid phase during eating or drinking. Orthonasal perception of odourants, have been thought to be quantitatively different experiences in comparison with retronasal (Rozin, 1982). The aim of the present study was to examine olfactory perception as intraoral liquid stimulation as during eating.

The presented data cannot be directly compared with published information because of the difference in methodologies used and the matrix differences, but it may provide information how the taste thresholds relate to odour thresholds. The major research was undertaken to establish odour thresholds for tomato (Buttery et al., 1971), but this task was achieved using orthonasal measurements where volatile compounds were usually diluted in water (Buttery et al., 1971) or other evaluation media (Tandon et al., 2000) and sniffed. Tandon *et. al* (2000) has found an odour threshold of E-2-hexenal in deodorized tomato homogenate of 592 ppb, which is similar to our taste detection threshold (663 ppb). According to Lesschaeve *et al.* (1997), the odour threshold of citral (from lemon) is 0.6 ppm in water. Our results gave us 0.52 ppm. Buttery and colleagues (1989) have found an odour threshold of E-2-pentenal in water of 1500 ppb; our threshold was 1789 ppb, which is close to their estimate. Earlier research by Buttery (1987) suggests an odour

threshold in water for methyl salicylate of 40 ppb. This value is much lower than from our data, which is 548 ppb. The difference between these two values can be explained by matrix (water vs. tomato juice) and also the methodology (orthonasal vs retronasal).

The data from detection threshold determination may not be strictly compared with instrumental data for tomato fruits (obtained via GC-MS SPME), but it can provide a general view about volatiles in tomato fruits and how it relates to detection threshold in tomato juice. Full analysis of the ILs by GC-MS for volatile levels was undertaken and the results can be compared with tomato juice. Levels of E-2-hexenal (1.23-4.65ppm from GC-MS) are well within the detection threshold in tomato juice at 0.66ppm. In contrast, levels of E-2-pentenal (0.06-0.42ppm) and citral (0.01-0.02ppm) are well below the detection threshold at 1.79ppm and 0.52ppm accordingly. Therefore, information from sensory science experiments indicates levels needed in tomato to obtain a more desirable fruit flavour. In addition, experiments revealed that none of the tested volatiles has a normal distribution with respect to detection threshold experiments e.g. citral.

4.3.2 Hedonic evaluation of selected volatiles

The results from hedonic profiling were analyzed using *Design Expert Software* (Statease, Minneapolis, USA). In the design of experiment we used thresholds obtained from both modified BS and modified ASTM method multiplied by a group of values from 0.5 to 1.5, so we had a range of concentrations below and above our thresholds. In *Design Expert* the four factors were as presented: A (methyl salicylate), B (citral), C (E-2-hexenal) D (E-2-pentenal). Two responses were monitored: liking (based on mean values from hedonic test) and relative liking (based on median from hedonic test).

Hedonic evaluation of tomato juice indicates that non-spiked samples are scored higher that spiked samples (Table 4.8). In addition the samples with lowest scores are the ones that have a high concentration of methyl salicylate present. This discovery is also supported by the data presented in Table 4.9 and in Figure 4.7.

Table 4.8 Hedonic test results. MS = methyl salicylate, C = citral, H = E-2-hexenal, p = E-2-pentenal (numbers 0.5, 0.83, 1.17 and 1.50 correspond to concentration below and above experimental taste detection thresholds; mean= mean liking from all panelists on a scale from 0 = dislike extremely to 8 = like extremely; median=relative liking based on mean values).

Run	MS	C	H	P	Mean	Median
129	0	0	0	0	5.28	5
167	0	0	0	0	4.44	5
857	0	0	0	0	4.12	4
917	0	0	0	0	5.00	5
687	0.50	0.50	0.50	0.50	4.60	5
351	0.50	0.50	0.50	1.50	4.52	4
483	0.50	0.50	0.50	1.50	4.92	5
234	0.50	0.50	1.50	1.50	4.64	5
931	0.50	0.50	1.50	0.50	4.20	4
464	0.50	0.83	0.50	0.83	4.68	5
249	0.50	0.83	1.17	0.50	3.40	3
466	0.50	1.00	0.50	1.50	4.48	4
921	0.50	1.50	0.50	1.00	3.76	4
876	0.50	1.50	0.50	0.50	4.60	5
615	0.50	1.50	0.50	1.50	4.84	5
487	0.50	1.50	1.00	1.50	3.60	4
792	0.50	1.50	1.50	0.50	4.96	5
085	0.50	1.50	1.50	1.50	4.24	4
897	0.75	1.25	1.25	1.00	4.12	4
872	0.83	0.50	0.83	0.50	3.64	4
709	0.83	0.83	1.50	1.50	3.84	3
626	1.00	1.00	1.00	1.00	3.84	4
339	1.00	1.00	1.00	1.00	4.44	5
162	1.00	1.00	1.00	1.00	3.84	4
596	1.00	1.00	1.00	1.00	4.08	5
313	1.00	1.00	1.00	0.50	4.76	5
708	1.17	1.17	0.50	0.50	4.08	4
265	1.17	1.50	1.17	0.50	4.28	5
908	1.25	0.75	0.75	1.00	3.56	3
013	1.50	0.50	0.50	1.50	3.52	3
561	1.50	0.50	0.50	0.50	3.12	3
950	1.50	0.50	0.50	0.50	3.36	3
223	1.50	0.50	1.50	0.50	4.04	4
134	1.50	0.50	1.50	1.50	3.32	3
098	1.50	0.50	1.50	1.00	3.80	5
468	1.50	1.17	0.83	1.50	4.36	5
634	1.50	1.17	1.50	0.50	3.24	3
571	1.50	1.50	0.50	0.50	4.28	5
350	1.50	1.50	0.50	0.50	4.32	5
544	1.50	1.50	0.50	1.50	3.60	4
331	1.50	1.50	0.50	1.50	3.12	3
757	1.50	1.50	1.50	0.83	3.56	4
785	1.50	1.50	1.50	1.50	3.24	2
068	1.50	1.50	1.50	1.50	2.96	3

Table 4.9 Outcome from Design Expert. P values are presented for the four volatiles based on liking (mean). The model was created to evaluate impact of four volatiles; each volatile was a separate factor. Only one factor was significant and it was methyl salicylate. The model was significant only for liking but not for relative liking (mean was significant, but median was not). NS - not significant. ** - significant at P <0.01

LIKING
Model**
Factor A (methyl salicylate)**
Factor B (citral) NS
Factor C (E-2-hexenal) NS
Factor D (E-2-pentenal) NS

Table 4.9 summarizes the results from hedonic test using Design expert. Liking corresponds to mean results from hedonic test while relative liking is associated with median. Only one factor (volatile) is significant in this model, at P<0.01, as presented in the Table 4.9, it is methyl salicylate. Data from design expert presented in Table 4.9 and Figure 4.7 indicate that only one categorical factor is significant for the model. Only methyl salicylate has an effect on overall liking. With increase of methyl salicylate samples are less and less liked. This makes sense taking into account that methyl salicylate or oil of wintergreen has a pharmaceutical flavour (Goff and Klee 2006). Other volatiles might not be that strongly perceived due to a masking effect of the cocktail or it might be a matrix effect.

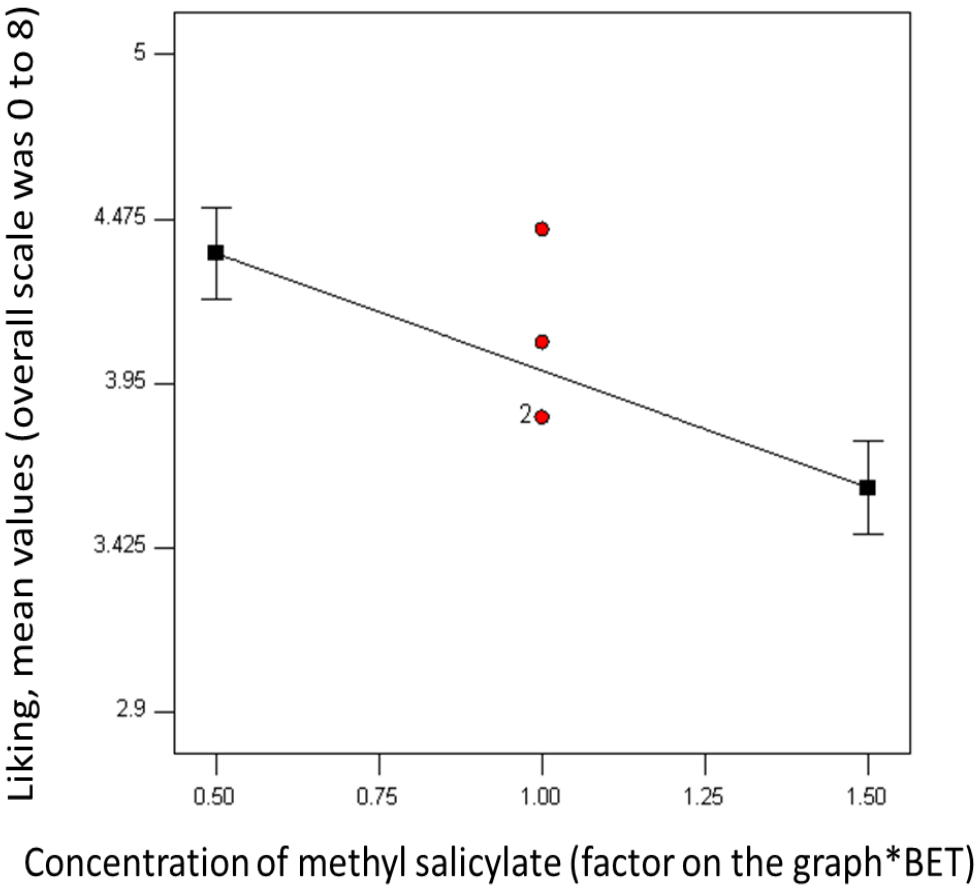


Figure 4.7 An interaction between liking of tomato juice spiked sample with methyl salicylate and concentration of this particular volatile. Increased amount of methyl salicylate (from 0.5 up to 1.5 x over the estimated threshold) leads to reduced liking.

The outcome of the results from Hedonic test, were described in terms of the mean and median. The results from hedonic test clearly indicate that the most liked sample is a reference (non-spiked tomato juice) and that the impact of volatiles is not significant with exception of methyl salicylate, which had a negative impact. One of the major constraints in the experiment was that the tomato juice was a complex mixture and we are adding a complex mixture of volatiles into this background. This makes the samples very challenging for panelists to score. In addition, masking effects of compound interactions are not that well known. Secondly the addition of volatiles at highest level (1.5 over detection threshold), gives a total of a six fold increase (1.5x4), and this also may be contributing to the fact that such a mixture is not liked. Finally, for this test we selected people who like tomato fruit, because we needed 100 panelists. It is very difficult to find enough tomato juice-liking panelists to run a large-scale tomato hedonic test. A complementary study was undertaken at the end of the test, only for statistics. 15% of the panel had never drunk tomato juice. 24% of the panel drunk tomato juice less than once a year, 32% several times a year, 11% several times a month, and 6% several times a week. As most of the panelists were not usual tomato juice consumers, their non-appreciation of the juices might be explained. All the panelists were used to eating tomato fruits.

4.3.3 Flash profile analysis

In the flash profile study, concentrations of volatiles within a sample are matched with various sensory responses given for each sample by the panel member using a GPA analysis (see Methods section in this chapter). The relationship between different volatile mixtures is calculated based on the attribute scores as a GPA. The relationship between the distribution of the mixtures on the GPA and the assignment of sensory characteristics can be

overlaid on this distribution. The repeatability of the judges between the two evaluation sessions was tested by the Spearman correlation test. An attribute is considered as repeatable if the evaluated attribute from both the first and the second evaluation session are significantly correlated at the level of significance $P<0.1$. All identified attributes for the judges are presented in Table 4.10

Table 4.10 Identified attributes for flash profile judges. Each of the judges used their own vocabulary to recognize attributes. Each of the judges found different number of attributes. AT = aftertaste.

Panelist	Number of identified attributes	Identified attributes
Sarah	3	Herbs flavour, Peppery flavour, Tangy flavour
Diane	7	AT (tomato), Flower (perfume), AT perfume (rose), AT (plastic, metallic), AT (green), Flavour tomato, Fruity
Carole	5	Flavour ripe tomato/sweet, Texture grainy, AT tinny, Taste sour, Flavour sweet/sour
Dawn	6	Aroma medicine, Taste salty, Texture smooth, AT (germolene), AT spicey, Taste fruity
Sue S	3	Aroma vinegary, Flavour tomato, AT bitter
Maureen	4	Texture thickness (runny/watery), AT earthy, Texture smooth, Aroma bland
Sue K	3	Texture blobby/grainy, Aroma metallic/tinny, Flavour chemical
June	4	Texture watery, Taste bitter/acidic, Texture (thick/thin), AT (bitter metallic)
Chris	1	Tomato flavour

Correlation of the samples is presented in Fig. 4.8 and Fig 4.9. In Fig. 4.8 the F2 axis separates all seven samples from the reference (129). We also noticed a negative correlation between samples with low methyl salicylate (483, 687, 931) in comparison with samples with high methyl salicylate (013, 134, 544, 757), well described in axis F1. Judges attributes were also presented and we could find attribute relating to specific volatiles. Increased level of methyl salicylate (013, 134, 544, and 757) can be linked with attributes such as aroma medicine, aftertaste earthy, and aftertaste germolene. These attributes were well separated by F1. E-2-pentenal (483) can be linked with attributes aftertaste perfume (rose) and aftertaste plastic, metallic. F2 axis separated a reference (129) from the other samples and reference can be linked with attributes flavour tomato ripe/sweet, herbs flavour, taste fruity and also aroma bland. Figure 4.8 well separated all samples from the reference. Both Figures 4.8 and 4.9 separated well methyl salicylate samples. Samples that contain high concentration of methyl salicylate correlated negatively with samples that had low quantities of methyl salicylate. E-2-pentenal was described by attributes like aftertaste perfume, aftertaste green aftertaste plastic (sample 483). E-2-hexenal was linked with fruity flavour attribute as well as aftertaste green (sample 931). Citral along with the other volatiles was responsible for bringing sample closer to centre of F1 axis. It may be responsible for transition from flavour sweet/sour and bringing a salty taste. Citral (sample 757) does not seem to have an obvious impact on attributes or maybe this impact is masked by the presence of other volatiles. It may have an impact on texture. The reference sample, which does not contain any volatiles was most liked in hedonic test and was well described in Flash profile.

On Figure 4.8, F1 axis separated the reference well from samples with low concentration of volatiles, but it was closer to samples with high concentrations. F2 axis gave better separation, where reference was positioned much further than the other samples. Attributes like flavour tomato ripe/sweet, aroma bland; characterised well this sample.

