CHAPTER 2 : LITERATURE REVIEW

2.1 Tomato history

The tomato probably originated in Central and South America since prehistoric times and distributed most widely in Peru and Chili. The tomato was introduced into Southern Europe soon after the discovery of New World, America (Davies and Hobson, 1981). The cultivation of tomato originated in the coastal strip of Western South America (Andean region) and reached a fairly soon before being taken to Europe. The plant known as *tomatl* from the Nahua tongue of Mexico, became the origin of the modern name of tomato (Goodenough, 1990).

The first evidence of tomato plants in the Old World was published by the Italian herbalist, Pier Andrea Mattiolus in 1544. The fruit was described as a *Pomi d' oro* or *Mala aurea* (golden apple) which had golden rather than red colour and in France, *Pomme d' amour* (apple of love) (Davies and Hobson, 1981).

The acceptance of tomato for consumption was very slowly gained because of its relationship to poisonous like deadly night shade in several poisonous plants such as *Atropa belladonna* (Rick, 1978). This originally led people to believe that tomato was poisonous and inedible. Their consumption was frowned upon such superstitions persisted until the 20th century in many areas, including North America (Goodenough, 1990). For this reason, most tomato plants were grown as ornaments. When the fruit was accepted and popularized, it became heavily consumed in European countries and North and South America. The widespread use of tomatoes in the USA as a food began during the second half of the 9th century (Friedman, 2002). Although the nutrient content of tomato is not particularly high compared with other fruit and vegetable crops, tomato was ranked in sixteenth place. However, its contribution was ranked in the first place in total contribution of vitamins and minerals requirement according to the USA diet (Rick, 1978).

excellent scientific model for molecular study in the role of a wide range of ripening genes.

2.2 Botanical details

The tomato *(Lycopersicon esculentum)* is a member of the same family of the *Solanaceae*, as potato and tobacco. There are about 1500 tropical and subtropical species in this family. Within the *Solanaceae*, the genus *Lycopersicon* ("wolf peach" from Greek) consists of relatively few species and it is subdivided into two subgenera, the *Eulycopersicon* and the *Eriopersicon.* The characteristics of these subgenera are shown in Table 2.1 (Davies and Hobson, 1981).

According to the botanical aspect the tomato is designated as a fruit since it develops from the ovary of the flower after pollination and the seeds are formed within a fleshy mesocarp. Although the tomato is almost universally assumed to be a vegetable, it is actually a fruit, often called tomato fruit (Friedman, 2002).

Table 2.1 Characteristics of two subgenera of genus *Lycopersicon.*

Subgenera	Species
Eulycopersicon	*L. pimpinellifolium* (currant tomato)
- Glabrous at maturity, normally yellow or red colour.	*L. esculentum* (normal species cultivated)
	L. esculentum var. *cerasiforme* (cherry tomato)
Eriopersicon	*L. cheesmanii*
- Hairy, whitish-green colour with purplish stripes.	*L. peruvianum* var. *typicum*
	L. peruvianum var. *humifusum*
	L. hirsutum var. *glabratum*
	L. chilense
	L. chmielewskii
	L. parviflorum

2.3 Structure of fruit

The main divisions of tomato fruit can be divided as skin, pericarp and locular contents (Figure 2.1). The skin or exocarp consists of four or five layers of cells under a thin cuticle. The heavily cutinized outer surface is the epidermal layer, which is rather thick wall like the underlying collenchyma layer. The pericarp is divided into the outer wall, radial wall and columella inner wall. It becomes very large (100 to 500 μm) and thin wall during the fruit matures (Davies and Hobson, 1981). Vascular bundles radiate from the stem end of the fruit, both round the pericarp and down the columella to the blossom end. The locular cavities show as gaps in the pericarp and contain the seed embedded in a jelly-like parenchyma tissue, originating from placenta. The number of locules is depended on each variety, normally varies from two upwards.

In early stage of fruit development, the locules are filled by externally grown of placental cells, which engulf the seed and fill the entire locular cavity. The walls of these parenchymatous cells become thin and waxy in maturity stage and become jelly-like material in the locular cavities when fruit is designated mature green. In the red ripe fruit, the locular jelly is often seen as a pinkish tinge (Goodenough, 1990). Differences in composition between locular and pericarp tissues could have dramatic effects on flavour. Cultivars with large locular portion and with high concentration of acids and sugars are those which have been found to be a good flavour quality (Stevens *et al.*, 1977).

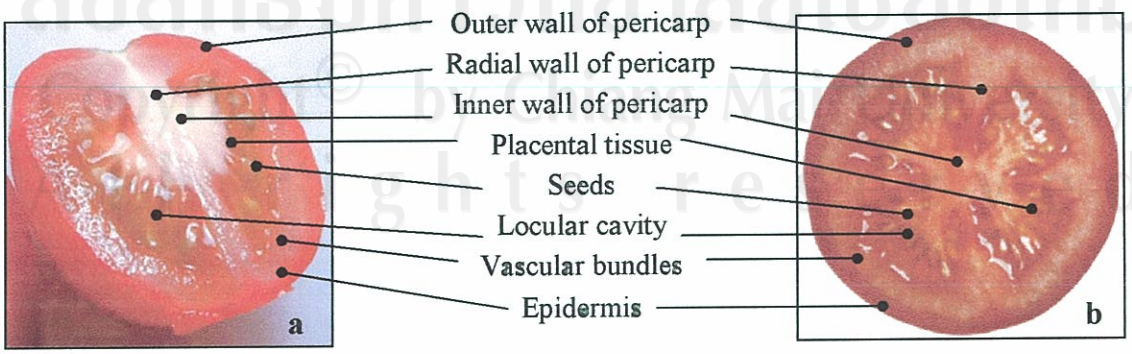


Figure 2.1 Cross-sections of (a) a bilocular and (b) a multilocular tomato fruit.

Tomato fruit is designated a climacteric fruit as apple, avocado, cucurbit, peaches, melon and banana. Its ripening is accompanied by a peak in respiration and a concomitant of ethylene biosynthesis (Alexander and Grierson, 2002). All cultivated tomatoes are self-pollination. Moreover, tomato plant is easy to grow with a short life cycle. In temperate countries with short growing season and sunlight limits, the cultivation of varieties is quick maturing and fruits tend to be small with few locules and uniform shape. On the other hand, in most countries, which have non-limiting of sunlight, it is possible to grow the larger and multilocular fruits (Davies and Hobson, 1981).

The suitable conditions for tomato growing and produce well flavoured fruit are intensively controlled at 12-16 hours of the daylight, similarly at day and night temperatures are 16-25°C and 11-18°C, respectively with 70-90% relative humidity (Goodenough, 1990).

2.4 Tomato production

Tomato is one of the most important vegetable crops in the world. It is the second only to citrus as a fruit crop. Tomatoes are used in many processed foods such as canned and sun-dried tomatoes, juice, ketchup, pastes, purees, salads, sauces and soups (Friedman, 2002). World production of the fresh and processed tomatoes is respectively valued at $3 billion and $13 billion annually. According to the FAO annual report, the world tomato production in 2002 was estimated at 108 million tons and China, the main producer of tomato production, produced 27.5% (FAO, 2003). The proportion of total world tomato production and yield in 2002 in some of the major tomato-producing countries are summarized in Table 2.2. While, the average world tomato yield in 2002 was 27.0 tons.ha^{-1}. The tomato yield of the leading country, China shown a very low yield (26.1 tons.ha^{-1}) compared to the United Kingdom (408.6 tons.ha^{-1}). These figures show the impact of new technology development in plant breeding, cultivation, postharvest physiology and molecular

characterized developmental mutants, relatively short life cycle and its economic importance as a crop species (Alexander and Grierson, 2002).

Table 2.2 Total world production and yield in 2002 in some of the major tomato producing countries.

Country	Production (million tons)	Yield (tons.ha^{-1})
China	25.47	26.1
USA	10.25	62.5
Turkey	9.00	40.0
India	8.50	17.0
Italy	7.00	53.8
Egypt	6.33	35.0
Spain	3.60	59.2
Brazil	3.52	56.4
Mexico	2.10	28.0
Greece	2.00	51.9
Russian	1.95	13.7
Thailand	0.24	23.8
United Kingdom	0.11	408.6

FAO (2003).

2.5 Tomato composition

The constituents of tomato fruit have been studied. The dry matter content of the ripe fresh fruit is between 5 and 7.5%. About half of this dry matter is in the form of reducing sugars and about 27% are in form of alcohol-insoluble solids. Other constituents are amino acids, pigments, vitamins, polyphenols and minerals (Davies and Hobson, 1981). The typical composition of a ripe cultivated tomato fruit is given in Table 2.3. Tomatoes contribute antioxidants, carbohydrates, fiber, flavour compounds, minerals, proteins, and vitamins to diet. Glycoalkaloids, N-containing secondary plant metabolites, involved in disease resistance in tomato plant, known as tomatine. In green tomatoes, tomatine concentration is about 48 mg kg^{-1} of fresh

Overall flavour of foodstuff is generally agreed to consist of volatile and nonvolatile compounds. Volatile compounds are detected in the nose and are responsible for aroma, while nonvolatile compounds are present in the mouth, which perceived by the tongue and adjacent tissues and responsible for taste (Taylor, 1996; Taylor and Linforth, 1996). The characteristic flavour of tomato is the complex effect of the many volatile and non-volatile components interaction, which beyond the individual component flavours.

Table 2.3 Typical compositions of ripe tomato fruit.

Component	% Fresh weight
Dry matter	6.50
Total carbohydrate	4.70
Fat	0.15
Protein, N x 6.25	0.40
Reducing sugar	3.00
Sucrose	0.10
Total soluble solids	4.50
Malic acid	0.10
Citric acid	0.20
Ascorbic acid	0.02
Fiber	0.50
Potassium	0.25
Calcium	0.15

Hobson and Grierson (1993).