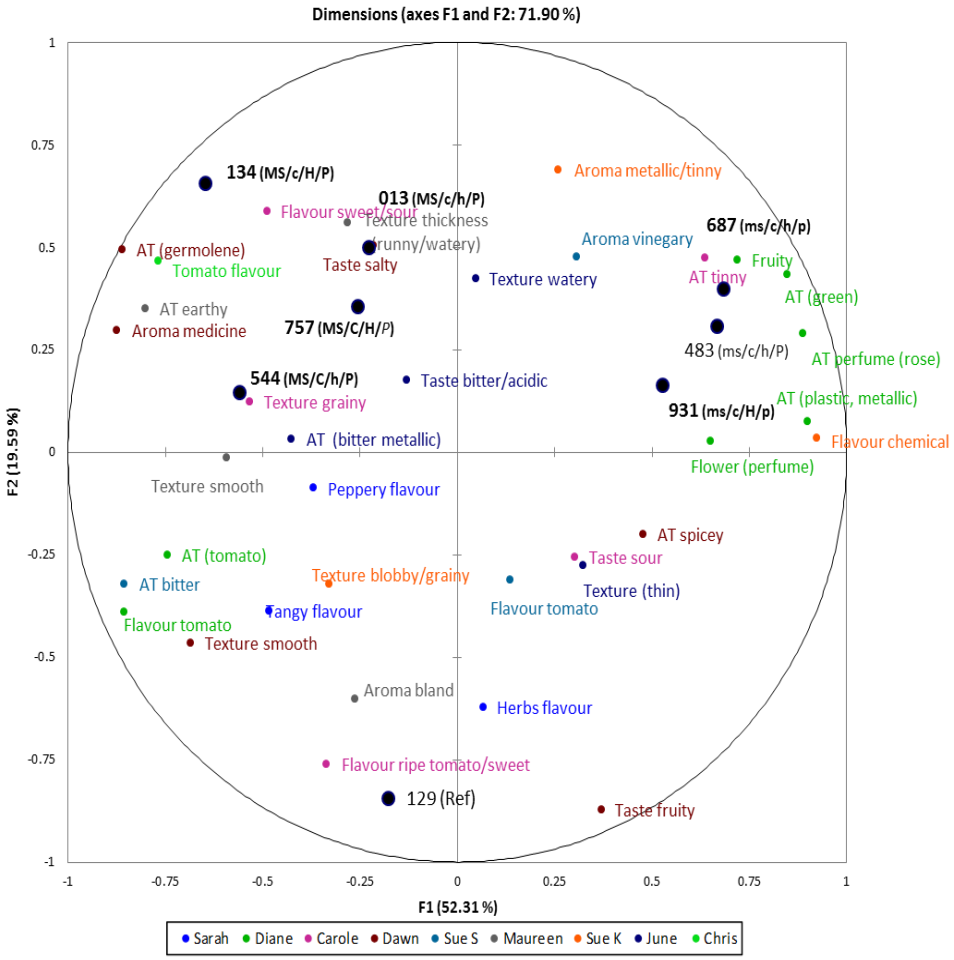


Figure 4.8 Variable plots determined by the first two axes of the GPA. Configuration of samples determined by the first and second axes of GPA. 71.9% of variation is described by these two axes. F1 is responsible for 52.31% while F2 account for 19.59%. Distribution of attributes for all the judges is shown. Black dots correspond to samples evaluated by the panelists. Each colour corresponds to attributes identified by different judge. Similar attributes cluster together, especially aftertaste green and rose along with flower attribute. Also aftertaste germolene clusters with aroma medicine. MS: methyl salicylate, C: citral, H: E-2-hexenal, P: E-2-pentenal. Capital letters mean higher concentration, small letters mean lower concentration.

In Figure 4.9 we had samples separated into 3 groups on F1 axis. There were samples with low methyl salicylate (483, 687, and 931) negatively correlated to samples with high methyl salicylate (134 and 544). Some samples separated closer to the middle of the graph (013, 129) and can be linked with sweet/sour and tomato flavour. We could identify significant attributes described by axes F1 and F3 and link them to the samples. We have found out that a presence of E-2-hexenal (931) gave fruity flavour with green aftertaste. Citral alone or in combination with other volatiles brought the samples closer to the center and reference. It may have an impact on texture as well (757), but it also may be a joined effect of volatiles. Tomato juice experiments were inconclusive in terms of E-2-hexenal, E-2-pentenal, and citral. The impact of methyl salicylate was strong enough to mask other compounds that were added into a tomato juice.

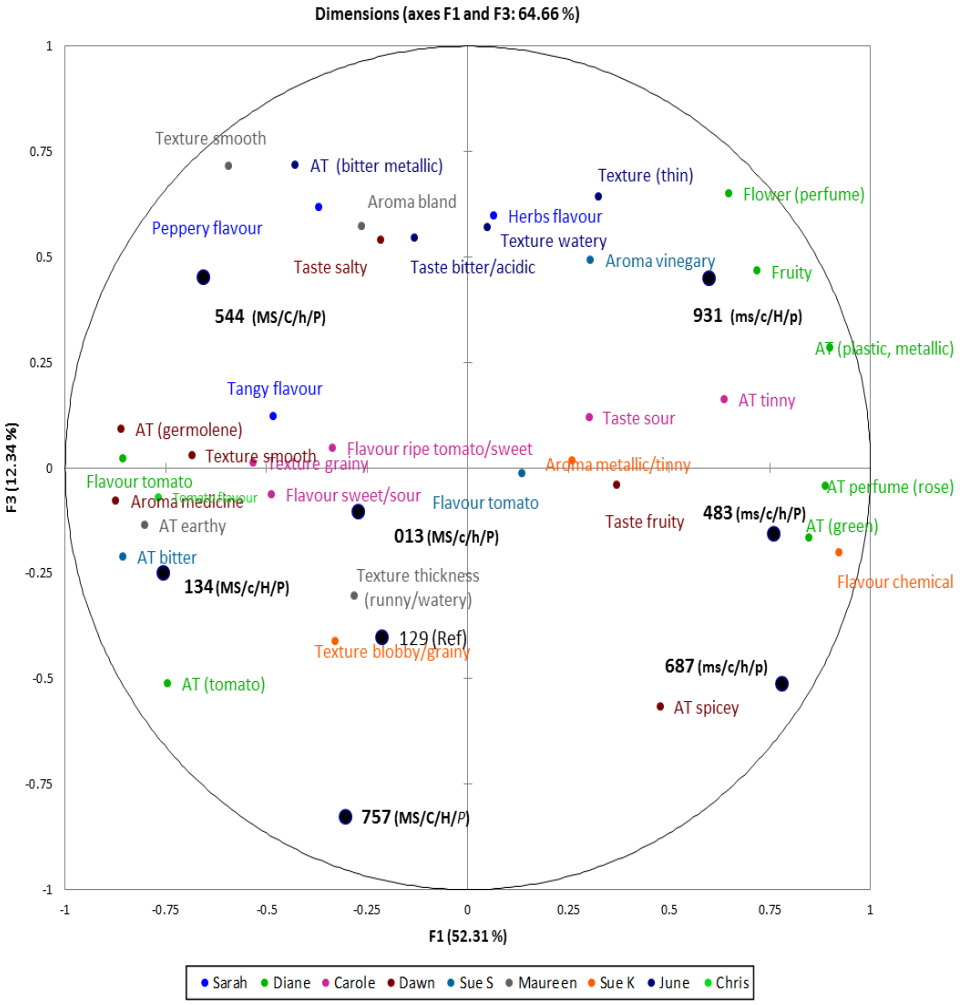


Figure 4.9 Variable plots determined by the first and third axes of the GPA. Configuration of samples determined by the first and second axes of GPA. 64.66% of variation is described by these two axes. F1 is responsible for 52.31% while F3 account for 12.34%. Distribution of attributes for all the judges is shown. Black dots correspond to samples evaluated by the panelists. Each colour corresponds to attributes identified by different judge. Similar attributes cluster together, especially aftertaste green and rose along with flower attribute. Also aftertaste germolene clusters with aroma medicine. MS: methyl salicylate, C: citral, H: E-2-hexenal, P: E-2-pentenal. Capital letters mean higher concentration, small letters mean lower concentration.

4.3.4 Sensory analysis using tomato purée

The other part of the chapter focuses on single volatile addition into a tomato purée sample, followed by sensorial evaluation. Simplification of the design aims for better understanding of selected volatiles on tomato purée. Despite constrains mentioned above, we decided that purée represented the best compromise for our base, although it is to be assumed that tomato purée is different in composition from fresh tomatoes and this might be a potential limitation. To minimize the effect of purée-to-purée variation, we ordered tomato fruits from the same supplier, the same the same class and type. In our study, we spiked tomato purée with three selected volatiles. The volatiles were chosen based on their flavour characteristics and we included here E-2-hexenal, E-2-pentenal, and citral. In tomato purée experiments, we used 10 trained panelists and the volatiles were evaluated separately, in three sessions, based on difference from reference sample. The experimental set-up and methodology are described in detail in section 4.2; the following section in this chapter presents the outcome of sensory experiments.

Impact of E-2-hexenal on tomato purée

In this study, the impact of E-2-hexenal on tomato flavour was evaluated in a sensory session where panelists were asked to taste spiked tomato purée containing this volatile at different levels (Figure 4.10). Four of the judges found the samples to be significantly different from the reference with the probability of $P<0.1$. There was a reference sample (non-spiked) and spiked samples were within 1.2-9.5ppm range, but only four out of ten panelists were able to discriminate between them reasonably well. All ten panelists show different and individual trends in sample recognition (Fig. 4.11), the general trend, calculated as a mean of all performances, is also

presented. The overall mean is a good indication of volatile perception because it corresponds to the predicted trend for sample recognition. R^2 (coefficient of determination, 0 = no fit, 1 = good fit with predicted model) value of 0.69 is reasonably close to the value of 1.

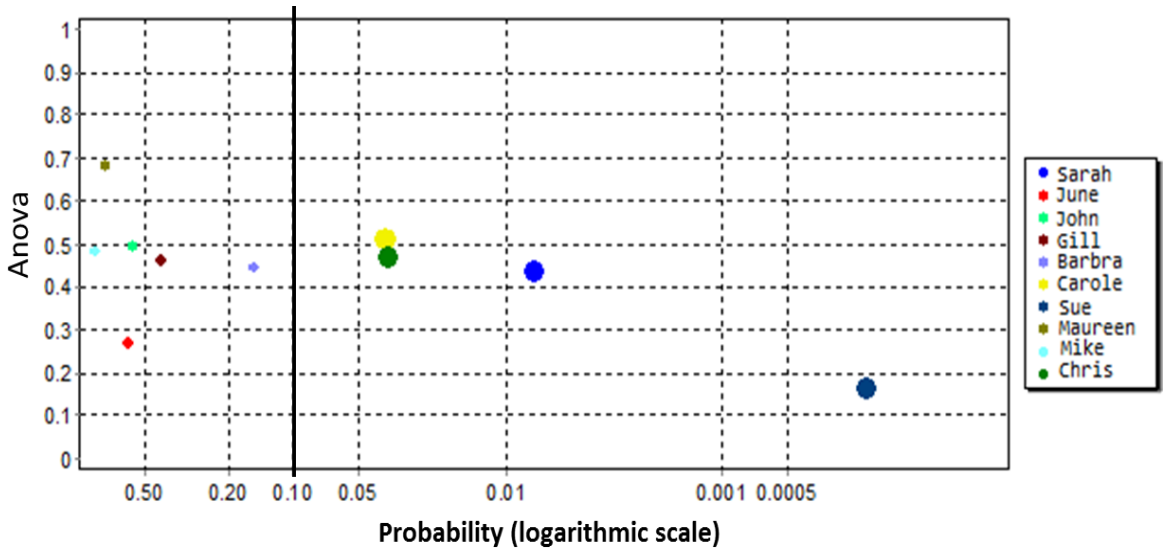


Figure 4.10 Panel performances for E-2-hexenal. Four judges found the samples significant with a probability of P<0.1 (There is only 10% chance that this happened by random). Y axis presents coefficient of variation in panelists individual responses while X axis descries probability on a logarithmic scale. ● significant at 10% • not significant. Black vertical line indicates a level of significance.

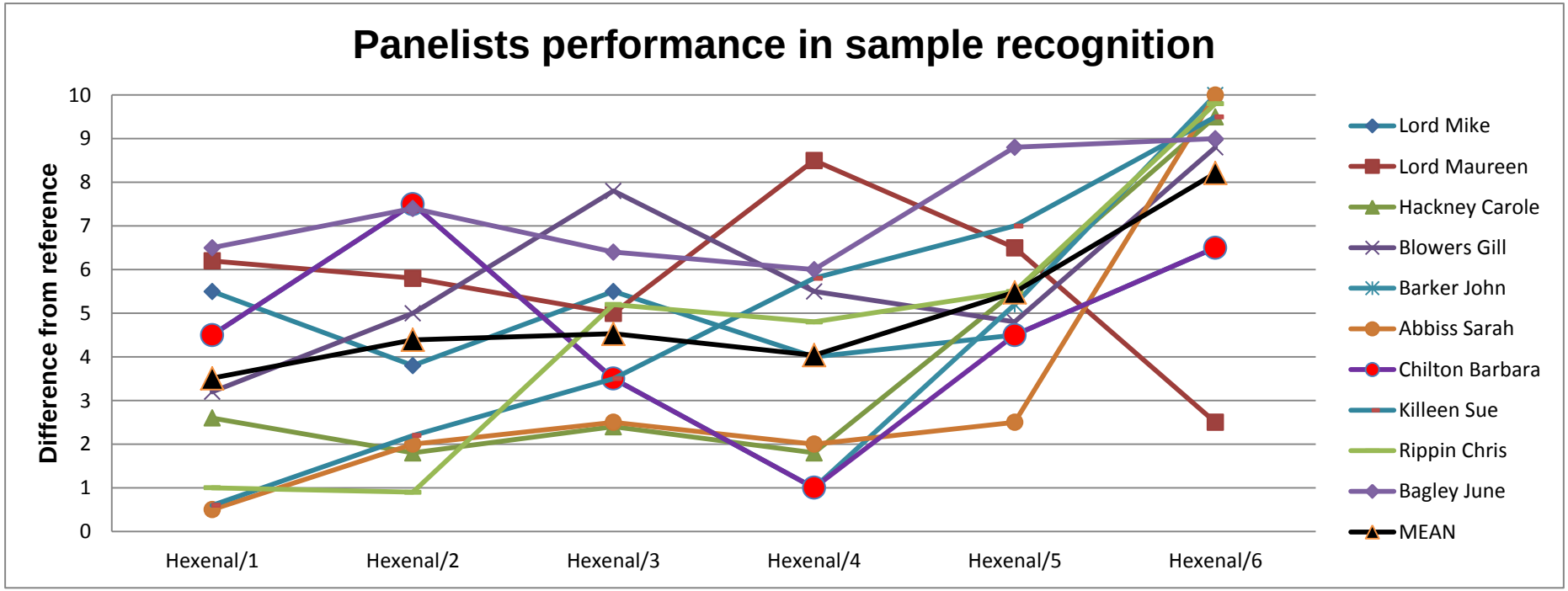


Figure 4.11 Performance for each panelist in sample recognition for E-2-hexenal. In black, overall mean for all panelists. Coefficient of determination is R^2=0.69.

Impact of E-2-pentenal on tomato purée

E-2-pentenal is a C5 volatile compound with a fresh floral aroma, positively contributing towards fresh tomato flavour. Its influence on tomato flavour was evaluated in a sensory session where panelists were asked to taste spiked tomato purée containing this volatile at different levels (Figure 4.13). The spiked samples were within 1.2-9.1ppm range, but only three out of ten panelists were able to discriminate between them reasonably well. Three of the judges found the samples to be significant with the probability of $P<0.1$. Other panelists found the test to be difficult. This range of volatile concentrations was close to the odour threshold in tomato purée.

As with E-2-hexenal the perception of spiked samples is difficult for some of the panelists (Fig. 4.12). All of the panelists show different and individual trends in sample recognition (Fig. 4.13), the general trend, calculated as a mean of all performances, is also presented. The overall trend does not correspond to the predicted trend for sample recognition with a low R^2 value.

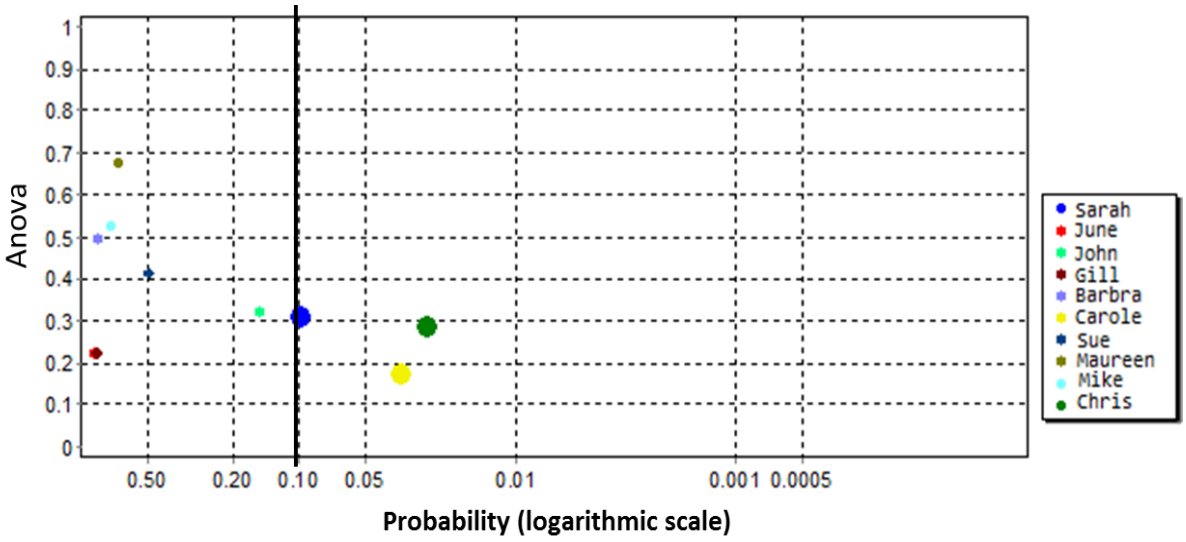


Figure 4.12 Panel performances for E-2-pentenal. Three judges found the samples significant with a probability of P<0.1 (There is only 10% chance that this happened by random). Y axis presents coefficient of variation in panelists individual responses while X axis descries probability on a logarithmic scale. ● significant at 10% • not significant. Black vertical line indicates a level of significance.

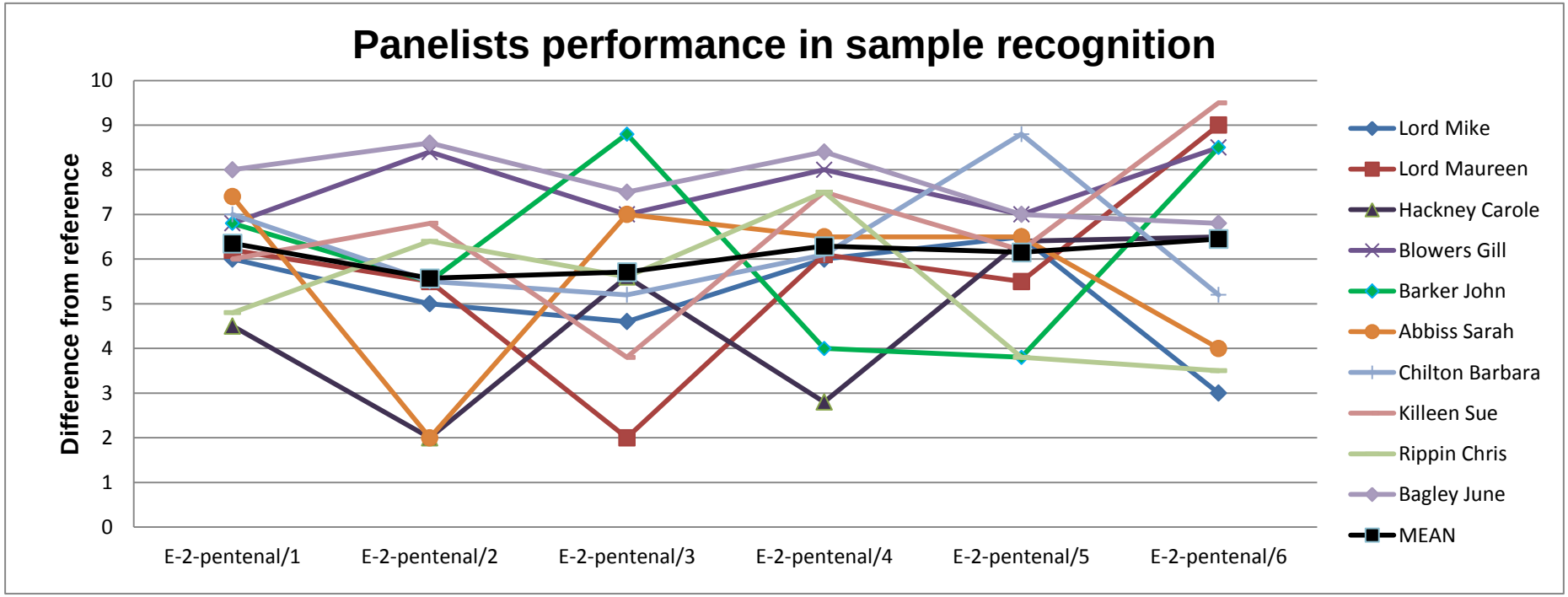


Figure 4.13 Performance for each panelist in sample recognition for E-2-pentenal. In black, overall mean for all panelists. Coefficient of determination is R^2=0.17.

Impact of citral on tomato purée

Citral is a carotenoid derived compound with a fresh citrus aroma, similarly to E-2-pentenal and E-2-hexenal, citral also positively contributes towards fresh tomato flavour. Its impact on tomato flavour was evaluated in a sensory session where panelists were asked to taste spiked tomato purée containing this volatile at different levels. Figure 4.14 presents the outcome of judges' performance for this volatile. Four of the judges have found the samples to be significant with the probability of $P<0.1$. Other panelists found the test to be difficult. The spiked samples were within 1.0-7.6ppm range but only four out of ten panelists were able to discriminate between them reasonably well. This range of volatile concentrations was near the odour threshold for tomato purée. The overall mean for citral corresponds well with the predicted trend for sample recognition (Figure 4.15). The R^2 value of 0.96 is very close to the value of 1.

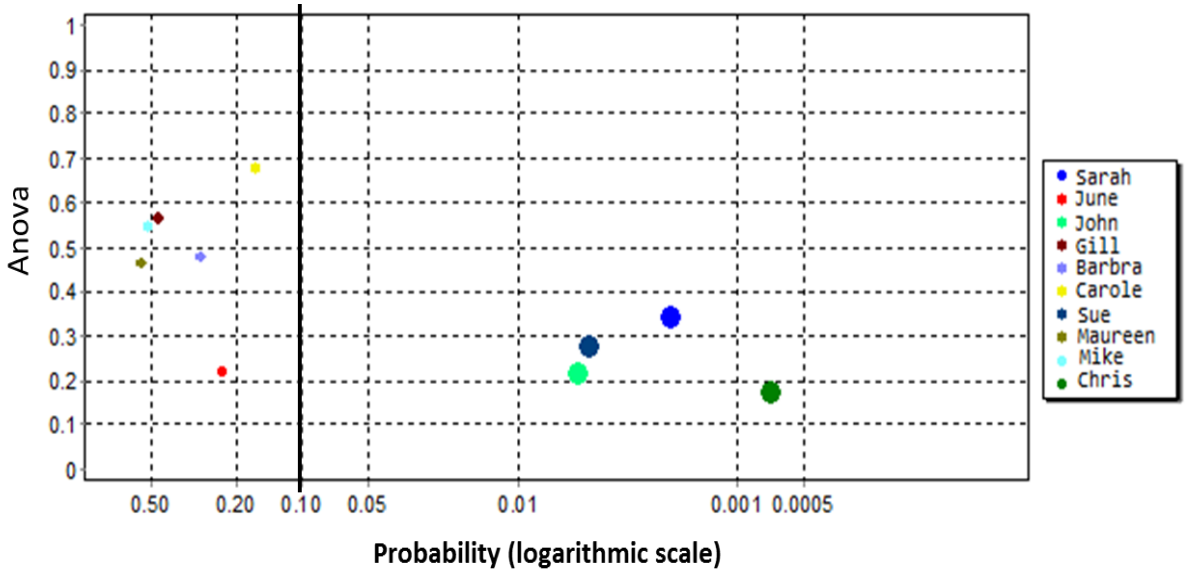


Figure 4.14 Panel performances for citral. Four judges found the samples significant with a probability of P<0.1 (There is only 10% chance that this happened by random). Y axis presents coefficient of variation in panelists individual responses while X axis descries probability on a logarithmic scale. ● significant at 10% • not significant. Black vertical line indicates a level of significance.

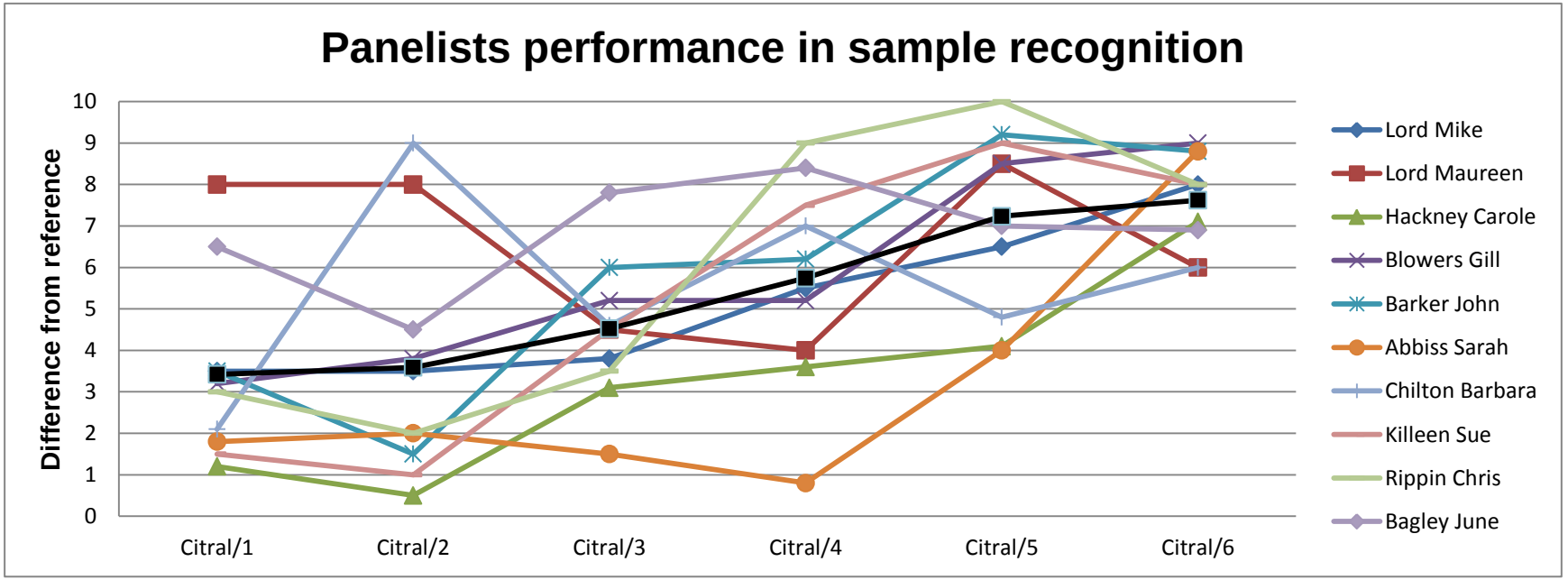


Figure 4.15 Performance for each panelist in sample recognition for citral. In black, overall mean for all panelists. Coefficient of determination is R^2=0.96.

The tomato purée experiments addressed key questions in terms of volatile impact on flavour. It was observed that tomato purée has a bigger masking effect on volatiles in comparison with tomato juice probably due to the higher viscosity of tomato purée. As a result possible odour threshold for tomato purée are higher in comparison with tomato juice.

In summary, the current study indicates that E-2-hexenal has a taste threshold of around 0.66ppm in tomato juice and between 1 and 9 ppm in purée. These values compare with 0.017ppm and 0.27ppm for water and fresh tomato as reported by Buttery (1993). Analysis of our ILs pericarp extracts by GC-MS SPME revealed levels between 1.2-4.6ppm. This suggests that for E-2-hexenal it may be possible to elevate levels of this volatile using wild species variation and hence improve tomato flavour. Experiments revealed that E-2-pentenal has a taste threshold of about 1.79ppm in tomato juice and within 1.20 to 9.11 in purée. These numbers can be compared with 1.5ppm and 0.14ppm for water and fresh tomato as described in Buttery (1993). Furthermore, GC-MS SPME data showed levels between 0.06-0.42. It implies that for E-2-pentenal it may be difficult to raise levels of this compound using ILs and therefore create a tomato with better flavour. Citral has a taste threshold of around 0.52ppm in tomato juice and the threshold in purée is bewtween 1 and 7.59ppm. These values are much higher than reported by Buttery (1993) values in water (0.039ppm) and fresh tomato (0.002-0.012). The data from GC-MS SPME for citral is within a 0.01-0.02ppm range. This leads to a conclusion that for citral it may be very difficult to increase levels of this volatile using ILs and improve tomato flavour.