2.5.1 Taste

The characteristic taste of tomato is mainly determined by the sweet-sourness. Sweetness induced by reducing sugars (glucose and fructose) and sucrose whereas sourness caused by the organic acid content (citric, malic and ascorbic acids). Bitterness is not a characteristic of ripe tomato taste, but coincidentally present caused by some phenolics and alkaloids, especially in green tomato. The free amino acids

high acids are being flavoured for consumer to diet (Hobson, 1981; Stevens, 1986). The perception of sweet and sourness is affected by levels of aroma compounds (Baldwin *et al.,* 1998).

2.5.1.1 Sugars

Sugars constitute between 65 and 70% of the total soluble solids in tomato fruit (Hobson and Grierson, 1993). In ripe tomato fruit, sugar content averages 3.25% (Davies and Hobson, 1981). The free sugars are almost entirely reducing sugars consisting of glucose and a slight preponderance of fructose. Sucrose in tomato fruit is rarely in amounts exceeding 0.1% of fresh weight. Sucrose is found the highest content in mature green stage and remained low throughout the ripening (Islam, 1997). Sucrose is rapidly converted by the enzyme invertase into glucose and fructose in the fruits. During ripening the sugar content rapidly increases and gradually decreases the glucose to fructose ratio. The total sugar content of ripe tomato fruit is between 1.7 and 4.7%, depending on the cultivars, and the environmental factors such as light and season. The more sunlight that reaches the fruit, the higher the sugar content (Petro-Turza, 1987). In a study of 55 divergent tomato lines, total solids content range from 4.13 to 6.83% and sugar content range from 1.66 to 3.99% (Stevens, 1972). Total sugar content was an important parameter to distinguish the quality levels of tomato in a model of quality assessment (Maul *et al.*, 2000).

2.5.1.2 Organic acids

The most abundant organic acids in tomato fruit are citric and malic acids (Davies and Hobson, 1981; Stevens, 1986; Goodenough, 1990). For typical cultivars, acid contents vary from 70-130 meq.L^{-1} (Stevens, 1986). Citric acid usually constitutes 40-90% of total organic acids, whereas malic acid ranges from 10-60% of that of citrate, depending largely on genotype, environment, fruit maturity and postharvest treatment. The potassium content of the soil or fertilizer most affects the

decomposition product of glutamine also occurs (Petro-Turza and Teleky-Vamossy, 1989).

In mature green tomato fruit, the predominant acid is malic acid, while citric acid forms only about 25% of total acidity. The malic acid contribution to the acidity falls quickly as the tomato fruit turn red, on the other hand, the concentration of citric acid rise to a peak as first stage of ripening and drops back at full ripening. Not only malic acid but also fumaric acid concentrations decreased significantly during later stages of ripening while citric, oxalic and succinic acids generally increased (Baldwin *et al.*, 1991a). The ratio of malic to citric acid rapidly decrease from above 1 in the mature green to 0.5 or below in the ripe red tomato fruit (Davies and Hobson, 1981). These changes involved in catabolism and anabolism of acids initiated of glycolytic and citric acid cycle enzymes including malate decarboxylating enzymes, which led to, a phenomenon called the malate effect (Goodenough, 1990). Buescher (1975) observed that malic acid concentration declined during chilling of tomato fruit at 2°C for 21 days, while citric acid tended to be increased.

The overall range of pH for different tomato cultivars is widely from 3.9 to 4.9. The pH of tomato is also concerning the effects of the quality and quantity of organic acids (Petro-Turza, 1987).

2.5.1.3 Free amino acids

Free amino acids formed about 2.0-2.5% of the total dry matter content in tomato. Glutamic, aspartic and γ -aminobutyric acids, together with glutamine compose 80% of the total free amino nitrogen-containing compounds. In fresh tomato, glutamic acid is the major part of free amino acids, contributed in tomato flavour. The concentrations of glutamic acid are particularly variable and range between 200-500 mg.100g^{-1} fresh weight depending on the nitrogen and phosphate contents in the soil or fertilizer. The higher nitrogen in the fertilizer has resulted in high glutamic acid level. During ripening, the quantity of only four of the amino acids (glutamic acid, asparagine, glutamine and alanine) increased (Kader *et al.*,

2.5.1.4 Minerals

Minerals constitute about 8% of the dry matter content of tomato fruit (Davies and Hobson, 1981). These inorganic constitutes are important to the taste of tomatoes and become of their buffering which effected on the pH and acidity of tomato fruit. Potassium is the predominant inorganic cation in tomato fruit, forming about 80-90% of the total amounts of cation. Potassium deficiency may result in poor colour and lower acid content in the fruit (Stevens, 1986). In addition, calcium is present in normal tomato fruit in limited amounts and adverse conditions of growth which will lead to deficiency symptoms in the form of "blossom-end" rot (Hobson and Grierson, 1993). Phosphorous, an anion forming about 50-60% of the total anions is also important to tomato taste as its buffering effect. During the development, growth and ripening of tomato fruit, the mineral content shows an increase in absolute value, but the concentration related to dry matter content remains unchanged (Davies and Hobson, 1981). An increase in fertilization with nitrogen and potassium reduced sensory flavour scores and increased levels of titratable acidity, soluble solids and several volatiles, including hexenal, 2-hexanone, benzaldehyde, phenylacetaldehyde, β-ionone and 6-methyl-5-hepten-2-one (Wright and Harris, 1985).

2.5.2 Aroma

2.5.2.1 Key volatile characterization

The volatile composition in the ripening tomato fruit has been studied extensively (Kazeniac and Hall, 1970; Buttery *et al.*, 1971; Buttery *et al.*, 1987; Buttery *et al.*, 1988a; Buttery *et al.*, 1988b; Buttery *et al.*, 1989a; Linforth *et al.*, 1994; Maneerat *et al.*, 2002). Although over 400 compounds have been identified as volatile components of fresh tomatoes and tomato products (Petro-Turza, 1987), but of these compounds only a small numbers have been studied and to be responsible for the key characteristic of fresh tomato aroma. Several reviews have indicated the following compounds play major volatiles in fresh tomato such as hexanal, *(Z)*-3-

fresh ripe tomatoes using GC-analysis with the dynamic headspace sampling method, along with their concentrations, odour threshold and log odour units were shown in Table 2.4 (Buttery *et al.*, 1989b; Buttery, 1993; Buttery and Ling, 1993).

In addition, methional, eugenol and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol) were further important adourants contributed to both fresh and processed tomato aroma volatiles (Guth and Grosch, 1999; Buttery *et al.*, 2001). Recent study by Bezman *et al.* (2003) found that the tomato matrix effects on the concentrations of the volatility of certain fresh tomato odourants in the headspace. *(E,E)*-2,4-decadienal, *β*-damascenone and *β*-ionone were particularly retained by the tomato matrix and hence their odour potency was significantly reduced.

2.5.3 Biogenesis of tomato aroma compounds

There are two classes of the production of volatiles in tomato fruit. The first class encompasses those volatiles that are direct metabolites produced in the intact plant tissue by intracellular biosynthetic pathways during the ripening period. The formations of carotenoid-derived, amino acids-derived and glycoside hydrolysis volatiles are the major routes involved in this class. The second class encompasses secondary products not occurring in intact cells, but formed during cell disruption by crushing, cutting and slicing. Lipid oxidation process is the major biogenesis pathway for volatiles generation in tomato fruit after maceration.

Biogenesis of volatiles in tomato fruit is a complex process involving other factors, which are ripeness dependent. It is well known that aroma formation in tomatoes requires certain biochemical pathways with different enzymes and substrates. Subcellular conditions like cellular stimulus, location of the reaction, availability of enzymes and substrates which are separated in different compartments of the cells, physical properties of fatty acids substrates and activity of enzymes are also important factors for generation and consequent liberation volatiles compounds (Yilmaz *et al.*, 2002).

Table 2.4 Tomato volatiles present in fresh ripe tomato, their concentration, odour threshold (in water) and log odour units.

Compound	Concentration (ppb)	Odour threshold (ppb, in water)	Log odour units[a]
(Z)-3-Hexenal	12,000	0.25	3.7
β-Ionone	4	0.007	2.8
Hexanal	3,100	4.5	2.8
β-Damascenone	1	0.002	2.7
1-Penten-3-one	520	1	2.7
3-Methylbutanal	27	0.2	2.1
(E)-2-Hexenal	270	17	1.2
2-Isobutylthiazole	36	3.5	1.0
1-Nitro-2-phenylethane	17	2	0.9
(E)-2-Heptenal	60	13	0.7
Phenylacetaldehyde	15	4	0.6
6-Methyl-5-hepten-2-one	130	50	0.4
(Z)-3-Hexenol	150	70	0.3
2-Phenylethanol[b]	1,900	1,000	0.3
3-Methylbutanol	380	250	0.2
Methyl salicylate	48	40	0.08
Geranylacetone	57	60	-0.02
β-Cyclocitral	3	5	-0.2
1-Nitro-3-methylbutane	59	150	-0.4
Geranial	12	32	-0.4
Linalool	2	6	-0.5
1-Penten-3-ol	110	400	-0.6
(E)-2-Pentenal	140	1,500	-1.0
Neral	2	30	-1.2
Pentanol	120	4,000	-1.5
Pseudoionone	10	800	-1.9
Isobutyl cyanide	13	1,000	-1.9
Hexanol	7	500	-1.9
Epoxy-*β*-ionone	1	100	-2.0

2.5.3.1 Amino acid-related volatile compounds

Amino acid metabolism generates aliphatic and branched chain alcohols, acids, carbonyls and esters that are important to the fruit flavour. Although, these compounds seem to be formed in the intact tomato during ripening process, there are very little different formations of these compounds after maceration. The concentrations of these compounds in the tomato fruit at different stages of ripening in the mature green, breaker and table ripe stage have been observed. It was found that the main formation of most volatile compounds seems to occur between the breaker and table ripe stages (Buttery and Ling, 1993). Valine, leucine, isoleucine, alanine, phenylalanine and aspartic acid could be serve as the precursors for the production of certain short chain carbonyl compounds (Yu *et al.*, 1967; Yu *et al.*, 1968a, 1968b, 1968c). Fresh tomato volatiles related to amino acids and their concentrations are listed in Table 2.5.

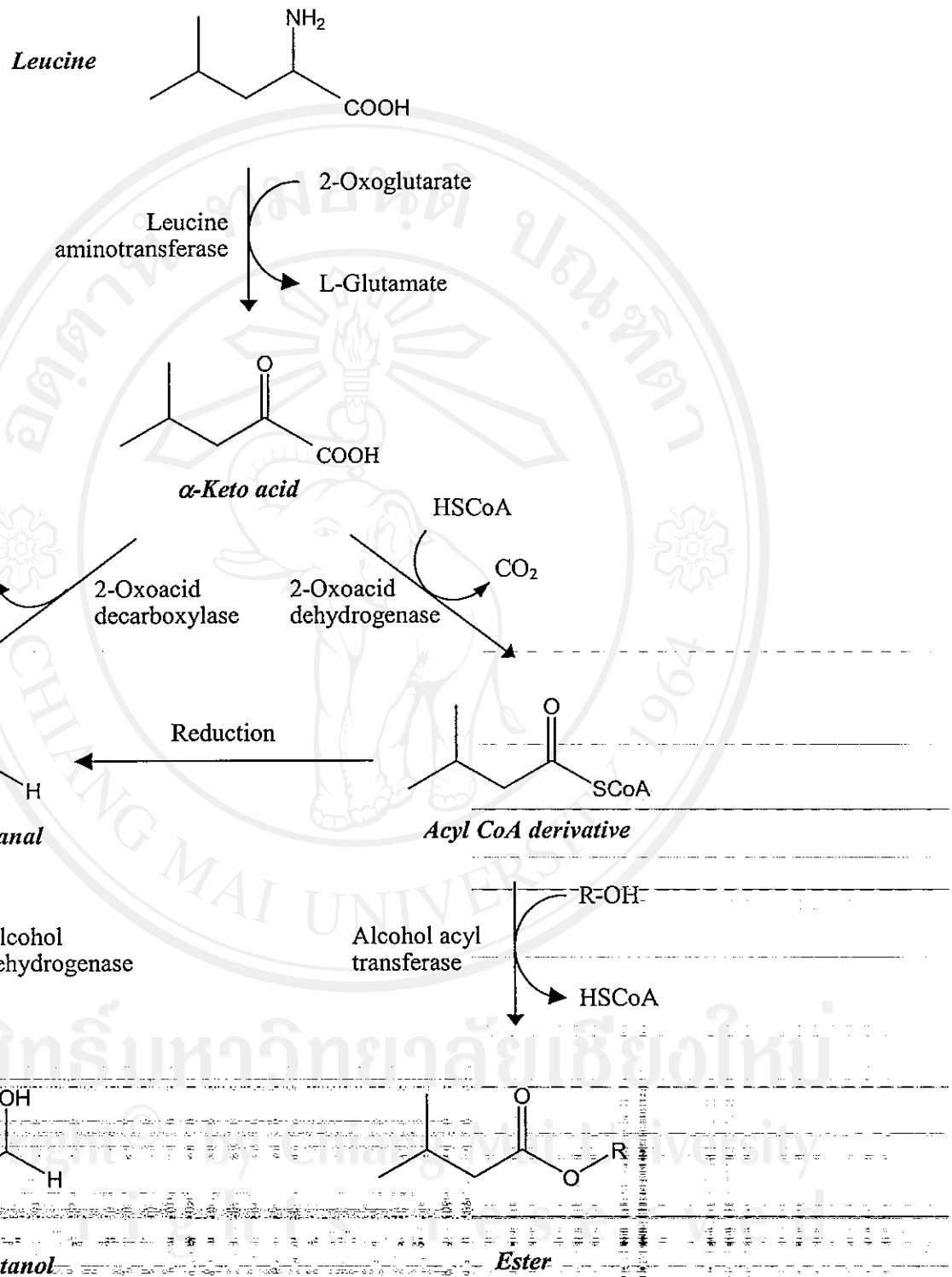
Table 2.5 Volatiles in fresh tomato fruit related to amino acids.

Amino Acid	Volatile compound	Concentration (ppb of 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-2-phenylethane	17-54
[illegible]	[illegible]	[illegible]

The biosynthetic pathway of 3-methylbutanal and 3-methylbutanol from leucine is shown in Figure 2.2. In the regular biosynthetic pathway, leucine is enzymatically deaminated to α–keto acid (4-methyl-2-oxopentanoic acid) by an amino transferase and then converted into aldehyde, 3-methylbutanal via at least two biochemical pathways. One possible route, α–keto acid is catalysted by 2-oxoacid decarboxylase. Other pathways, a keto acid is catalysted by 2-oxoacid dehydrogenase, resulting in 3-methyl-CoA derivative, which is converted into 3-methylbutanal, by acyl CoA dehydrogenase. The aldehyde in its turn can be converted into the corresponding alcohol by alcohol dehydrogenase and finally converted into various esters (van der Hijden and Bom, 1996; Bauchot *et al.*, 1998).

Nitro-compounds and nitriles forms do occur in tomato for the leucine and phenylalanine derived compounds. Biosynthetic pathway for nitro-compounds in tomato is possibly a variation of the known pathway to cyanogenic glycosides (Buttery and Ling, 1993). The usually accepted pathway for the formation of amino acid derived aldehydes in cooked foods is by Strecker degradation. However, in the fresh tomato these aldehydes are present at a relatively high concentration. One alternative pathway is by enzymatic hydrolysis of glycoside occurring during ripening. Another possibility is an enzymatic version of so called Nef reaction, which involves conversion of nitro-compounds to the corresponding aldehyde. The conversion of 1-nitro-2-phenylethane to phenylacetaldehyde via the Nef reaction at pH of 10.0 and 4.5 is given in Figure 2.3 (Buttery, 1993; Buttery and Ling, 1993).

Alkylthiazoles are well known in cooked products, but they are one of the few fresh foods in tomato fruits. 2-Isobutylthiazole is the only one that has been found to be important to the flavour of tomato. It occurs in the ripe tomato fruit at about 100-300 ppb and unable to detect any in the leaves or the other parts of the tomato plants (Buttery and Ling, 1993). The amounts of 2-isobutylthiazole did not appear to be dependent on the crushing procedure or on oxygen (Kazeniac and Hall, 1970). It is



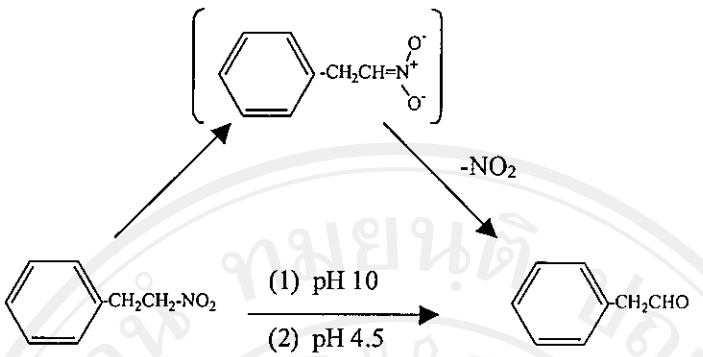


Figure 2.3 The ready conversion of 1-nitro-2-phenylethane to phenylacetaldehyde via the Nef reaction (Buttery, 1993).

2.5.3.1.1 Terpene-related volatile compounds

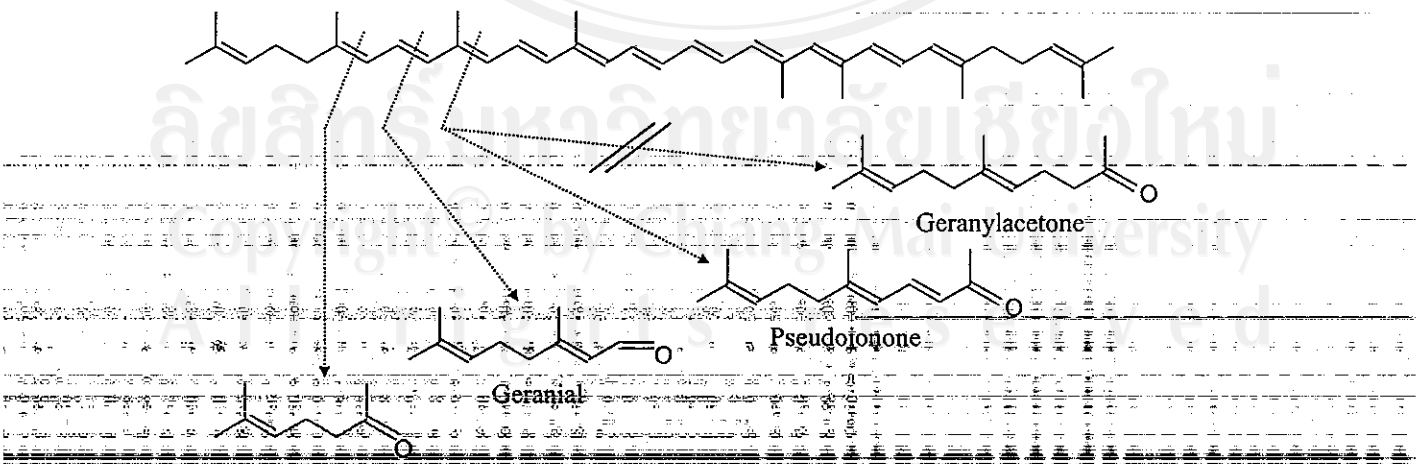
Many of the terpenoids are stored in plants as non-volatile glycoside. Terpenoid hydrocarbons occur in leaves and other parts of tomato plants, but not in the fruit. The postulated terpene formation is involved mevalonic acid, synthesized from acetate (Heath and Reineccius, 1986). The only C10 oxygenated terpenoids is formed from the breakdown of lycopene in the blend fresh fruit. There are linalool, neral and geranial. Linalool has commonly been formed in cooked fruits and a product of glycoside hydrolysis. The major terpenoids those are found in the blended leaves and fruit are listed in Table 2.6.