4.3 Conclusions

- Using modified BSM and ASTM methods it was possible to identify taste detection thresholds in tomato juice for selected volatiles.

- Hedonic evaluation of four volatiles indicated methyl salicylate had a significant, but negative effect, affecting other volatiles and as a result, the overall sample perception.

- Flash profiling in tomato juice revealed volatile attributes.

- Initial experiments with tomato juice and mixture of four volatiles were to complex and difficult for panelists to discriminate between positive and negative attributes of particular compounds.

- Tomato purée experiments resulted in an estimation for the taste detection thresholds for three volatiles, E-2-hexenal, E-2-pentenal and citral.

- Comparison of taste detection thresholds for E-2-hexenal, E-2-pentenal, and citral with levels found in ILs suggested that E-2-hexenal was the best target for further study to locate genomic regions for enhanced volatile production in the *S. pennellii* lines and identify candidate genes.

CHAPTER 5:

Fine mapping of volatile QTL

5.1 Introduction

In the previous chapters, analysis of volatile emissions by fruit from tomato ILs was performed initially using APCI-MS and then followed by GC-MS SPME The sensory profile of selected volatiles in tomato juice and tomato purée was also evaluated. The next steps, was to more fully resolve selected vQTL, especially those shown to have positive sensory effects and where levels in the ILs are substantially above taste detection thresholds (see Chapter 4). The eventual aim is to narrow the genetic interval of the vQTL to permit marker assisted selection (MAS) or if possible identify candidate genes using the tomato genome sequence (http://solgenomics.net/). The strategy adopted in the current study was to focus on ILs containing significant vQTL for E-2-hexenal, Z-3-hexenal, E-2-pentenal, hexanal, methyl salicylate and citral volatiles. Then to examine gene expression profiles from ripe fruits of selected ILs and compare these with the M82 control and then link with volatile production. Candidate genes will be nominated by their altered expression patterns and that they map within the selected IL boundaries. The vQTL were also resolved for candidate gene nomination by measurement of selected volatiles in a range of Sub-ILs provided by Dani Zamir (Hebrew University of Jerusalem).

5.2 Materials and Methods

5.2.1 Microarray analysis of RNA material from selected ILs

5.2.1.1 RNA extraction

Total RNA extraction was performed using chloroform and isopropanol method (Chang *et al.,* 1993, modified). 1 g of ground frozen tomato fruit tissue was added to 10 mL of extraction buffer in the mortar and ground vigorously for 30 s. 9 mL was transferred into an Oakridge tube, which was incubated at 65ºC for 10 min. This was extracted with an equal volume of chloroform, vortexed for 15 s, and centrifuged for 10 min at 13200 rpm. 7 mL of the supernatant was transferred into a new 30 mL oakridge tube, 0.8 volume of cold isopropanol was added, mixed and left on ice for 5 min before a 10 min spin at 13200 rpm. 10 mL of 70% EtOH was added, and samples were spun for 2 min at 13200 rpm. Supernatant was removed and pellet was air dried for 5 min. The pellet was re-dissolved in 9 mL of sodium chloride-sodium dodecylsulphate-tris-EDTA buffer (SSTE) at 37ºC for 10 min. Then it was extracted with 9 mL of chloroform, vortexed for 15 s, centrifuged for 10 min at 13200 rpm. 8 mL of the supernatant was transferred to a new 30 ml oakridge tube. 6.4 mL of cold isopropanol was added, mixed, and left on ice for 5 min before 10 min spin at 13200 rpm. Washed with 10 mL of cold 70% EtOH and 10 mL of cold 100% EtOH. Air-dried, on tissue paper. The pellet was dissolved in 1 mL of DEPC treated water and kept on ice. 200 µL of 8M Lithium chloride (LiCl) was added to the supernatant and mixed thoroughly, incubated for 2 h on ice and harvested by centrifugation at 12000 rpm for 20 min at 0ºC. The pellet was washed twice with 10 mL of cold 70% and 10 mL of cold 100% EtOH. After air-drying 50 µL of diethylpyrocarbonate (DEPC) treated H_2O was added and vortexed. Quality of total RNA was checked with Nanodrop (ND 100, Thermo Fisher Scientific, Loughborough, U.K) and if required amount of

RNA was not achieved, RNA was re-extracted from relevant tomato material. Purity of RNA was checked using 1%, agarose gels. The components of RNA extraction buffer and SSTE buffer are presented in the Appendix (Tables 8.4 and 8.5)

5.2.1.2 GeneSpring

GeneSpringGX is a statistical tool that is used in biology to analyse and visualise expression and genomic data. Based on the earlier findings from GC-MS, four ILs 1-4, 9-1-2, 11-2 12-1 and the control (M82) were chosen for the microarray analysis. Total RNA was extracted from fruit at four developmental stages mature green, breaker, breaker+3 and breaker+7; with three technical replicates at each stage. The extracted RNA was converted to cDNA and hybridised to the Syngenta Tomato GeneChip and results were delivered back to UK. The array data, was used to identify candidate genes responsible for the altered volatile composition.

Normalisation was performed, so it is possible to compare gene expression across different arrays. Data was normalised using summarisation algorithm called Robust Multi-array Analysis. It is a method for normalising and summarizing probe-level intensity measurements from Affymetrix Gene Chips. Median of all samples was used as a baseline. Median is more robust to outliers than a mean and helps centering the array data to the same level.

GeneSpringGX ver. 11.0 (Agilent, U.K) was used to analyse genotypes: M82D and ILs: 1-4, 9-1-2, 11-2, 12-1; 4 developmental stages: mature green, breaker, breaker+3, breaker+7. There were three technical replicates each. Total number of probes was 22821.

5.2.1.3 Statistical analysis

Statistical tests were performed. Initially, samples were analysed as four separate projects in GeneSpring, where ILs were compared with a control (1-4 vs. M82D, 9-1-2 vs. M82D, 11-2 vs. M82D, 12-1 vs. M82D). This was achieved using unpaired t-test, followed by Benjamini-Hochberg procedure for multiple testing correction. In addition, a fold change with cut-off value 2.0 was applied to identify highly expressed genes.

5.2.2 Speed DNA extraction

A small leaf (approximately 40 mg) was placed in labelled Eppendorf tube on ice. It was ground in a small volume of extraction buffer with a blue Eppendorf grinder and gradually more extraction buffer was added up to 600 µL. The samples were vortexed for 5 s and centrifuged at full speed (12000 rpm) in a micro-centrifuge for 5 min. 500 µL of the supernatant was transferred into a new tube and 500 µL of isopropanol was added. The mixture was incubated at room temperature for 2 min. Samples were centrifuged at full speed (12000 rpm) in a micro-centrifuge for 5 min. The supernatant was discarded and the pellet was washed in 70% ethanol (1 mL and vortexed to dislodge the pellet). Next, the samples were centrifuged at full speed in a micro-centrifuge for 5 min. 150 µL of 100% ethanol was added to the pellet. The pellet was air dried for at least 20 min and later re-suspended in 50-100 µL tris-EDTA (TE) buffer for at least 2 h at 4°C. Components of the Speed DNA extraction buffer are presented in the Appendix in Table 8.6.

5.2.3 Markers development to identify sub-IL boundaries

Bin H was chosen as most likely to contain sub-IL 1-4-1. A set of markers was developed, specifically for this region. These markers were the size of about 900bp and the distance between each of them was about 350

kbp. Initially markers were tested by PCR to check if they amplify correctly and give desired product.

The PCR reaction mix is stated below. The PCR buffer and dNTPs were from NEB (Hitchin, U.K), primers were ordered from Eurofins (Ebersberg, Germany). Polymerase and cresol were made in Plant Sciences labs.

PCR reaction mix (1x)	Volume (μL)
10xBuffer (with $MgCl_2$)	1.5
dNTPs	0.5
Solyc01g105130_F	0.5
Solyc01g105130_R	0.5
Cresol	2.5
Taq polymerase	0.5
Water	12

PCR programme used to identify correct elongation temperature of DNA fragment of interest is shown below.

PCR programme	Temperature	Time length
Initial denaturation	95 °C	3 min
Denaturation	95 °C	30 s
Annealing	60 °C	45 s
Gradient	10 °C	
Elongation	72 °C	1min
Step 2	95 °C	30 s (35 cycles)
Final elongation	72 °C	10 min
Hold	10 °C	

The gradient PCR was used to identify optimal temperature and the outcome was presented on Figure 5.1. Only one marker was tested - Solyc01g105130 and it gave the required band only for M82D parent. The annealing temperature was chosen to be 59°C for all markers. Nine out of ten markers worked well with both parental DNA samples, only one mentioned above amplified well with M82D DNA. The agarose gels for each of the marker sets are presented in the Appendix. All markers that amplified with both parental DNA material, were then subject of sequencing alongside with DNA material from sub-IL 1-4-1.

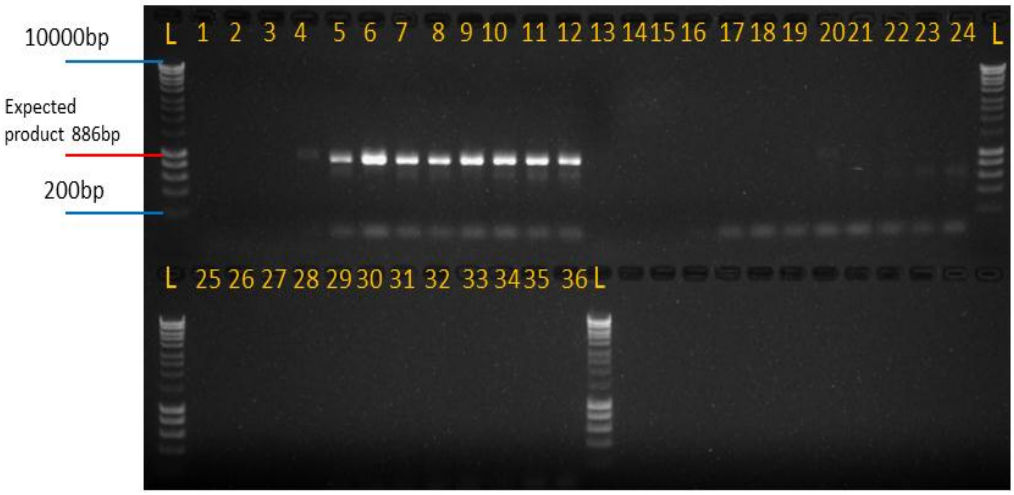


Figure 5.1 Gradient PCR to identify optimal annealing temperature. PCR was run for marker Solyc01g105130 with expected product of 886bp within temperature range of 55.5°C to 70.5°C. Wells 1 to 12: M82D parent, 13 to 24: *S. pennellii* parent, 25 to 36: negative control. Temperature gradient was as follows: Lane 1: 50.0°C, lane 2: 50.3°C, lane 3: 51.4°C, lane 4: 53.2°C, lane 5: 55.6°C, lane 6: 58.1°C, lane 7: 60.8°C, lane 8: 63.5°C, lane 9: 66.0°C, lane 10: 68.1°C, lane 11: 69.7°C, lane 12: 70.5°C. The same gradient was used accordingly for lanes 13-24 and 24-36.

5.2.4 Sequencing of parental DNA and sub-IL DNA to identify region for candidate genes

Purification of samples for sequencing using QIAquick PCR purification kit (QIAGEN, UK).

100 µL of sodium phosphate buffer (PB) was added to 20 µL of the PCR reaction and mixed. QIAquick column was placed in a provided 2 mL collection tube. The samples were centrifuged for 1 min at 13000 rpm in a table top centrifuge. The flow through was discarded and a QIAquick column was placed back in the same tube. To wash the samples 750 µL of protein extraction (PE) buffer was added to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and the column was put back in the same tube. Residual wash buffer was removed by another centrifugation step for 1 min and 13000 rpm. Each column was placed in a clean 1.5 mL microcentrifuge tube. The DNA was eluted using 30 µL of elution buffer (EB) added to the centre of the QIAquick membrane. The column was left to stand for 1 min and then centrifuged at 13000 rpm.

Sequencing data was analysed using BioEdit software (Ibis Biosciences, Carlsbad, CA, U.S.A). Initially, the sequences were checked for quality and then they were analysed. Complimentary sequences for both parents were aligned and single nucleotide polymorphisms (SNPs) were identified for short size markers (TG237 and TG528). Other markers (Solyc01g103040-106190) were aligned using nucleotide blast tool on NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Similar procedure was performed for sub-IL1-4-1 against both parents.

5.3 Results and discussion

5.3.1 Affymetrix GeneChip analysis of IL expression patterns

The major ILs selected for further studies were IL1-4, 9-1-2, 11-2, and 12-1 based on APCI and GC-MS SPME analysis. High quality RNA samples were generated for the GeneChip analysis. Figure 5.2 presents significant genes after application of fold change. Tables 5.1-5.4 present top five differentially expressed genes for all four ILs.

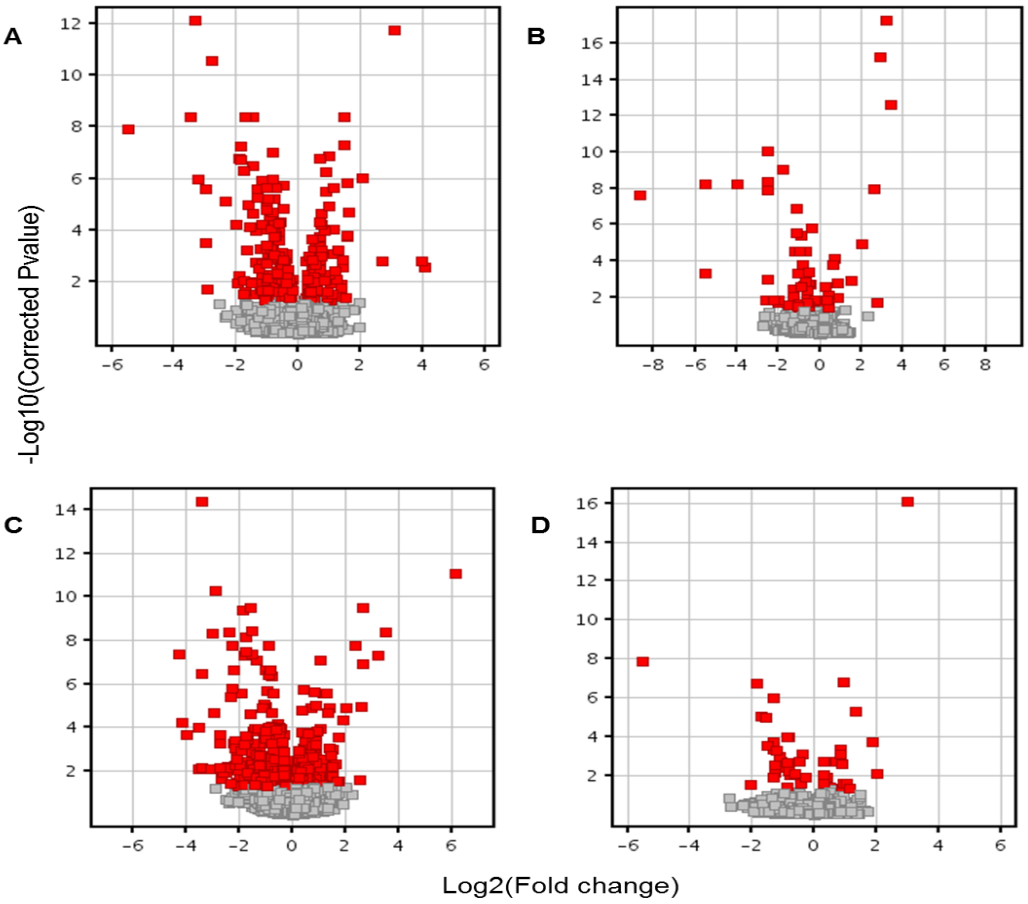


Figure 5.2 Volcano plots presenting the significant genes. Initially t-test was undertaken to identify significantly different genes. After application of t-test, fold change with cut off value 2 was applied (in red). A) IL1-4, 85 out of 263 genes are shown. B) IL9-1-2, 31 out of 62 genes are shown. C) IL11-2, 190 out of 563 genes are shown. D) IL12-1, 21 out of 48 genes are shown.