Table 2.6 Volatile C10 and C15 terpenoids in blended tomato leaves and fruit.

Compound	Concentration, ppb (leaves)	Concentration, ppb (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

2.5.3.1.2 Carotenoid-related volatile compounds

In the fully ripe tomato, the carotenoids consist of 50-80% lycopene and 2-7% β-carotene (Davies and Hobson, 1981). The oxidative decomposition of carotenoids leads to the formation of terpenes and terpene-like compounds. There are two types of carotenoid related compounds, linear and cyclic (Table 2.7). The comparison is also shown for the concentrations found in the intact tomato and those are formed in the macerated tomato. There seems to be a different principle mechanism for those compounds. 6-Methyl-5-hepten-2-one and geranylacetone are the main volatiles from lycopene degradation. The branched chain ketonic volatile compounds from thermal breakdown of lycopene are presented in Figure 2.4. The production of C9 to C13 cyclic compounds from β-carotene breakdown is shown in Figure 2.5. The C13 norisoprenoids-like, such as β-ionone and β-damascenone are generally regarded as carotenoid degradation products, resulting from an oxidative cleavage of the polyene chain in the 9 and 10 position (Winterhalter, 1992). β-Carotene is found in tomato homogenate with a very low concentration. β-Damascenone, one of the rose ketone compounds can be formed in fruits from hydrolysis of glycosides via an intermediate acetylenic compound of megastigm-5-en-7-yne-3,9-diol (Buttery and Ling, 1993; Pickenhagen, 1999). This compound has been identified as a major component volatile from thermal pH 4 hydrolysis of tomato glycosides (Kazeniac and Hall, 1970; Buttery and Ling, 1993).



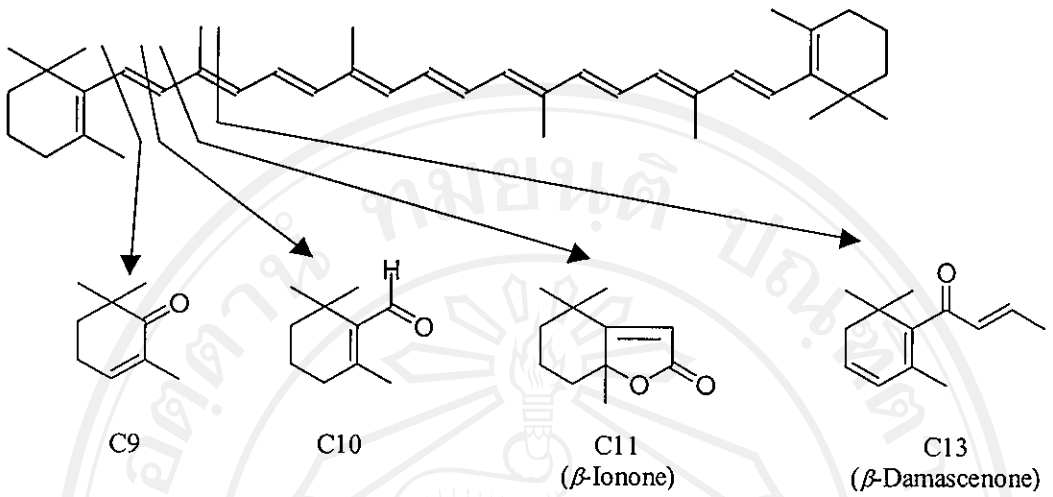


Figure 2.5 Oxidation of β-carotene resulting in the formation of C9 to C13 compounds (Winterhalter, 1992).

Table 2.7 Carotenoid-related volatile compounds in fresh tomato fruit.

Compound	Concentration, ppb (intact)	Concentration, ppb (macerated)
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-Trimethylcyclohexanone	<5	<5
β-Cyclocitral	3	5
β-Damascenone	<5	<5

2.5.3.2 Lignin-related volatile compounds

Although the lignin-related compounds are not numerically a major class of tomato volatiles, they should be considered. Aromatic amino acids, phenylalanine and tyrosine are formed by the Shikimic acid pathway, generated several aromatic lignin-related compounds such as benzaldehyde and cinnamaldehyde (Fisher and Scott, 1997). Other lignin-related compounds, eugenol, guaiacol (2-methyloxy-phenol) and methyl salicylate, have been identified in tomato volatiles (Kazeniac and Hall, 1970). Ferulic acid and *p*-coumaric acid are also main precursors of these compounds (Whitfield and Last, 1991).

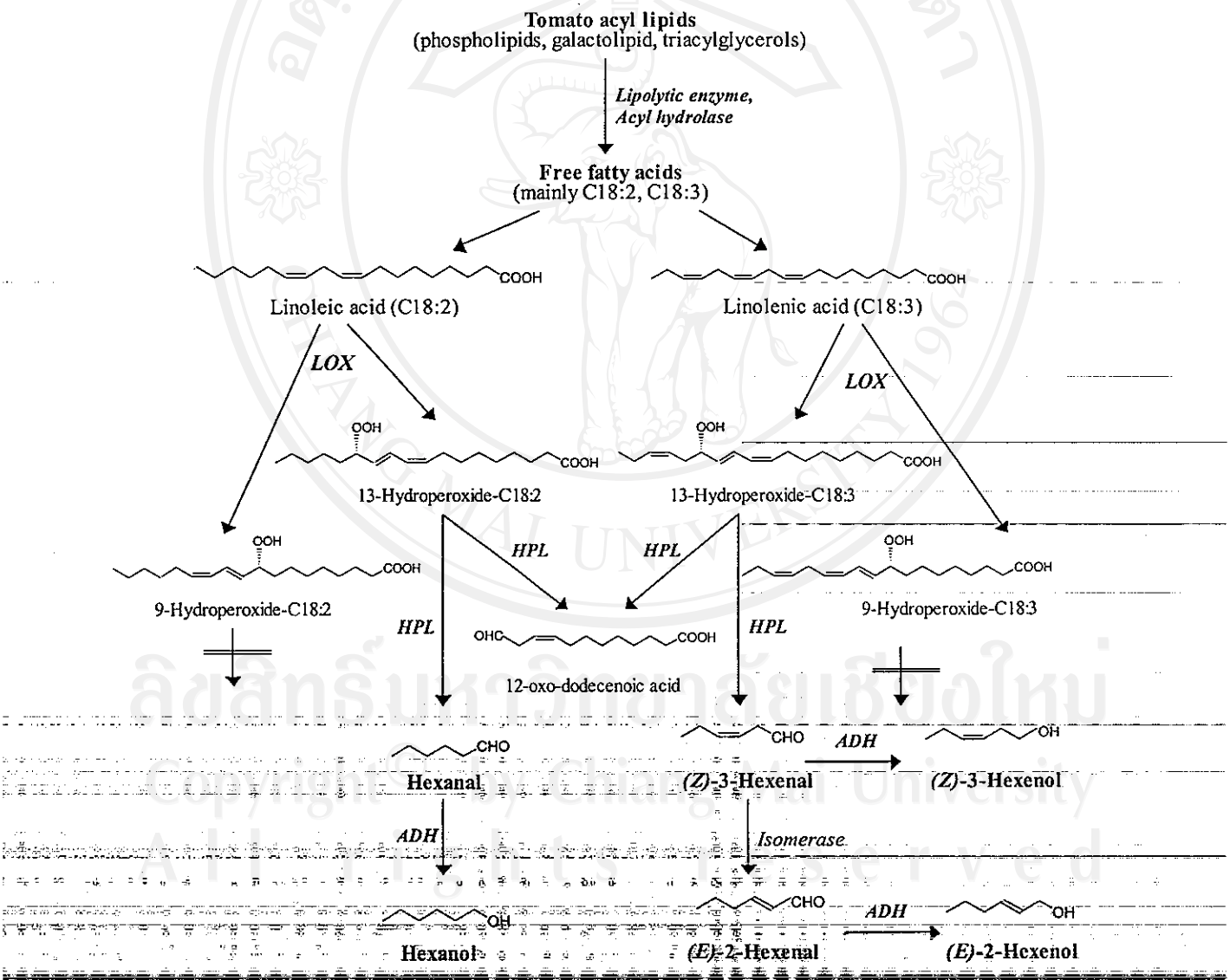
2.5.3.3 Lipid oxidation

The C6 aldehyde compounds are an important part of tomato flavor volatiles. They are the predominant compounds identified in both the blended leaves and tomato fruit (Buttery and Ling, 1993). These compounds are principally derived from unsaturated fatty acids. The lipid oxidation pathway for the formation of carbonyl compounds in disrupted tomato fruit is well known and has been proposed by Galliard and co-workers (1977) (Figure 2.6).

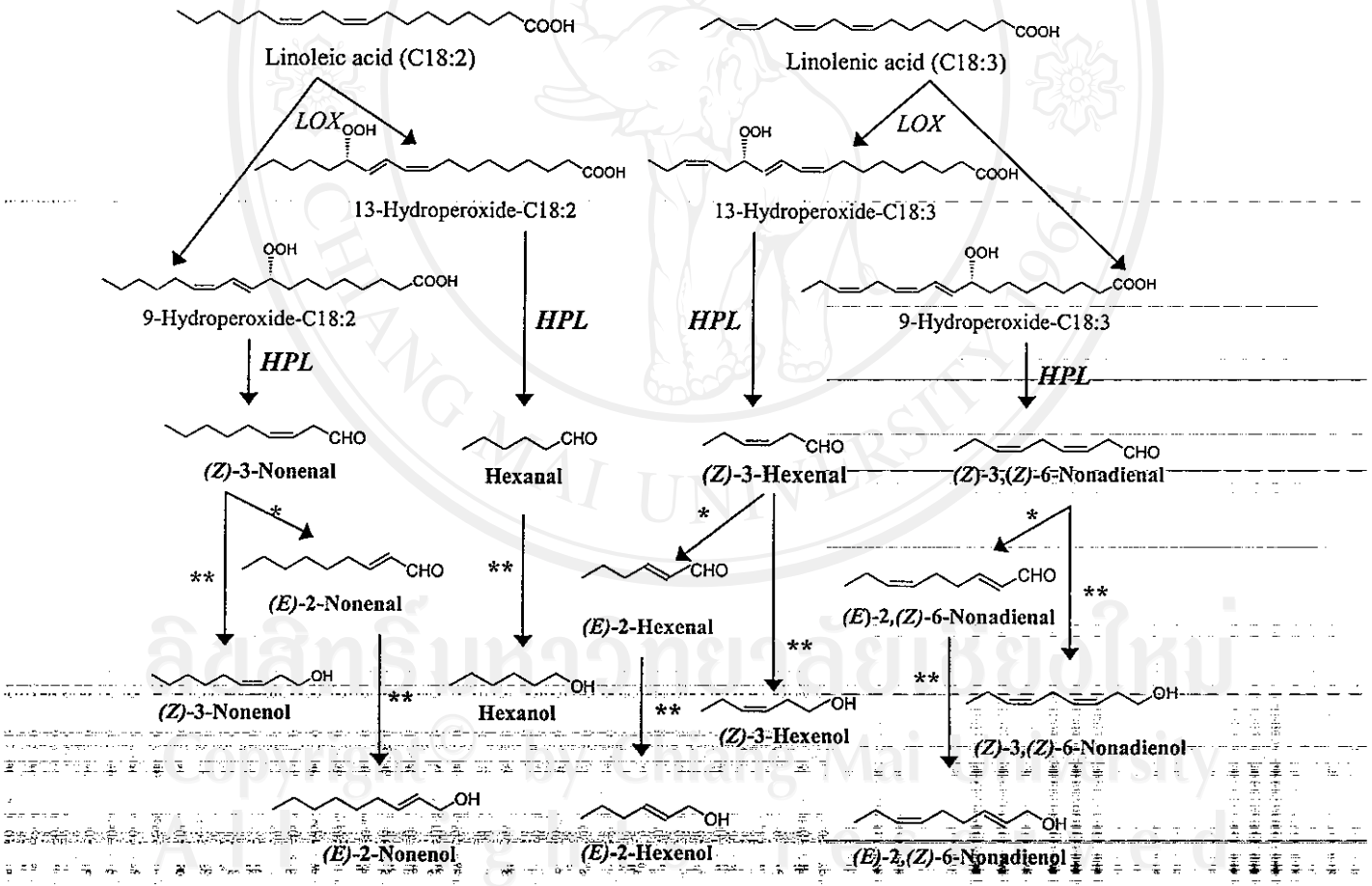
In fresh ripe tomato, lipid content has been shown to be about $1 g.kg^{-1}$. About 50% of total lipid content was found to be phospholipid/phosphatidyl choline and phosphatidyl ethanolamine (1:1), with free and acylated-sterol-glycosides (about 18%) and galactosyl diglycerides (about 16%) as the other two most important groups of components. Within these groups the polyunsaturated fatty acids, particularly linoleic acid (40%) and linolenic acid (26%) were found to predominate (Galliard *et al.*, 1977). During tomato tissue disruption, these free fatty acids are released by the action of hydrolytic and oxidative enzymes and are further converted by lipoxygenase (LOX) into the 9- and 13-hydroperoxides of both linoleic and linolenic acids. The formation of 9-hydroperoxides is favored over the 13-hydroperoxides by 24:1 by tomato LOX (Galliard and Matthew, 1977; Regdel *et al.*, 1994). Although, LOX favors the formation of 9-hydroperoxides, 13-hydroperoxides are cleavaged by

(Grechkin, 1998). The specific enzyme for 9 hydroperoxides is not present in tomato tissue, and the fate of the 9-hydroperoxides is unknown (Hatanaka *et al.*, 1992).

Hexanal is produced on the cleavage of 13-hydroperoxide linoleic acid while *(Z)*-3-hexenal is the volatile cleavage product when 13-hydroperoxide linolenic acid is the substrate. *(Z)*-3-hexenal is rapidly converted into a *(E)*-2-hexenal by isomerase. In most plant *(E)*-2-hexenal rather than *(Z)*-3-hexenal is found (Eriksson, 1979). These aldehydes can then be further converted into hexanol and hexenol by the action of alcohol dehydrogenase (ADH) (Figure 2.6).



The production of C6 and C9 aldehydes in cucumber, the pathway being found to be similar to that in tomato. An important characteristic between volatile formation in cucumber and tomato fruit is that in the former, both 9- and 13-hydroperoxides of both linoleic and linolenic acids are cleaved to yield of the C9 and C6 aldehydes, respectively (Figure 2.7). While, in tomato fruit only 13-hydroperoxide of linoleic and linolenic acids are specifically cleaved by HPL, giving rise to C6 aldehydes alone. *(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal are responsible for an odour of fresh cucumber. These aldehydes were found to increase stepwise during ripening stage and the increase occurred when the cucumbers were half ripe (Sekiya *et al.*, 1977).



2.5.3.4 Other pathways

Ethanol and acetaldehyde were the first two volatile compounds to be detected in tomato (Petro-Turza, 1987). The amount of ethanol in tomato volatile was rather lower than, when fresh, firm ripe tomato with handle quickly (Kazeniac and Hall, 1970). The pathway of these volatiles involved lactate and pyruvate by a combination of the action of lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) under oxygen stress is described in Figure 2.8. In lactic acid fermentation, pyruvate is converted to lactate by LDH, whereas in ethanolic fermentation, pyruvate is converted to acetaldehyde by PDC and subsequently converted to ethanol by ADH. In many plant species, both lactate and ethanol have been shown to accumulate during hypoxia and anoxia (Tadege *et al.*, 1999).

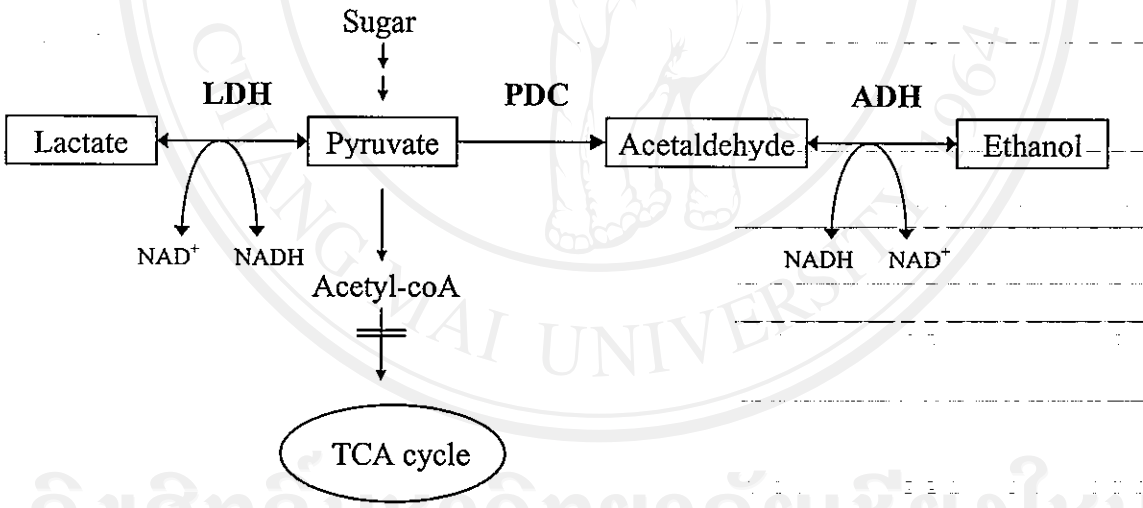


Figure 2.8 Metabolic routes of acetaldehyde and ethanol formation (Tadege,1999).

2.5.3.5 Ripening and storage

Effects of ripening and storage on tomato volatiles have been studies

to be the most important factor in determining levels of volatiles produced (Stern *et al.*, 1994). Tomato fruit stored at chilling temperature before ripening at 20°C were generally low in volatile content and loss of characteristic tomato-like flavour (Kader *et al.*, 1978a). Two tomato cultivars (cvs. Sunny and Solar Set) were significantly increased in the following *(Z)*-3-hexenol, *(E)*-2-hexenal, acetaldehyde, hexanal, acetone, 6-methyl-5-heptenone, geranylacetone and 2-isobutylthiazole during ripening (Baldwin *et al.*, 1991b). Tomato fruit stored at 5, 10, and 12.5°C were significantly low in ripe tomato aroma and flavour compared to those stored at 20°C when analyzed by trained sensory panelists (Maul *et al.*, 2000). However, storage at recommended temperatures of 10°C to 13°C might have a significant effect on tomato flavour even before any visual injury symptoms expressed. The mechanism of volatiles decrease is not known, but it could be related to reduce ethylene synthesis at low temperature (Baldwin *et al.*, 2000). A study by Ishida *et al.* (1993) showed that the synthesis of flavour volatiles in the calyces of tomato fruit were similar to those of ripening tomato fruit.