Table 5.1 Top 5 candidate genes for IL 1-4 based on t-test corrected with Benjamini-Hochberg procedure for multiple testing correction. In addition, fold change with cut-off value 2.0 was applied to identify highly expressed genes. Top 5 genes out of 85 differentially expressed. Fold change, regulation, and gene description are presented.

Gene	Fold change	Regulation	Description
Le012550_at	45	down	Similar to CAA71784.1 Glycine max; glutathione transferase; G.max mRNA for glutathione transferase
Le001179_at	16	up	Similar to AAK30143.1 Capsicum annuum; pathogenesis-related protein PR-1 precursor; *Capsicum annuum* pathogenesis-related protein PR-1 precursor, mRNA, complete cds
Le016384_s_at	15	up	Similar to AAK30143.1 *Capsicum annuum*; pathogenesis-related protein PR-1 precursor; Capsicum annuum pathogenesis-related protein PR-1 precursor, mRNA, complete cds
Le022158_at	11	down	No Description
Le019966_s_at	9	down	No Description

Table 5.2 Top 5 candidate genes for IL 9-1-2 based on t-test corrected with Benjamini-Hochberg procedure for multiple testing correction. In addition, fold change with cut-off value 2.0 was applied to identify highly expressed genes. These are 5 genes out of 31 genes that were differentially expressed. Fold change, regulation, and gene description are presented.

Genes	Fold change	Regulation	Description
Le001924_at	406	down	Similar to gi|548587|sp|P04284|PR06_LYCES Pathogenesis-related leaf protein 6 precursor (P6) (Ethylene-induced protein P1) (P14) (P14A) (PR protein)
Le012550_at	45	down	Similar to CAA71784.1 Glycine max; glutathione transferase; G.max mRNA for glutathione transferase
Le001548_s_at	45	down	Similar to gi|548586|sp|Q04108|PR04_LYCES Pathogenesis-related leaf protein 4 precursor (P4)
Le004994_at	15	down	Similar to AAL24262.1 *Arabidopsis thaliana*; At5g03380; *Arabidopsis thaliana* At5g03380/C160EPL23M mRNA, complete cds; unknown protein
Le019985_at	10	up	No Description

Table 5.3 Top 5 candidate genes for IL 11-2 based on t-test corrected with Benjamini-Hochberg procedure for multiple testing correction. In addition, fold change with cut-off value 2.0 was applied to identify highly expressed genes. These are 5 genes out of 190 genes that were differentially expressed. Fold change, regulation, and gene description are presented.

Gene	Fold change	Regulation	Description
Le011783_at	68	up	Similar to BAB02115.1 *Arabidopsis thaliana*; ; *Arabidopsis thaliana* genomic DNA, chromosome 3, TAC clone:K17E12; gene_id:K17E12.2 unknown protein
Le001197_s_at	19	down	Similar to gi|1351018|sp|P47926|RSI1_LYCES RSI-1 protein precursor (TR132)
Le001373_at	18	down	Similar to gi|131026|sp|P17642|PRS2_SOLTU Pathogenesis-related protein STH-2
Le001168_at	15	down	Similar to CAA54561.1 *Lycopersicon esculentum*; cell wall protein; *L. esculentum* mRNA for cell wall protein
Le000753_s_at	11	down	Similar to AAF18450.1 *Nicotiana glutinosa*; proteinase inhibitor type II precursor NGPI-1; *Nicotiana glutinosa* proteinase inhibitor type II precursor NGPI-1 mRNA, complete cds

Table 5.4 Top 5 candidate genes for IL 12-1 based on t-test corrected with Benjamini-Hochberg procedure for multiple testing correction. In addition, fold change with cut-off value 2.0 was applied to identify highly expressed genes. These are 5 genes out of 21 genes that were differentially expressed. Fold change, regulation, and gene description are presented.

Genes	Fold change	Regulation	Description
Le012550_at	46	down	Similar to CAA71784.1 Glycine max; glutathione transferase; G.max mRNA for glutathione transferase
Le019985_at	7	up	No Description
Le012483_at	4	down	Similar to AAA34073.1 *Nicotiana plumbaginifolia*; ; *N. plumbaginifolia* extensin (ext) gene, complete cds; extensin precursor
Le009778_at	4	up	Similar to AAL38336.1 *Arabidopsis thaliana*; unknown protein; *Arabidopsis thaliana* unknown protein mRNA, complete cds; unannotated coding sequence from BAC F5O24
Le017437_at	3	down	No Description

Within top 20 genes for each of the ILs, there were some similar trends. IL1-4, 9-1-2 and 12-1 had two common genes: Le012550_at, similar to glutathione transferase and Le019985_at with no description. IL1-4 and 11-2 had one common gene Le001179_at, similar to PR-1 pathogenesis related protein. IL9-1-2 and 11-2 had a common gene Le003735_at, similar to *Arabidopsis thaliana* Skp1 homolog.

It is difficult to identify obvious candidate genes. Certain volatiles could discourage insects and other pathogens from invading fruits. Bate and colleagues (Bate and Rothstein 1998) suggested that C6 volatiles induce a set of defence related genes. C6-volatiles including E-2-hexenal, E-2-hexenol, Z-3-hexenol and hexenol are key volatiles in plant defence signalling. This group of volatiles is among the earliest to be released from damaged tissue

(Hatanaka *et al.,* 1987; Turlings *et al.,* 1995). However, treatment of *Arabidopsis* tissue with E-2-hexenal did not induce the accumulation of mRNA for PR-1 or PR-2 within 24 h of treatment (Bate and Rothstein 1998). C6-volatiles form the basis for 'green note' flavour recognition by herbivores indicating that they act as signal molecules to animals, possess the general physical characteristics of a volatiles signal molecule and are released in sufficient quantity to be detected by animals (Hatanaka, 1993). Recent research confirms that volatiles emitted by plants are involved in the defence against pathogens and herbivores, abiotic stress, and play important role in the process of attraction of pollinators and seed dispersers (Pickersky & Gershenzon 2002; Reinhard *et al.,* 2004; Walter *et al.,* 2010).

5.3.2 Targeted volatile analysis of specifically selected ILs and sub-ILs to further resolve genomic regions containing vQTL

It has been possible to obtain relevant seed material from Prof Dani Zamir at the Hebrew University of Jerusalem that represent a sub-population of IL1-4 and 11-2. These two introgressions were subject to more detailed analysis using GC-MS approach and looking at introgression boundaries exploiting available sub-IL populations created for these ILs. The focus was on C5 volatile (E-2-pentenal), C6 volatile (hexanal), citrals (isoforms A and B) and methyl salicylate. In addition to these volatiles, data for E-2-hexenal and Z-3-hexenal was also obtained as these are also major contributors to tomato flavour (Buttery *et al.,* 1990, Yilmaz 2001).

Many ILs, including IL1-4, have been backcrossed to M82 to generate even smaller introgressed segments of wild species genome. In this study, 13 sub-ILs or QTL-NILs spanning the IL1-4 region were obtained from Dani Zamir and assessed for volatile emissions.

In terms of E-2-pentenal (Figure 5.3), sub-IL1-4-3 was significantly low ($P<0.05$), sub-IL1-4-1 was significantly high ($P<0.05$), while sub-IL1-4-13 was exceptionally high in terms of E-2-pentenal ($P<0.001$) when compared with control fruit M82D. Sub-IL 1-4-13 was also exceptionally high ($P<0.001$) when compared with IL1-4. Other sub-ILs did not present such a strong trend for this volatile. In terms of methyl salicylate, sub-IL1-4-11 was significantly high ($P<0.01$) in comparison with control M82D (2.4 times higher) and IL1-4 (2 times higher). Other sub-ILs did not show a strong trend for this particular volatile.

In addition to these mentioned above volatiles, citral A was significantly low in IL1-4 ($P<0.05$), while not significant in sub-ILs, the highest sub-IL was 1-4-1; however, not significant. Citral B did not have any significant results, but high in this volatile were sub-ILs 1-4-8, 1-4-9, 1-4-13 and the lowest was sub-IL 1-4-10. Data for E-2-hexenal indicated that sub-ILs 1-4-1 and 1-4-11 were significantly high ($P<0.05$). Analysis of Z-3-hexenal data revealed only significantly low ILs. These were M82, 1-4-4, 1-4-8, 1-4-17 ($P<0.05$).

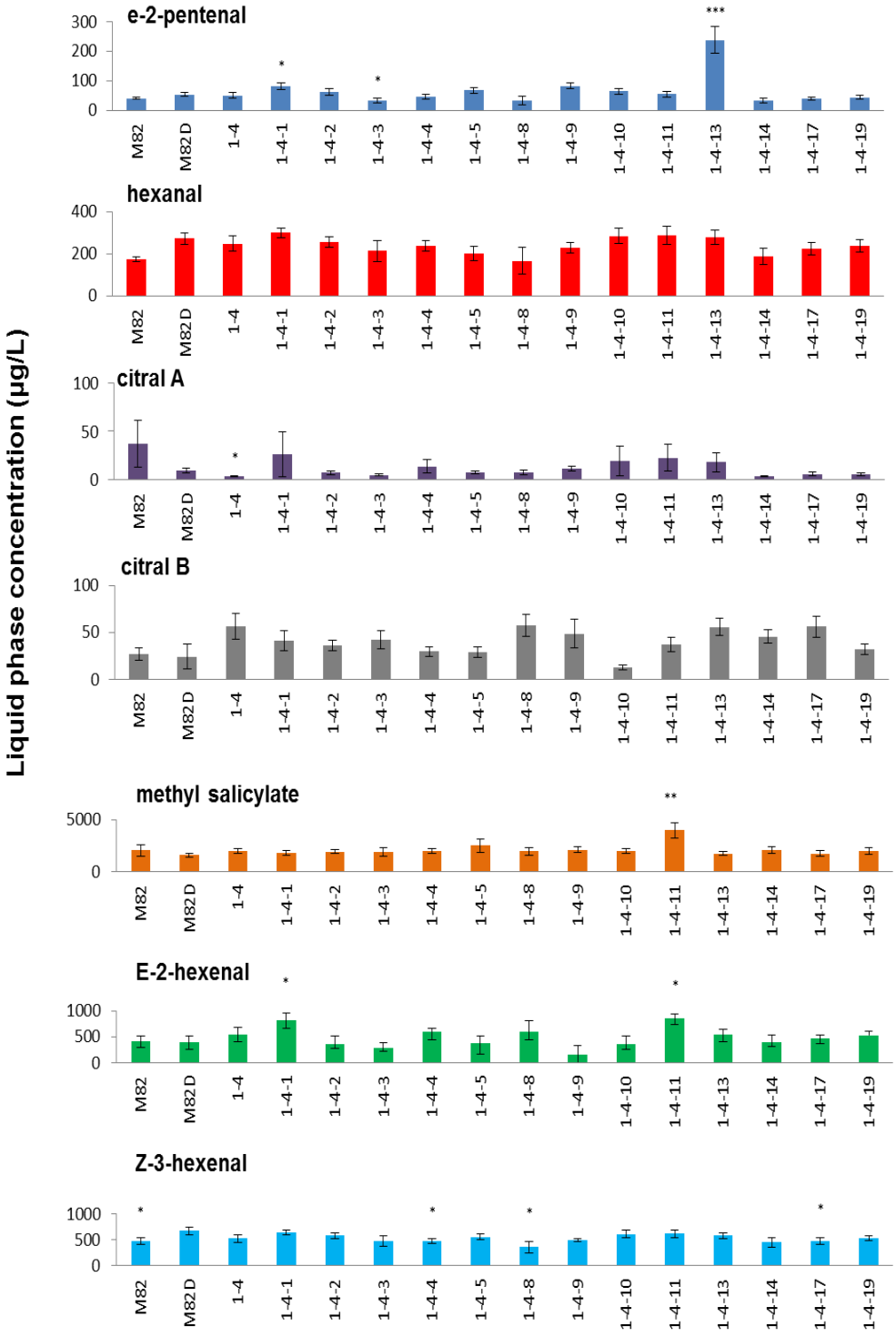


Figure 5.3 Levels of E-2-pentenal, hexanal and citrals, methyl salicylate and E-2-hexenal in IL1-4 introgression and its sub-ILs. 10 fruits were collected for majority of the lines except sub-IL1-4-8 (7), Sub-IL1-4-9 (3) and sub-IL1-4-14 (9).Error bars are standard errors of the mean (n = 10, unless otherwise stated). Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

A similar approach was undertaken using sub-lines from IL11-2 (Figure 5.4). For E-2-pentenal IL 11-2 was significantly higher ($P<0.01$), while other sub-IL did not present any significant results. Sub-IL11-2-5 was significantly ($P<0.05$) higher in hexanal emissions, but other sub-ILs or IL11-2 were not significant, with exception of M82 being significantly low ($P<0.05$).In terms of methyl salicylate, only sub-IL11-2-1 was significantly ($P<0.05$) higher, but other lines were not significant. In addition, the levels of citrals were not significant in any of the lines. Sub-IL 11-2-6 had significantly higher levels of E-2-hexenal ($P<0.01$) in comparison with control fruits M82D. In case of Z-3-hexenal M82 and sub-IL 11-2-3 were both significantly low ($P<0.05$), when compared with M82D, however, sub-IL 11-25 was significantly high ($P<0.05$).