2.6 Biochemical and physiological changes during tomato ripening

The ripening of tomato fruit corresponds to a series of biochemical, physiological and structural changes, aimed in making the fruit attractive to consumers and thereby increasing chances for seed disposal (Hoeberichts *et al.*, 2002). These changes, although variable among species, generally include modification of cell ultrastructure and texture, conversion of starch to sugars, increased susceptibility to postharvest pathogens, alterations in pigment biosynthesis and accumulation, and heightened levels of flavour and aroma volatiles.

2.6.1 Changes in cell ultrastructure and chemical compositions

Tomato ripening can be considered as several changes in fruit's attributes

these changes, there is a breakdown of α-tomatine, a steroidal-glycoalkaloid, by tomatinase. Mature green tomatoes had more 100 times of tomatine levels than the red tomatoes (Friedman, 2002). The soluble solids content of ripening tomatoes is largely a reflection of the sugar content. During maturation, substantial amounts of sugars are transported to the fruit from the leaves in the form of sucrose. The breakdown of sucrose, which is probably mediated by the action of invertase, converted to fructose and glucose. Glucose is either synthesized to starch, or stored as hexoses depending on the stage of fruit development (Grierson and Fray, 1994). There is an increase in sweetness as starch is degraded and glucose and fructose are accumulated. The concentration of glutamic acid also increases from the onset of ripening, as does the ratio of citric acid to malic acid together with a general acidity decrease (Grierson and Kader, 1986). Sugar and acid profiles mainly determine for fruit taste. High sugars and relatively high acids are required for the best flavour. High acids and low sugars will produce a tart tomato while high sugars and low acids will result in a bland taste. When both sugars and acids are low, resulting a tasteless, insipid tomato (Grierson and Kader, 1986). Finally the fruit softens, primarily as a result of soluble pectin increases, consequently cell wall softening and degradation (Tucker and Grierson, 1982). Volatile compounds making up the tomato aroma complement the taste components to give the overall flavour of the whole fruit. More than 400 compounds have been identified and those compounds contribute to the odour, but no single or simple combination of compounds has a smell reminiscent of the ripe fruit (Hobson and Grierson, 1993).

2.6.2 Changes in gene expression during tomato ripening

The physiological changes in tomato fruit are the result of the co-ordinated expression of many genes. Analysis of proteins synthesized *in vitro* translation provides the first evidence for changes in mRNA during tomato ripening (Rattanapanone *et al.*, 1978). Further investigation showed that several abundant mRNAs in green fruit disappear and several mRNAs appear as ripening begins and

related genes (Gray *et al.*, 1994). Although ethylene is the dominant trigger for ripening in climacteric fruits, it has been suggested that both ethylene-dependent and ethylene-independent gene regulation pathways co-exist to co-ordinate the process in climacteric and non-climacteric fruits (Alexander and Grierson, 2002).

Two systems of ethylene regulation have been proposed to operate in climacteric fruits. The first system includes functional during normal vegetative growth, ethylene autoinhibitory and responsible for producing basal ethylene levels that are detected in all tissues and non-climacteric fruits. The second system operates during the ripening of climacteric fruits and senescence of some petals when ethylene production is autocatalytic (Alexander and Grierson, 2002).

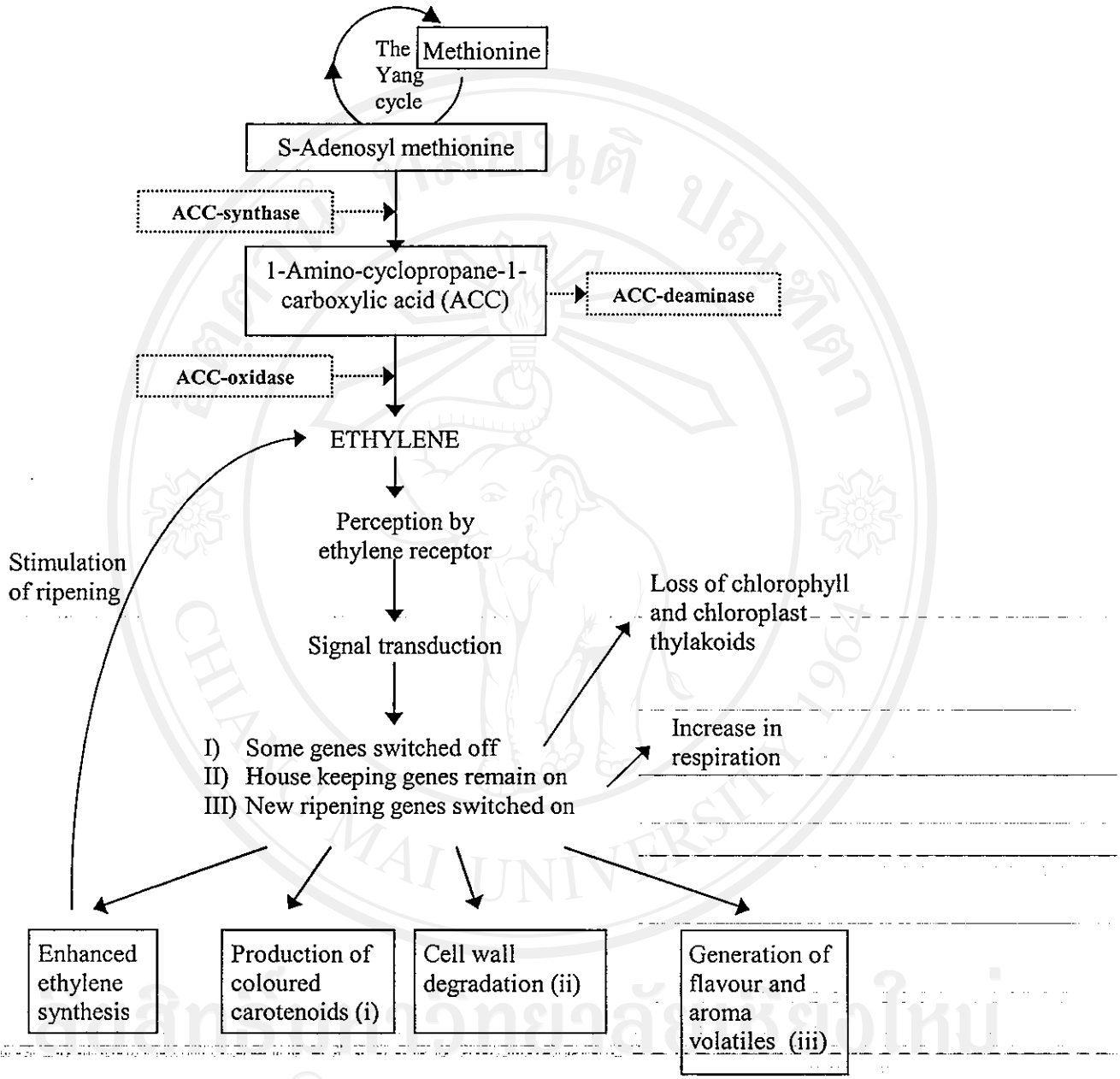
Schematic representation of the interaction between ethylene biosynthesis and tomato fruit ripening is shown in Figure 2.9 (Gray *et al.*, 1994; Grierson, 1998). Genetic manipulation in transgenic tomato plants can be achieved by the reduction levels of ethylene biosynthesis by expression of antisense ACC-oxidase or ACC-synthase genes or by sense suppression ACC-deaminase gene. Other ripening related processes have been manipulated in transgenic tomato plants include:

(i) the cell wall degradation enzymes (PG, PME, TBG and EXP),

(ii) an enzyme involved in the carotenoids production (PSY) and

(iii) enzyme involved the flavour volatile generation (LOX and ADH).

These led to the concept of ripening as being controlled at partially and the level of gene expression.



ACC-oxidase	Phytoene synthase (PSY)	Polygalacturonase (PG)	Lipoxygenase (LOX)
ACC-synthase		Pectinmethyl esterase (PME)	Alcohol dehydrogenase (ADH)
ACC-deaminase	Dehydrogenase	β-Galactosidase (TBG)	
		Expansin (EXP)	Hydroperoxide lyase (HPL)

2.6.2.1 Colour development

The colour development of red ripe tomato fruit is due to the deposition of lycopene and β–carotene, which are associated with the change from green to red colour. These are resulted from transformation of chloroplasts to chromoplast.

Carotenoids are formed by the condensation of two molecules of geranyl-geranyl pyrophosphate (GGPP), the ubiquitous isoprenoid precursor, to produce phytoene, which catalyzed by the enzyme phytoene synthase. A series of dehydrogenation reactions, carotenoid by phytoene and ζ–carotene desaturases, converted phytoene into lycopene (Hobson and Grierson, 1993). These observations suggested that the pTOM5 gene product is involved in the production of carotenoid pigments during tomato fruit ripening. The formation of the *Psy* gene was confirmed by the production of transgenic plants with altered levels of *Psy* expression (Bird *et al.*, 1991; Fray and Grierson, 1993). Treatment of tomato fruit with 1-methylcyclo-propane (1-MCP), a potent inhibitor of ethylene production, delayed colour development by decreasing the mRNA abundant of phytoene synthase 1 (PSY1) (Hoeberichts *et al.*, 2002).

2.6.2.2 Cell wall softening

During ripening partial disassembly of the fruit cell wall is largely responsible for softening and textural changes. As ripening progresses, the cell wall becomes increasingly hydrated as the pectin rich middle lamella is modified and partially hydrolyzed. The changes in cohesion of the pectin gel govern the ease which one cell can be separated from another, which in turn affects the final texture of the ripe fruit (Alexander and Grierson, 2002). These changes are thought to be brought about a wide range of cell wall degradation enzymes such as polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase (TBG), and expansin (Exp) (Grierson *et al.*, 1985; Hall *et al.*, 1993; Brummell *et al.*, 1999; Carey *et al.*, 2001).

A cDNA clone (pTOM6) encoding the cell wall metabolizing enzyme, PG was first cloned and sequenced by Grierson *et al.*, 1986, and subsequently the

and PG2b (Bird *et al.*, 1988). Analysis of low ethylene transgenic tomato plants has been shown that induction of PG-mRNA occurred at very low ethylene levels (Sitrit and Bennett, 1998). Although PG activity in homozygous transgenic plant lines was reduced to 1% of the normal value, the ripe fruits were only slightly firmer leading to the conclusion that PG is not the main determinant of tomato fruit softening (Grierson and Schuch, 1993). The softening rate of PG-antisense transgenic tomato was not significantly inhibited during ripening but was retarded in the over ripening stage. This study showed that tomato softening involved several biochemical processes. The action of PG is just one part of a joint attack on the cell wall mediated by different enzymes (Quiroga and Fraschina, 1997).

Pectin methylesterase is responsible for de-esterification of the highly methyl-esterified polygalacturonans in the cell wall. PME is present as a small gene family in tomato, some members of which are highly homologous. PME protein is formed in most plant tissue with the isoform being specific to fruits: PME1 (Hall *et al.*, 1993). Exo-galactanase and β-galactosidase activities are thought to be responsible for the loss of galactosyl residues from the cell wall of ripening tomatoes. β-Galactosidase (TBG1), encoding by a gene family at least seven members, displayed in different patterns of expression during fruit ripening. This enzyme is responsible for poly-merization of polymeric galactose within the cell wall (Carey *et al.*, 2001).

Cell wall localized enzymes, expansin results in the cell wall loosening by reversibly disrupting the hydrogen bonds between cellulose microfibrils and matrix polysaccharides (Cosgrove, 2000). In tomato, the expansin gene (LeExp1) showed ripening-related accumulation of mRNA and protein. The transgenic silencing of the expression of this gene results in tomato fruit, which is significantly firmer than corresponding controls throughout ripening (Brummell *et al.*, 2002). There are at least six different expansin genes expressed during tomato fruit development (Brummell *et al.*, 1999).

2.6.2.3 Volatile production

maceration. Thus, the flavour of the fruit depends on the complex interaction of sugars, organic acids and more flavour compounds. More than 400 compounds, have been identified including phenols, hydrocarbons, ethers, aldehydes, alcohols, ketones, esters, lactones, sulphur containing compounds, amines and varieties of heterocyclic compounds (Petro-Turza, 1987). Nevertheless, only small numbers of these compounds is among the most important contributors to tomato aroma. These flavour volatiles are formed by different pathways such as deamination and decarboxylation of amino acids and carotenoids during fruit ripening such as 3-methylbutanal, 3-methylbutanol (Yu *et al.*, 1968c). The C6 aldehydes and alcohols (hexanal, hexenal, hexanol and hexenol) are formed by lipid oxidation of unsaturated fatty acids when tissue is disrupted (Galliard *et al.*, 1977).

In tomato fruit, lipoxygenase (LOX) plays a major role for generation of key tomato volatiles via lipid oxidation pathway. Linoleic and linolenic acids are the main substrate of LOX for forming of aldehyde volatile compounds. The biogenesis of these volatile compounds is formed via a sequential active of enzymes consisting of hydrolysis of lipids by lipolytic acyl hydrolase (LAH) to form free fatty acids, oxygenization of free fatty acids by LOX to form fatty acid hydroperoxides (HPOs) and cleavage of these HPOs to produce short chain aldehydes and oxo-acids by fatty acid hydroperoxide lyase (HPL).

There are at least five LOX genes in tomato fruit, namely *TomloxA*, *TomloxB* (Ferrie *et al.*, 1994), *TomloxC*, *TomloxD* (Heitz *et al.*, 1997) and *TomloxE* (NCBI Accession AY008278). Among these, only three LOX genes (*TomloxA*, *TomloxB* and *TomloxC*) express in the fruit but in different functions during tomato fruit ripening (Griffiths *et al.*, 1999a). Kausch and Handa (1997) reported that the 94-kD LOX is a fruit ripening-specific LOX, which is expressed mainly in pericarp and radial walls of red-ripe tomato fruit.

Levels of *TomloxA*-mRNA decrease as ripening progresses and this is delayed in mutant tomatoes (*Nr* and *rin*) as well as ACO1 sense suppression transgenic tomato (Griffiths *et al.*, 1999a; Griffiths *et al.*, 1999b). This indicated that LOX genes are

TomloxB expression increases during ripening stage. This gene is regulated by ethylene, due to the expression is reduced in mutant and low ethylene transgenic tomato fruits.

TomloxC is a fruit-specific gene with maximum expression at the breaker and red ripe stages. This gene is regulated by ethylene. However, ethylene treatment of mature green fruit does not induce its expression (Griffiths *et al.*, 1999a).

TomloxD expressed in leaves but not in fruit and its expression was increased in wounded leaves. The gene product of *TomloxD* may be involved in defense signaling in response to herbivore and pathogen attack by forming a component of octadecanoid signaling pathway. These considerations indicate that LOX appears to have a dual role in fruit development, including a defense component and contributors to aroma and flavour generation (Griffiths *et al.*, 1999a).

The studies of *TomloxA* and *TomloxB* antisense transgenic tomatoes revealed that levels of *TomloxA* and *TomloxB* were successfully down-regulated. However, it resulted in little changes in flavour profiles and did not alter the levels of *TomloxC*–mRNA. These findings suggest that either very low level of LOX is sufficient for the generation of C6 aldehydes or that a specific isoform such as *TomloxC* in the absence of *TomloxA* and *TomloxB* is responsible for the formation of these volatile compounds (Griffiths *et al.*, 1999b).

Alcohol dehydrogenase (ADH) has also been shown to play an important role for generation of hexanol and hexenol by interconversion of aldehydes into alcohols in ripening tomato fruit. There are two isoforms of ADH genes in tomato. ADH1 expressed only in pollen, seeds and young seedling, while ADH2 accumulated during the later stages in ripening fruit (Chen and Chase, 1993).

Genetic manipulation of ADH2 levels in ripening tomato fruit has been shown to affect the balance of some flavour aldehydes and alcohols. Fruits with increased ADH2 levels had a more intense "ripe-fruit" flavour (Speirs *et al.*, 1998). Tomato fruit with enhanced ADH2 activity had significantly increased level of ADH activity up to three times the activity of control plant whereas down-regulation of ADH

Moreover, the study of overexpression of the yeast Δ–9 desaturase gene in tomato showed that changes in the levels of fatty acids not only monoenoic fatty acids but also polyunsaturated fatty acids in tomato fruit and leaves. This result could be led to change in their profile of flavour compounds (Wang *et al.*, 1996; 2001).

2.7 Tomato genetic engineering

2.7.1 Genetic modification of ripening in transgenic tomato fruit

Tomato has long served as the primary model for climacteric fruit ripening, due to its relatively small genome, well-characterized developmental mutants, ease of genetic manipulation, relatively short life cycle and its economic important as a crop species (Gray *et al.*, 1994; Giovannoni, 2001; Alexander and Grierson, 2002). The application of molecular biology techniques to study of tomato ripening, such as mRNA synthesis, cDNA cloning and sequencing and gene identification led to major progresses in the study of gene regulation in transgenic plants. Five genes involved in changes in colour, texture and ethylene synthesis have now been manipulated in transgenic tomatoes either by inhibition or over expression (Table 2.8).

Table 2.8 Inhibition of ripening gene expression in transgenic tomato fruit.

Gene	Function	Important
Polygalacturonase	Cell walls	Longer shelf life (better flavour) Improved processing Enhanced disease resistance
Pectinesterase	Cell walls	Improved processing
Phytoene synthase	Carotenoids	Enhanced colour and vitamin A
ACC oxidase	Ethylene	Ripening control
ACC synthase	Ethylene	Prevented over-ripening Longer shelf life and reduced losses

Grierson and Fray (1994).

transgene of similar or identical DNA sequence. There are two main ways of inactivating plant gene. The first involves constructing an antisense gene to a cloned and identified gene and transferring it to the target mRNA. The antisense RNA operates post-transcriptionally and interferes with normal mRNA accumulation from homologous target gene by forming a RNA-RNA helix, which is rapidly degraded (Hobson and Grierson, 1993). The reduction in mRNA accumulation thus caused led directly to decrease in the amount of the target protein synthesized. Antisense genes have been shown to be stably inherited and to act in a gene-dosage-dependent fashion. However, there is still some doubt as to exactly how they bring about their effect.

The first publication-demonstrating stable down regulation of an endogenous gene in tomato fruit using an antisense PG gene was reported by Smith *et al.* (1988). Transgenic tomato with the PG antisense gene produced only 1% of the normal amount of PG. The degradation of this protein that is associated with normal ripening was greatly reduced (Smith *et al.*, 1988; Smith *et al.*, 1990b). Subsequently other genes have been silenced by down regulation of genes involved in ethylene biosynthesis using antisense transgene for ACC-oxidase (Hamilton *et al.*, 1990; Picton *et al.*, 1993), ACC-synthase (Oeller *et al.*, 1991), and ACC-deaminase (Klee, 1993). Moreover, the inhibition of pectinesterase has also been achieved using the antisense PE gene in transgenic tomato (Hall *et al.*, 1993). Transgenic tomato fruit containing pectin methylesterase antisense gene had greatly reduced levels of PME activity below 10% compared with normal tomato cultivars Rutgers (Tieman *et al.*, 1992). Phytoene synthase, encoded by cDNA clone TOM5 which involved the biosynthesis of carotenoid in tomatoes was inhibited by using antisense-TOM5 gene resulting yellow fruit and pale flowers (Bird *et al.*, 1991). Current study of the manipulation of ethylene synthesis in cantaloupe Charentais melons using ACC-oxidase antisense showed that ethylene production of transgenic melon was less than 1% of control untransformed fruit (Ayub *et al.*, 1996).