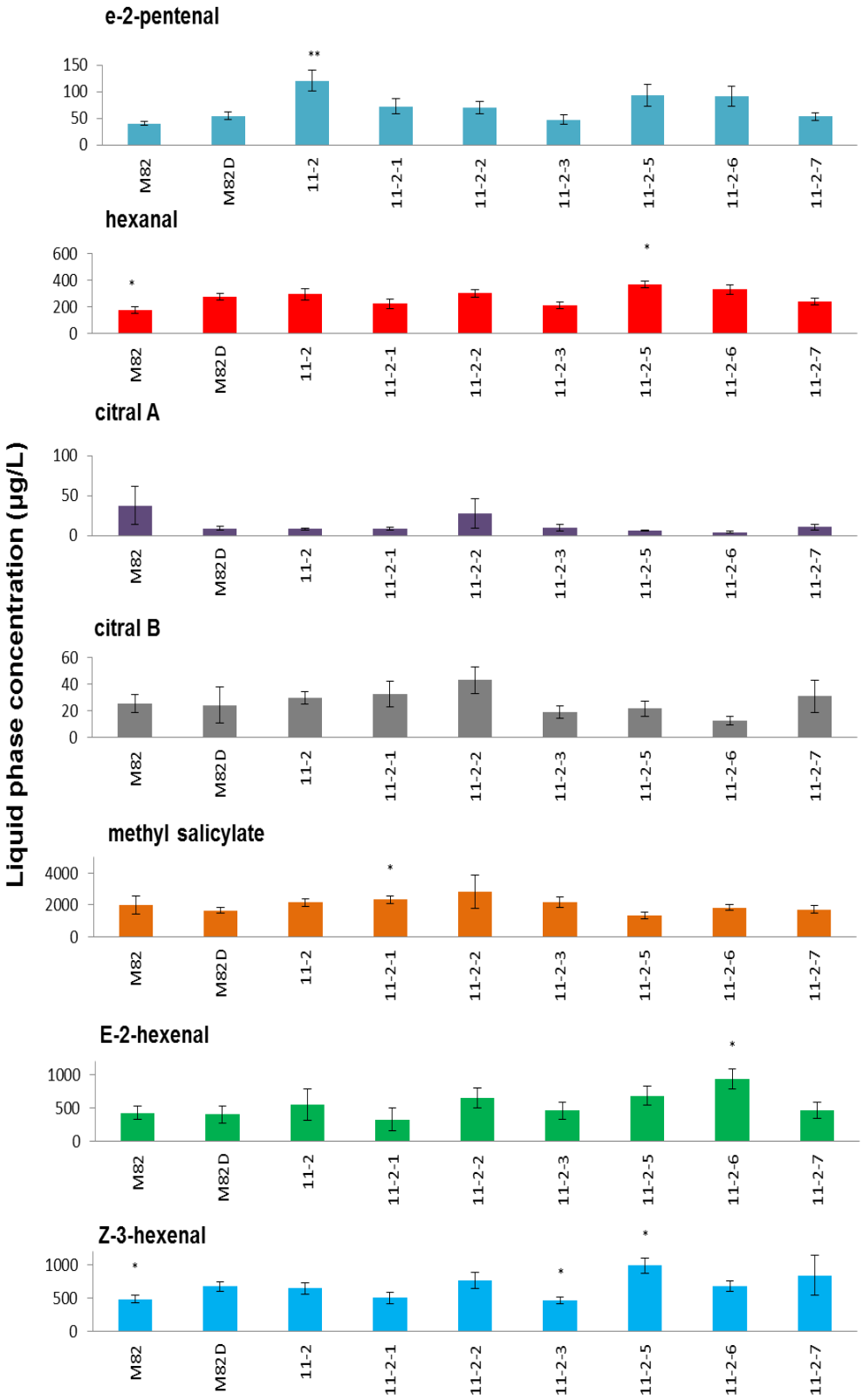


Figure 5.4 Levels of E-2-pentenal, hexanal and citrals, methyl salicylate and E-2-hexenal in IL11-2 introgression and its sub-ILs. 10 fruits were collected for all of the lines. Error bars are standard errors of the mean (n = 10). Level of significance labelled with stars; P<0.05(*), P<0.01(**), P<0.001(***).

5.3.3 Mapping of the volatile QTL

Extensive analysis of tomato ILs by GC-MS SPME indicated two promising vQTL on ILs 1-4 and 11-2. In depth GC-MS analysis using sub-ILs indicated that sub-IL 1-4-1 harbours effects on levels of C6 volatiles hexanal and E-2-hexenal.

To identify possible candidate genes within QTL-NIL 1-4-1, it is critical to determine the location of the genomic region covered by this sub-IL. However, this information was not available within the marker boundary. IL1-4 has a size of 6.5 Mbp and we assumed that our target sub-IL is on the top part of this introgression corresponding to bin H. We created a set of markers, to define the location of the introgression. Summary of single nucleotide polymorphisms (SNPs) for both parents and sub-IL is presented in Table 5.6.

Table 5.6 Summary table of SNPs based on sequencing of 9 markers and comparison between parents M82D and *S. pennellii.* For M82D parent only M82D DNA is present, for S*. pennellii* parent only its DNA is present. Sub-IL1-4-1 has a *S. pennellii* parental DNA for markers Solyc01g103040 and Solyc01g103980, indicating a region for candidate genes.

Marker	**M82D Parent**	***S. pennellii* Parent**	**Sub-IL1-4-1**
Solyc01g103040	M82D	*S. pennellii*	*S. pennellii*
Solyc01g103980	M82D	*S. pennellii*	*S. pennellii*
Solyc01g104260	M82D	*S. pennellii*	M82D
Solyc01g104490	M82D	*S. pennellii*	M82D
Solyc01g105460	M82D	*S. pennellii*	M82D
Solyc01g105490	M82D	*S. pennellii*	M82D
Solyc01g105870	M82D	*S. pennellii*	M82D
Solyc01g106190	M82D	*S. pennellii*	M82D
Solyc01g104850	M82D	*S. pennellii*	M82D

Some of the identified genes within 750kbp region on chromosome 1 are presented in Table 5.7. These gene models were part of 122 genes identified in the region. They were compared with 290 genes identified using GeneSpring analysis. Many genes were unknown proteins and their function still needs to be discovered.

Table 5.7 Gene models within 750kbp region on chromosome 1 compared with microarray data for 85 and 263 genes found in IL1-4 using GeneSpring.

Gene description	85 genes (ttest and 2 fold change)	263 genes (ttest without 2 fold change)
Unknown protein	Found	Found
Dynamin	Not found	Found
Cytochrome	Found	Found
Phosphoribosylamine-glycine ligase	Found	Found
40S ribosomal protein	Not Found	Found
Phospholipase D	Not Found	Not Found
Legumin like protein	Not Found	Not Found
Transcription factor protein	Found	Found
Transferase	Found	Found
Dehydrogenase/Reducatse	Found	Found

Cytochrome P450 (Howe *et al.,* 2000; Froehlich *et al.,* 2001) is involved in metabolism of oxylipins especially allele oxide synthase (AOS) and formation of jasmonic acid (JA). Products of the AOS pathway are essential signals for plant defence against pests (Staswick and Lehman, 1999),

mechanical responses (Weiler *et al.,* 1993). 13-HPOT metabolism may be regulated alternatively, by fatty acid hydroperoxide lyase (HPL). Short chain aldehyde products of HPL, together with their corresponding reduced alcohols, are important volatile constituents of the characteristic odour of fruits, vegetables, and green leaves (Hatanaka, 1993).

Pathogenesis related protein may also be 40S ribosomal protein (http://ted.bti.cornell.edu/TFGD/digital/supplement/diff/disease.html) found both in Tomato and Arabidopsis. A number of pathogenesis related proteins were found among top 20 highly expressed genes using GeneSpring in all four ILs. PR-1 precursor was found in IL1-4, leaf protein precursors (P4 and P6) were found in IL9-1-2. STH-2 protein was identified in IL11-2. However, in the top 20 highly expressed genes for IL12-1, there were no pathogenesis related proteins. Engelberth *et al.,* (2004) showed that green leaf volatiles (GLVs) emitted from herbivore-infested corn plants can prime intact plants against insect herbivore attacks. Bate and Rothstein (1998) showed that E-2-hexenalinduced several defence-related genes in *A. thaliana*, such as chalcone synthase (CHS), allene oxide synthase (AOS) and lipoxygenase 2 (LOX2). Arimura *et al.,* (2001) also showed that E-2-hexenal, Z-3-hexenol or Z-3-hexenyl acetate could induce genes encoding basic pathogenesis-related proteins (PRs) or LOX.

Dynamins are mostly involved in the scission of newly formed vesicles from the membrane of one cellular compartment and fusion with another compartment, at the cell surface and at the Golgi apparatus. Dynamins are also important for many processes including division of organelles, cytokinesis, and microbial pathogen resistance. In addition, dynamins are involved in plant in signal trafficking (Jin *et al.,* 2001) endocytosis and pathogen resistance (Praefcke and McMahon 2004).

Interestingly, Phospholipase D has been implicated in a variety of plant processes, including signalling (Laxalt *et al.,* 2001); however, Barsan (Barsan *et al.,* 2010) found Phospholipase D chromoplast proteome. It is involved in lipid metabolism in LOX pathway and generation of volatile compounds. Boukobza (Boukobza *et al.,* 2001) also used phospholipase in her study of lipid oxidation derived volatiles.

Glutathione transferase was identified in IL1-4, 9-1-2, 12-1 and was in all cases down regulated with fold change over 45. Kishimoto *et al.,* (2005) identified a number of genes induced by GLVs to include glutathione-S-transferase1 (GST1). The studied GLVs were E-2-hexenal, Z-3-hexenal and Z-3-hexenol. The ability of E-2-hexenal to induce defence gene expression can be attributed to the chemical reactivity of this compound. Z-3-hexenal, an aldehyde with a β, γ-unsaturated carbonyl part, is also a good inducer of the defence genes, however not that good as E-2-hexenal. In some cases the isomerization of the β, γ-unsaturated carbonyl moiety to the corresponding α, β-form can occur. As a result, a portion of Z-3-hexenal might be converted into E-2-hexenal in plants, which in turn might induce the defence genes. The chemical reactivity of Z-3-hexenol is even lower; however, the ability of the alcohol to induce defence gene expression was comparable with those of E-2- or Z-3-hexenal. Especially with GST1, the alcohol was most potent in inducing its expression. The role of hexanal in induction of GST has not been established.

In summary of this section, it is very difficult to nominate strong candidate genes without further research; however, the information discovered so far provides a good indication about possible candidate genes. They are probably involved in pathogenesis response or lipoxygenase pathway.

5.4 Conclusions

- Microarray analysis was undertaken on four ILs 1-4, 9-1-2, 11-2 and 12-1; however, it proved difficult from the array data alone to identify vQTL candidate genes.

- Additional GC-MS SPME analysis for IL1-4 and IL11-2 allowed improved resolution of vQTL for E-2-hexenal and hexanal indicating genomic regions spanned by sub-IL 1-4-1 and sub-IL 11-2-6 as the strongest candidates for further study.

- Major effects were observed for E-2-hexenal on sub-IL 1-4-1 so this sub-IL was subject of border mapping for identification of vQTL

- Marker analysis defined borders for sub-IL and focused the effects to 750kbp on the bottom of Chromosome 1 in an area containing 122 gene models.

- The possible candidate gene may be related to PR protein, phospholipase, or glutathione transferase.

CHAPTER 6:

General discussion and future work

Tomato is one of the most popular items of fresh produce in supermarkets; however, from consumer point of view; tomatoes often lack characteristic taste and flavour. Customers are willing to pay extra for full flavoured tomatoes that will meet their expectations (Bruhn *et al.,* 1991). This project investigated the genetic basis of volatile flavour attributes in tomato with the aim of identifying novel variation in wild species crop relatives that could be used to improve fruit quality.

Initial screening of tomato introgression lines to identify vQTL

The first task was to identify volatile QTLs in tomato plant using a genetically diverse and well-characterized set of *S. penn*ellii and *S. habrochaites* ILs. Chromosomal regions from crop related wild species can be used to increase of genetic diversity without losing any desirable traits (Zamir, 2001). The initial studies aimed to identify introgression lines (ILs) that had potential vQTL. This was undertaken by screening ripe fruits from each of the various ILs using direct mass spectrometry in the form of APCI-MS. The screen of the *S. habrochaites* lines indicated that they had a very limited number of vQTL based on trials over two years. However, the *S. pennellii* population proved much more promising with vQTL on a wide range of lines.

The IL screen revealed a range of vQTL many of which has been observed in previous studies. Tieman *et al.,* (2006) used GC-MS in contrast to APCI-MS that was used in the present study. A number of bins on different chromosomes have been identified in both studies for different volatiles.

Methylbutanals were identified in bins 2G, 5A, 7B, 9J, and 10G, which agrees with Tieman *et al.* (2006). Similar results were obtained for C5 unsaturated volatiles (1-penten-3-one and E-2-pentenal), which were identified in bins 2K, 8A and 10B. In addition, 2-phenylacetaldehyde was identified in bin 8D in both investigations. The most robust vQTL from APCI-MS and GCMS SPME were those related to IL1-4 (bin 1I) and IL9-1-2 (bin 9B) for hexanal and hexenals. However, Tieman *et al* (2006) failed to report any significant vQTL within this region, but they reported hexenals associated with the neighbouring bin 9D.

In this study, we also discovered a range of novel vQTL with our APCI-MS strategy. We related 1-penten-3-one and E-2-pentenal to chromosome 5 (bin A), in contrast to Tieman *et al.,* (2006) who correlated these volatiles with bin D on Chr. 5. Methylbutanals were associated with Chr.7 bin A in our study, but were linked to bin B by Tieman *et al.,* (2006). Moreover, we also identified phenylacetaldehyde on Chr. 5 (bin A) and Chr.7 (bin A). In addition a number of volatile compounds reported by Tieman *et al.,* (2006) were not identified by our study, including here geranyl acetone, β-ionone. Furthermore, citral and methyl salicylate were not reported in Tieman *et al.* (2006), but a number of bins were identified in our research to include mainly regions on Chr.1, 9, 11 and 12. The different results reported by the two studies are likely due to and the methodologies used as well as the fact that plant material was obtained from two distinct sources (glasshouse in this study and field in Tieman *et al.,* 2006).

APCI-MS has several advantages over GC-MS, especially in relation to sampling high numbers of tomato fruits. Previous studies have indicated that this approach can mimic maceration of tomato tissue in the mouth (Linforth *et al.,* 1994) and capture the volatiles that are released (Boukobza *et al.,* 2001; Boukobza and Taylor 2002). This is important because it allows for real time monitoring of volatile release (Taylor *et al.,* 2000; Taylor and Linforth

2000), mainly volatiles emitted upon maceration. The synthesis of C6 volatiles requires the activity of 13-LOX. In immature fruits or leaves, these volatiles are released only upon tissue damage where they possibly act as wound signals. The 13-LOX enzyme is targeted to chloroplasts and probably is not exposed to its substrates under normal circumstances (Chen *et al.,* 2004). The limiting step in C6 volatile production is the action of an unidentified lipase and/or loss of integrity of membranes through cell disruption caused by chewing or ripening (Klee 2010). Therefore, ACPI-MS may be better tool to mimic chewing action and allow the detection of C6. In addition, the simplicity of this APCI-MS system and rapid sampling time (3-5 min) were the major reasons to use it for assessment of tomato volatiles vQTL.

Sensory analysis of vQTL volatile effects

Volatiles are the crucial element of flavour along with sugars and organic acids (Tieman *et al.,* 2006, Mathieu *et al.,* 2009, Klee 2010). More than 7000 flavour compounds have been identified and catalogued in food and beverages (Baldwin *et al.,* 2002; Goff and Klee 2006). There are hundreds of volatiles present in tomatoes, but only around 30 are really considered to be important contributors based on odour threshold studies (Buttery, 1993; Tandon *et al.,* 2003, Tieman *et al.,* 2006, Goff and Klee 2006, Klee 2010). The main focus of this study was on C6 volatiles, responsible for a fresh, green and ripe tomato flavour (Baldwin *et al.,* 2002, Goof and Klee 2006, Klee 2010). In addition, citral which is responsible for the floral note (Tandon *et al.,* 2001, Lewinsohn *et al.,* 2005) and methyl salicylate which is linked with pharmaceutical flavour (Tieman *et al.,* 2010), were also studied.

Sensorial experiments provided additional important information in terms of flavour thresholds as well as volatile interactions. The hedonic test

clearly indicated that the most liked sample is a reference (non-spiked tomato juice) and that the impact of volatiles is not significant with the exception of methyl salicylate. However, panelists rated samples within three to six points on a scale, not really finding a striking and desirable sample. Increased concentration of methyl salicylate decreased overall liking of the tomato juice mixture. This finding is in agreement with published work of Vogel *et al.,* (2010), where a negative correlation in relation to methyl salicylate was reported.

Further analysis of tomato juice was undertaken out using flash profiling. This is a rapid method for sample description and correlating it with specific attributes. Increased methyl salicylate was related to attributes such as an aftertaste of germolene, or aroma of medicine and an earthy aftertaste. We found a negative correlation for samples that contain high methyl salicylate. E-2-pentenal was described by attributes as having an aftertaste like perfume, or green plastic-like aftertaste. E-2-hexenal was characterised by fruity flavour attributes as well as a green aftertaste. Fruity and green attributes are characteristic for C6 volatiles and sensory investigations using related compounds to those in the current study were also described in Baldwin *et al.,* (2008). In their work hexanal and Z-3-hexenal, were used and analysed in combination with sugars and acids. Tropical and fruity flavours were found to be enhanced with added sugar and acid for Z-3-hexenal. In our study we hypothesised, that citral might be responsible for transition from sweet to sour flavour based on the flash profile analysis the GPA results.

Identification of candidate genes underlying vQTL

After identification of robust vQTL the aim of the project was to resolve these QTL sufficiently so that candidate genes could be identified or at least minimum genomic regions to facilitate marker assisted breeding. The robust C6 vQTL and methyl salicylate vQTL on IL 1-4 were the subject of further investigation. High-resolution linkage mapping was not possible during the project because of time limitations. Therefore, the strategy adopted was to identify expression QTL within the ILs of interest and determine if any of these could be reasonably linked to volatile emissions by knowledge of volatile pathways. The main precursors of volatiles are known to be lipids (Boukobza *et al.,* 2001; Boukobza and Taylor 2002), amino acids (Buttery and Ling 1993; Tieman *et al.,* 2007; Kovchenko and Fernie 2011) and carotenoids (Lewinsohn *et al.,* 2005).

In this study, the transcriptome analysis of ILs containing vQTL revealed a small number of candidate genes, the most promising introgression based on the APCI and GCMS work for a robust vQTL were IL1-4, 9-1-2, 11-2, 12-1.

There were a number of possible candidate genes. Within top 20 differentially expressed genes with fold changes higher than two for each of the ILs, there were some comparable trends. IL1-4, 9-1-2 and 12-1 had two common genes: Le012550_at, similar to glutathione transferase and Le019985_at with no description. IL1-4 and 11-2 had one common gene Le001179_at, similar to PR-1 pathogenesis related protein. IL9-1-2 and 11-2 had a common gene Le003735_at, similar to *Arabidopsis thaliana* Skp1 homolog. Numerous researchers have attempted to assess the function of PR-1 proteins in plants, but without much success (Buchel *et al.,* 1999; Edreva 2005) Comparative analysis of PR-1-type proteins from various plant taxa

suggests that PR-1 family is highly conserved in plants (Edreva 2005). The widespread presence of PR-1 family suggests that these proteins share an evolutionary origin and possess activity essential to the functioning and survival of living organisms (Van Loon, 2001). PR-6 is a proteinase inhibitor (Van Loon and Strien, 1999). Certain volatiles could discourage insects and other pathogens from invading fruits. Bate and colleagues (Bate and Rothstein., 1998) suggested that C6 volatiles may induce a set of defence related genes. The green leaf volatiles to include E-2-hexenal, Z-3-hexenal, and Z-3-hexenol induced several genes comprising glutathione-S-transferase1 (GST1), however the role of hexanal in induction of GST has not been determined (Kishimoto *et al.,* 2005). The way in which C6 synthesis is regulated remains unclear. Barsan *et al.,* (2010) found Phospholipase D in the chromoplast proteome. It is involved in lipid metabolism in LOX pathway and generation of C6 volatile compounds.

A number of genes controlling volatile biosynthesis have been identified in tomato by other workers. Volatile terpenoid compounds, potentially derived from carotenoids, are important components of flavour and aroma in fruits. The tomato genome contains two closely related genes encoding carotenoid cleavage dioxygenases, *LeCCD1A* and *LeCCD1B* (Simkin *et al.,* 2004). The over-expression of the antisense transcript led to reductions in mRNA levels of both LeCCD1A and LeCCD1B in the leaves as well as fruits resulting in reductions of β-ionone (a β-carotene-derived C13 cyclohexone) and geranylacetone (a C13 acyclic product) suggesting that *LeCCD1* genes play important role in the formation of these flavour volatiles in vivo (Simkin *et al.,* 2004). The other members of CCD gene family CCD4 and CCD7 can also cleave carotenoids to generate volatiles (Klee 2010). Changing expression of transcription factors can be an efficient way to modulate metabolic pathways in plants. It can also provide useful information

regarding the identities of genes that constitute metabolic networks (Dal Cin *et al.,* 2011). Expression of a MYB transcription factor, *Petunia hybrida* ODORANT1, to alter phenylalanine and phenylpropanoid metabolism in tomato have been a subject of the study by Dal Cin *et al.,* (2011). The authors concluded that the increased activity of the phenylalanine synthetic pathway was inadequate to increase synthesis of the most important flavour-related volatiles (2-phenylacetaldehyde, 2-phenylethanol), probably because phenylalanine was rapidly converted to other non-volatile phenylpropanoids. In addition, the lack of correlation between branched-chain amino acids and their corresponding volatiles in the *Solanum pennellii* introgression population has also been observed (Schauer *et al.,* 2006; Tieman *et al.,* 2006b; Maloney *et al.,* 2010). The first committed step in synthesis of the major flavour-associated volatiles is encoded by a small family of AADCs (Tieman *et al.,* 2006a). Moreover, analysis of transgenic plants with altered AADC expression, led to conclusion that the AADC enzymes are rate limiting to synthesis of these volatiles, however the substrate (in this case phenylalanine) is not a major control point for synthesis of phenylalanine derived volatiles (Dal Cin *et al.,* 2011). The C6 volatiles are abundant in tomato and their synthesis starts with C18 fatty acids, which are then converted to 13-lipoxygenase to relevant hydroperoxides. An antisense construct targeting the single tomato 13-LOX greatly decreases or eliminates synthesis of all C6 volatiles (Chen *et al.,* 2004). In addition, the lyases and isomerases involved in the pathway have yet to be identified in tomato (Klee 2010).

Future work

The use of *Solanum pennellii* introgressions led to a number of discoveries related to tomato taste and flavour. IL8-2 and 8-2-1 have been identified to contain high levels of 2-phenylacteldehyde and 2-phenylethanol

(Tadmor *et al.,* 2002; Tieman *et al.,* 2007). The use of *S. pennellii* introgression led to identification of soluble solids QTL identified in IL 9-2-5 (Baxter *et al.,* 2005). Our study led to identification of strong vQTL on chromosome 1, IL1-4. IL1-4 has a size of 6.5 Mbp and we assumed that our target sub-IL is on the top part of this introgression corresponding to bin H This was achieved by designing specific markers, followed by PCR and sequencing of plant material and finally by comparison and identification of SNPs between the IL and parental lines . The GC-MS analysis of nearly isogenic lines (QTL-NILs) led to identification of sub-IL 1-4-1 with elevated levels of C6 volatiles in comparison with control fruits. The region of interest has therefore a size of 750kbp and 122 gene models are located within this region.

The next step in analysis of the tomato volatiles and identification of vQTL would be the use of linkage mapping to further resolve the QTL and delineate a small mapping interval so a candidate gene can be nominated for functional analysis. The tomato genome has recently been sequenced and annotated (http://solgenomics.net/). Recent development and decreasing costs of Next Generation Sequencing (NGS) provide a method to sequence of marker boundaries and therefore precisely defining QTL-NIL regions. The low-cost production of large volumes of sequence data is the main advantage over conventional methods (Metzker 2010). This eliminates the need for expensive marker genotyping. This will be through both map-based cloning and developments in network inference that provide novel ways to link volatiles to candidate genes.

A powerful tool for functional analysis of vQTL could be VIGS (Orzaez *et al.,* 2006). However, use of VIGS to carry out functional analysis of genes involved in fruit metabolism can have appreciable technical limitations. Penetration of VIGS phenotypes in most plant species is only partial and

shows a patchy tissue distribution (Orzaez *et al.,* 2006). A visually traceable VIGS system was created for fruit, comprising two elements: a transgenic tomato line (Del/Ros1) expressing *Antirrhinum majus*, Delila and Rosea1 transcription factors under the control of the fruit specific E8 promoter, showing a purple-fruited, anthocyanin-rich phenotype. In addition, a modified tobacco rattle virus VIGS vector incorporating partial Rosea1 and Delila sequences was used, which was shown to restore the red-fruited phenotype upon agroinjection in Del/Ros1 plants (Orzaez *et al.,* 2009).

The wealth of tomato wild species variation has only just begun to be explored and presents new and exciting opportunities for improving tomato flavour. Natural variation within the *Solanum* complex includes a number of species to include *S. pennellii*, *S. habrochaites*, *S. chmielewskii*, *S. pimpinellifolium*, *S. neorickii* allowing that can be crossed with the cultivated tomato (Tadmor *et al.,* 2002; Tieman *et al.,* 2006, Mathieu *et al.,* 2009). This variation provides an excellent genetic pool for researchers and a source of potential candidate genes to improve quality traits. The advent of next generation sequencing combined with advances in quantitative genetics, including the use of introgression lines, provides new opportunities to accelerate crop improvement and hopefully lead to tomato fruit with improved taste.

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Appendix

Table 8.1 RNA samples list for GeneChip. Developmental stage and plant ID included. Fruit material was collected from three different plants for each IL for each developmental stage.

RNA extraction number	PID	IL	Developmental stage	RNA concentration from Nanodrop (ng/µl)
1	92	M82D	Br+3	497.8
3	234	9-1-2	Br+3	795.9
5	57	9-1-2	Br+3	868.1
6	12	M82D	Br+3	690.9
9	303	11-2	Br	622.6
13	18	1-4	MG	612.7
16	13	12-1	MG	1312.2
17	29	M82D	Br+3	687.3
23	302	1-4	Br	754.8
31	64	11-2	MG	1839.6
32	102	12-1	MG	1474
38	198	12-1	Br	690.2
39	303	11-2	MG	1622.6
41	198	12-1	MG	729.4
43	234	9-1-2	Br	1411.7
45	206	11-2	Br	597.3
49	302	1-4	MG	515.5
53	206	11-2	MG	713.1
55	234	9-1-2	MG	2341.5
58	64	11-2	Br+3	670.7
68	198	12-1	Br+7	1534.4
69	216	1-4	Br+7	544.6
72	26	9-1-2	Br+3	597.6
76	29	M82D	Br	4856.5
77	102	12-1	Br	750.1
78	26	9-1-2	Br	825.8
82	206	11-2	Br+3	628.4
83	29	M82D	Br+7	694
85	13	12-1	Br+7	542.9
86	216	1-4	Br+3	537.5
87	216	1-4	MG	1021.8
88	102	12-1	Br+3	774.3
90	13	12-1	Br	778.5
91	12	M82D	Br	519.8
92	92	M82D	Br	543
93	92	M82D	Br+7	815.8
94	57	9-1-2	Br	544.8
96	216	1-4	Br	1301.1

98	29	M82D	MG	1730.7
109	57	9-1-2	MG	2396.5
110	234	9-1-2	Br+7	1193.7
111	198	12-1	Br+3	1486
112	12	M82D	MG	831
113	102	12-1	Br+7	1230.7
114	13	12-1	Br+3	1267.7
115	92	M82D	MG	2589.3
116	64	11-2	Br	820.4
121	18	1-4	Br	702.4
124	302	1-4	Br+7	781.3
125	26	9-1-2	MG	858.1
127	57	9-1-2	Br+7	987.4
128	303	11-2	Br+3	831
129	64	11-2	Br+7	619.3
134	302	1-4	Br+3	926.5
136	12	M82D	Br+7	889
138	18	1-4	Br+3	810.6
140	303	11-2	Br+7	732.2
141	18	1-4	Br+7	763.7
143	206	11-2	Br+7	813.2
145	26	9-1-2	Br+7	452.9

Table 8.2 Markers developed for IL1-4 including the name, start/end, length, annealing temperature, GC content, sequence and expected product size.

	Primer	Start /End	Length	tm	GC%	seq	Product size
1	TG237	7	20	59.84	45	ACGCCTGATGATTTGCTTCT	
		252	21	60.05	47.62	GCACGCTTTTGTCAAAGTCTC	**246**
2	TG528	88	20	59.82	50	CACCCATTCCCTTGACTGTT	
		226	20	59.45	40	AACCCAAGTTCGAAAAACCA	**139**
3	Solyc01g103040	5148	20	60.1	50	TTTGGTCCCCTTGCAGTTAG	
		6073	20	60.01	45	GGATCTTTTGCTTCCATCCA	**926**
4	Solyc01g103980	1499	20	60.23	40	TGCATCGTTGGTTCTTTCAA	
		2495	20	59.93	50	GGGGCTGAATGTGTAAAGGA	**997**
5	Solyc01g104260	99	20	59.93	50	GACAACCTCAGCCAATCCAT	
		1007	20	60.6	55	AGGATTCCACCAAGCTCTCC	**909**
6	Solyc01g104490	913	20	60.1	50	GCTGAAGCTGGCTTTCATTC	
		1904	20	60.03	45	TGATGCAGAACACACAGCAA	**992**
7	Solyc01g104850	2008	20	59.96	50	CCTTGTTCCTAACGGGTGAA	
		2945	20	60.03	50	GCCTGCTGTCTCACAAAACA	**938**
8	Solyc01g105130	1032	20	59.97	50	ATGTGGGCACAAGAGGAAAC	
		1917	20	59.95	55	GTCTGCGAGGATTTCAGGAG	**886**
9	Solyc01g105460	1513	20	60.1	50	TTATTGACCGCTAGCCAACC	
		2413	20	60.23	50	GATTTGCAGCCTCCACAGAT	**901**
10	Solyc01g105490	543	20	59.9	50	TGCAACAAGTGGGCAGTAAG	
		1404	20	59.87	55	CGCCCAAAGGTAAGAGACAG	**862**
11	Solyc01g105870	378	20	59.98	50	GCGATTTAACGAGCTGAAGG	
		1296	20	59.83	55	AGAGAGTTGGACGGACTGGA	**919**
12	Solyc01g106190	1394	20	59.9	50	CAGCACACAAGTTGCTGGAT	
		2281	20	59.79	50	GGGTGAACGGGACAAGAATA	**888**

Table 8.3 Panelists preference for three volatiles. Information presented below is for guidance only. Sample preference test was not run so the data below is not statistically significant. In the training session, panelists were presented with three samples: reference, middle sample and highly concentrated sample. They were asked to choose their preferred one among the three given. For E-2-pentenal: 60% preferred the top sample while 20% preferred reference and other 20% preferred middle sample. For citral: 70% preferred middle sample, 20% preferred reference and only 10% preferred top (high) sample. The test for E-2-hexenal was similar as above, but after initial test, another sample was presented, with middle concentrations of three volatiles: E-2-hexenal, E-2-pentenal and citral. They were asked to choose their preferred one among the three given. Initially 50% preferred reference, 40% middle sample, and only 10% top sample. After introduction of a sample with all three volatiles, panelists preference changed. 60% preferred a mixed sample, 20% preferred reference, and 20% preferred middle sample for E-2-hexenal.

		E-2-PENTENAL	
JUDGE	**PREFERENCE I**	**PREFERENCE II**	**COMMENTS**
1	SAMPLE (202) HIGH	N/A	Sharper taste
2	SAMPLE (202) HIGH	N/A	More tomato flavour
3	REFERENCE	N/A	
4	SAMPLE (202) HIGH	N/A	More fruity
5	REFERENCE	N/A	
6	SAMPLE (202) HIGH	N/A	Appley
7	SAMPLE (202) HIGH	N/A	
8	SAMPLE (851) MID	N/A	
9	SAMPLE (851) MID	N/A	Sharper, distinct, acidic, less mellow
10	SAMPLE (202) HIGH	N/A	Sharper taste
		CITRAL	
JUDGE	**PREFERENCE I**	**PREFERENCE II**	**COMMENTS**
1	SAMPLE (114) MID	N/A	Fruity, flowery
2	REFERENCE	N/A	
3	SAMPLE (114) MID	N/A	
4	SAMPLE (114) MID	N/A	
5	REFERENCE	N/A	
6	SAMPLE (114) MID	N/A	
7	SAMPLE (981) HIGH	N/A	lemongrass
8	SAMPLE (114) MID	N/A	Fresh note
9	SAMPLE (114) MID	N/A	
10	SAMPLE (114) MID	N/A	
		E-2-HEXENAL	
JUDGE	**PREFERENCE I**	**PREFERENCE II**	**COMMENTS**
1	Reference	503 (3 volatiles)	
2	237 (MID)	503 (3 volatiles)	
3	237 (MID)	237 (MID)	
4	Reference	503 (3 volatiles)	503-better flavour
5	182 (HIGH)	503 (3 volatiles)	503-sharper taste, but not too strong
6	Reference	Reference	
7	Reference	Reference	
8	Reference	503 (3 volatiles)	503-fuller flavour
9	237 (MID)	237 (MID)	
10	237 (MID)	503 (3 volatiles)	

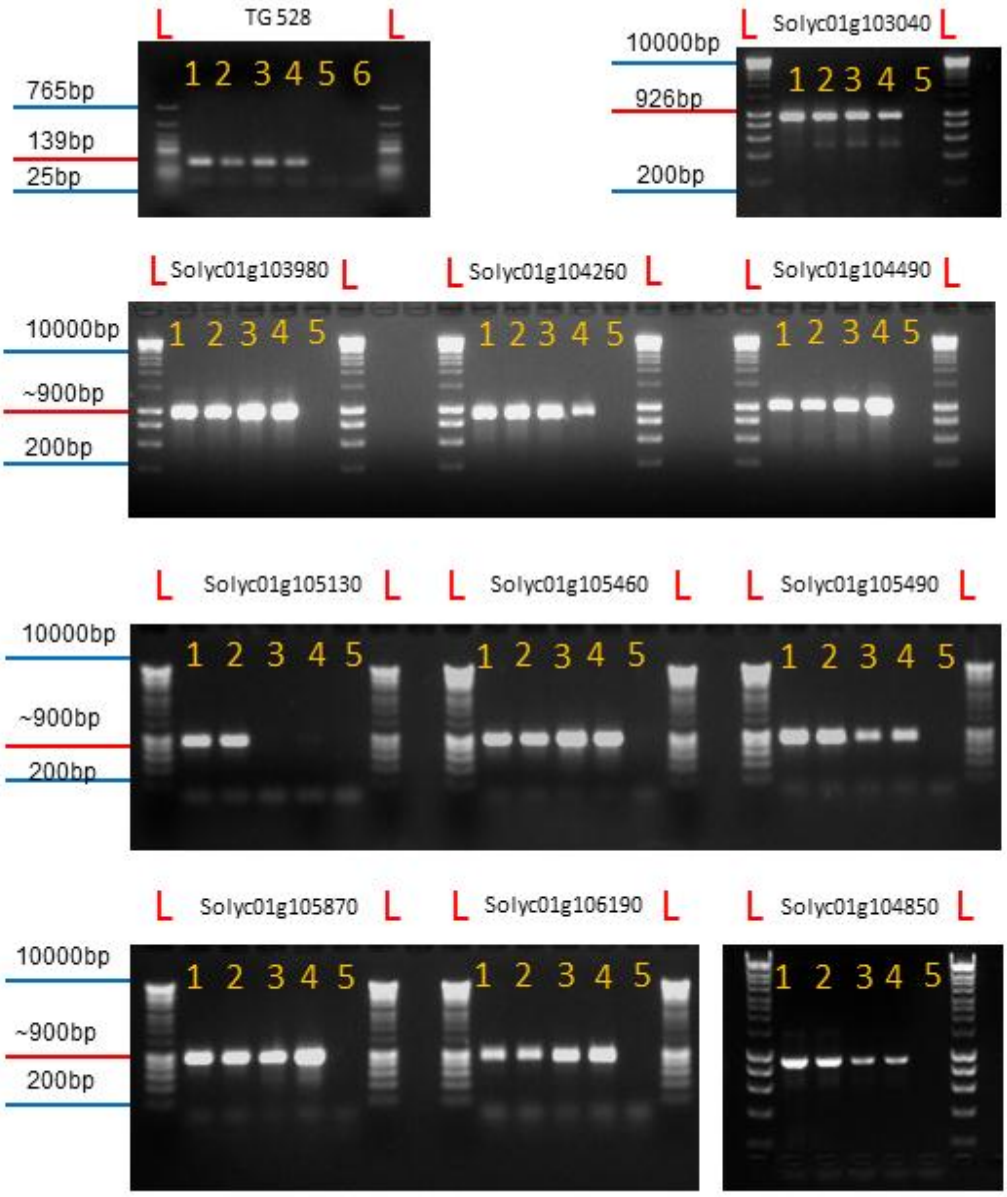

Figure 8.1 Agarose gels confirming expected PCR products for all the samples for all the markers. Number 1-2 M82D, 3-4 *S. pennellii* 5-6 PCR negative. *S. pennellii* did not amplify with Solyc01g105130 and this marker was not used in sequencing.

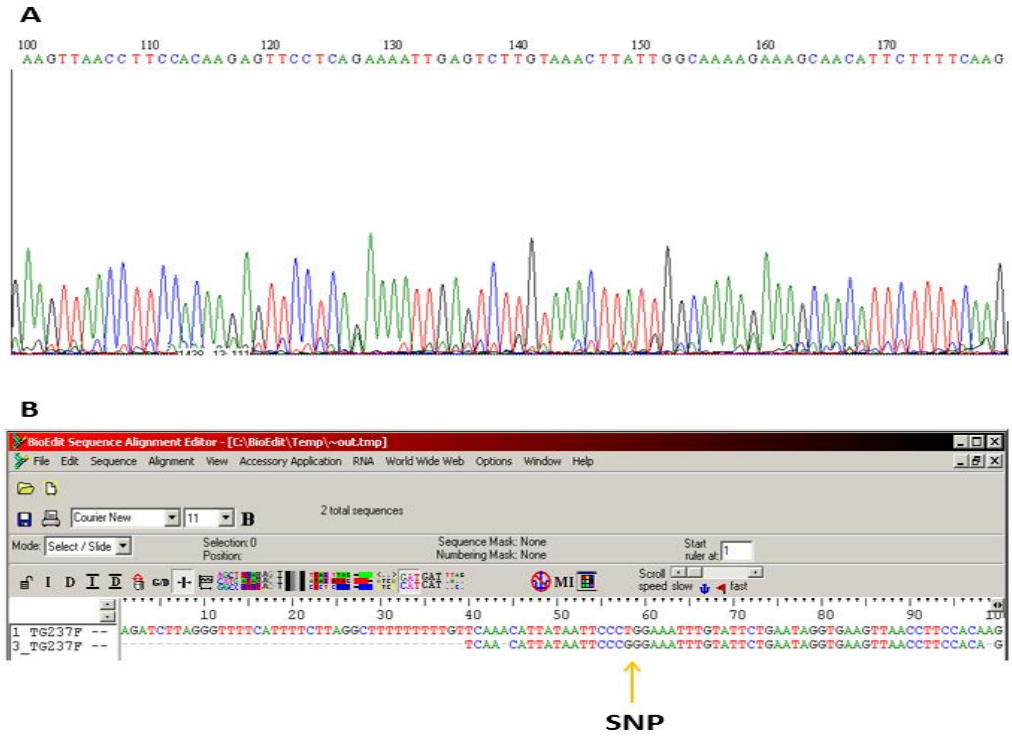

Figure 8.2 Sequence analysis using BioEdit. Quality of sequence can be inspected (A). Alignment of two parental sequences leads to identification of SNPs (B).

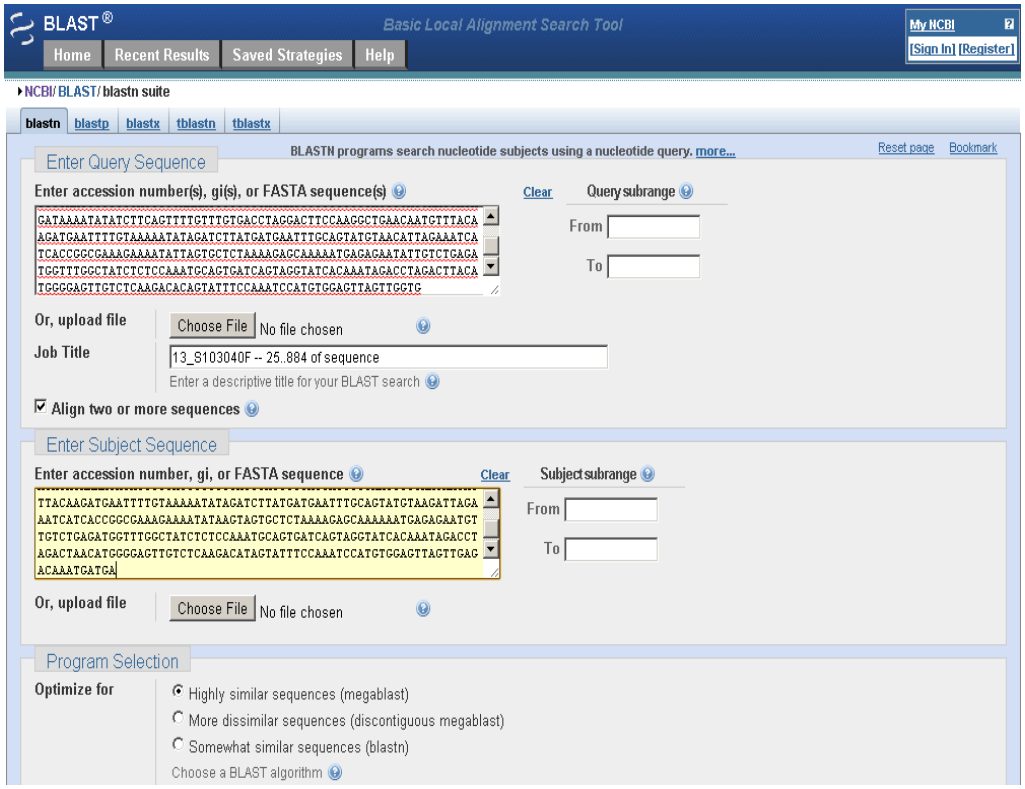

Figure 8.3 NCBI blast for larger sequences (800bp) to identify SNPs.

Table 8.4 Salts and solutions used for the preparation of the RNA extraction buffer

Salts and solutions	Weight and volume used
Cetyltrimethylammonum bromide (CTAB)	5 g
Polyvinylpyrrolidone K30 (PVP)	5 g
1M Tris hydrochloride (Tris-HCl) at pH 8.0	25 mL
0.5M Ethylenediaminetetraacetic acid (EDTA) at pH 8.0	1.25 mL
Sodium chloride (NaCl)	29.22 g
Water (top up to 250 mL)	
Total	**250 mL**

Table 8.5 Salts and solutions used for the preparation of the SSTE buffer

Salts and solutions	Weight and volume used
Sodium chloride (NaCl)	14.61 g
20% Sodium dodecyl sulphate (SDS)	6. 25 mL
1M Tris-HCl at pH 8.0	2.5 mL
0.5M Ethylenediaminetetraacetic acid (EDTA) at pH 8.0	0.5 mL
Water (top up to 250 mL)	
Total	**250 mL**

Table 8.6 Salts and solutions used for the preparation of the Speed DNA extraction buffer

Salts and solutions	Volume
1M Tris hydrochloride (Tris-HCl) at pH 7.2	200.0 mL
0.5M Ethylenediaminetetraacetic acid (EDTA) at pH 8.0	62.5 mL
4M Sodium chloride (NaCl)	50.0 mL
10% Sodium dodecyl sulphate (SDS)	50.0 mL
Sterile water	637.5 mL
Total	**1000.0 mL**