The second method is called sense-suppression or co-suppression where expression of endogenous genes can be inhibited by homologous sense constructs

generally renamed posttranscriptional gene silencing (PTGS) (Fagard and Vaucheret, 2000) or RNA interference (RNAi) (Hannon, 2002) because posttranscriptional RNA degradation can affect a wide range of transgenes expressing plant, bacterial or viral sequence. This silencing mechanism is known as "homology dependent gene silencing" (HDGS). It can be found at transcriptional or posttranscriptional level (Meyer and Saedler, 1996). Several models for co-suppression or HDGS have been proposed and it should be note that these models are still hypothetical. Nevertheless, these models might help in the development of new experimental strategies for elucidating the mechanism of co-suppression (Meyer, 1995).

There are several examples of sense-suppression for inactivation of homologous gene sequence in plants. Ethylene production has been inhibited by sense-suppression of ACC-oxidase and ACC-synthase (Lee *et al.*, 1997; Hamilton *et al.*, 1998) in transgenic tomatoes. Hamilton *et al.* (1998) reported that 96% of tomato plants transformed with 35S-ACC-oxidase sense gene containing two additional upstream-inverted copies of its 5′ untranslated region has reduced ACC-oxidase activity compared to wild-type plant. Alternatively, the interaction of truncated sense transgene, containing the PG-sense gene in tomato plants was inhibited in a substantial reduction in PGmRNA and enzyme accumulation (Smith *et al.*, 1990a).

Most of the genetic modification in tomato involved either the use of antisense or the posttranscriptional type of sense-suppression (Grierson, 1998). These evidences suggested that gene silencing both sense and antisense transgenes has become a common approach and useful tool in plant molecular biology and successfully application for plant improvement in the modern agriculture (Meyer, 1995).

2.7.2 Commercial applications of transgenic tomato fruit

The processes of fruit ripening, such as texture change, colour production and hormone synthesis have all been manipulated genetically. Several commercial products based on these modifications have already reached the market place.

modified ripening and processing characteristics. These have been quickly followed by several field crops such as corn and soybean (Dunwell, 1998).

FLAVR SAVR™ tomato was the first GM-plant food to enter the market and this first sold by Calgene Inc, of Davis, California under the MacGregor's trade name in the USA in May 1994. This is a low PG fresh market tomato, produced by antisense PG gene (Redenbaugh *et al.*, 1992; Kramer and Redenbaugh, 1994). The claimed benefits relate to the alterations in texture caused by low PG, which enables the tomato to be left to ripen longer on the plant before harvesting. The Food and Drug Administration (FDA) approved that the FLAVR SAVR™ tomato is as safe as tomatoes bred by conventional means (USDA, 2001). However, these tomatoes have been currently withdrawn from the markets. Low PG tomato was also used to produce GM-tomato purée with commercially desirable characteristics such as higher viscosity and higher solid contents (Schuch *et al.*, 1991; Porretta *et al.*, 1998). GM-tomato purée was produced by ZENECA and their collaborators. These products sold in Sainsbury and Safeway supermarkets in the UK since 1996 (Grierson, 1998).

Apart from these researches involved plant molecular biology and gene technology can greatly improve understanding of how plant function. They will be important for offering advantages to growers, retailers and consumers and generate new opportunities for enhancing the quality and nutritional values of food plants.

2.8 Measurement of tomato volatiles

2.8.1 Sensory evaluation methods

2.8.1.1 Odour threshold

Determination of odour threshold of components in water solution was an early method adopted by many researchers for evaluating the relative contribution of food aroma components (Buttery, 1999). The concept of utilizing odour threshold

value of a compound, also called the "odour unit" (U_o) is defined as the ratio of a flavour compounds concentration in the aroma extract to its odour threshold (Buttery, 1999; McGorrin and Gimelfarb, 2001):

$$U_o = \frac{\text{Compound concentration}}{\text{Odour threshold}}$$

Compounds with high U_o have been found by sensory panels is quite important to the flavour of foods. Odour unit > 1 is indicative of compounds present at a concentration that greatly exceeds their thresholds, and therefore is likely to contribute flavour impact. The logarithm of the odour unit ($\log U_o$) is calculated to represent changes in concentration, which are significant of olfactory discrimination. Consequently, logarithmic functions more significantly represent meaningful sensory differences. This technique has been successfully used to determine key flavour volatile compounds in tomato by determination of individual odour threshold in water and calculation of odour unit (Buttery, 1993).

2.8.1.2 Gas chromatography-olfactometry

Gas chromatography-olfactometry (GC-O) is an identified method of flavour volatile compounds. It combines GC to separate volatiles with a human nose as a detector. Since investigation of aroma must differentiate between odour active and non-odour active compounds, GC-O has become essential to study of flavour chemistry.

Several techniques have been developed to apply GC-O data and to estimate the sensory contribution of single aroma component. GC-O techniques such as AEDA (aroma extract dilution analysis) (Guth and Grosch, 1999) and CHARM (combined hedonic analysis response measurement) analysis (Acree, 1993) are often applied to elucidate the key odourants in isolated flavours.

CHARM analysis indicated the odour activity of eluting volatile by GC-O of serial dilution of extract obtained from the food sample and the outputs are expressed

The result obtained for each odourant using AEDA technique is expressed as flavour dilution (FD)-factor, reflecting the ratio of the concentration of the odourant in the initial extract to its concentration in the most dilution extract where an odour can be detected by GC-O. The dilution factor is represented for each individual as an indication of the strength of the odour in AEDA. AEDA technique was applied to evaluate the potent odourants in fruits and vegetables such as tomato fruits (Buttery, 1999) and cucumbers and muskmelons (Scieberle *et al.*, 1990). In fresh tomato, *(Z)*-3 hexenal showed the highest FD factor using AEDA analysis. This result correlated that the log U_o of *(Z)*-3 hexenal exceeded the log U_o of the other volatiles occurring in fresh tomato fruit (Buttery, 1993).

However, AEDA and CHARM analysis are rather time-consuming and require repetitive analyses. These techniques are also limited to odourants having a higher boiling point than the solvent used for extraction and used for dilution step. Moreover, quantitative data for statistical analyses are difficult to attain with such methods, but valuable indication can be obtained (Guth and Grosch, 1999).

2.8.2 Instrumental methods

2.8.2.1 Gas chromatography-mass spectrometry

Flavour research has benefited tremendously from the development of GC. This is advanced greatly in the mid-1960s, when GC became readily available to the flavour chemists. Only 500 compounds of food flavour, identified in food lists found by 1963. Fifteen years later this number had increased to over 3000 and over 7000 compounds have been identified to date (Reineccius, 2002).

GC is now the most widely used and ideally suitable method in flavour research. It has excellent separation power and extreme sensitivity. However, GC is still a time-consuming technique.

2.8.2.2 Electronic nose

Electronic nose (EN) is a sensor-based instrument capable of detecting volatile compounds present in the headspace over a sample of interest. The instrument

sensors to electrical signals into the brain. Finally, the software analysis requests the brain itself. The EN is therefore analogous to that of the human olfactory sensing system (Hodgins, 1997). This technique is particularly attractive for food quality control applications. However, this technique is relatively young and the inherent weakness of such instrument should be considered (Reineccius, 2002). An application of EN for study aroma volatile profiles from ripe tomatoes effected by physiological maturity at harvest has been carried by Maul and co-workers (1998).

2.8.2.3 Atmospheric pressure chemical ionization-mass spectrometry

In the flavour analysis of many fruits and vegetables, major changes in flavour can occur after harvesting due to the rapid metabolic changes and some volatile compounds are immediately formed when tissue is disrupted. In plant breeding programs typically produce many samples in a short time for analysis. A rapid technique for measuring the flavour volatile compounds is necessary for such situations.

Conventional GC-MS is still slow making analysis of large numbers of samples an unrealistic task. To overcome the slow analysis times associated with GC-MS method, an alternative is to introduce the mixture of volatile compounds directly into MS and resolve their ions entirely by mass. This gives a real time analysis and it possible to monitor the dynamic of volatile release in headspace directly with a millisecond time delay (Taylor and Linforth, 2003).

2.8.2.3.1 Introduction to APCI/MS

Atmospheric pressure chemical ionization (APCI) is a novel form of mass spectrometry. It was originally developed as an analytical technique for analysis of trace components in the gas phase (Taylor *et al.*, 2000). The technique of APCI is different from other ionization methods because sample ionization occurs at atmospheric pressure and outside the vacuum system (Mitchum and Korfmacher, 1983).

The concept of ionization process at atmospheric pressure for mass

first commercially available APCI/MS system for bioanalytical application by plasma chromatography-mass spectrometry (Carroll *et al.*, 1975). The use of commercially APCI-MS (TAGA™ 2000, Sciex Inc., Toronto, Canada) for analysis of trace components in breath reported by several researchers (Lovett *et al.*, 1979; Benoit *et al.*, 1985). This new technique has been provided real time analysis of volatile introduced into the interface, which designed and published in a European patent (Linforth and Taylor, 1998) application and in a US. Patent (Linforth and Taylor, 1999). At the present time, APCI-MS system is now commercially available as the MS-Nose™ by Micromass, Manchester, UK (Taylor *et al.*, 2000). This technique has been successful that APCI and electrospray ionization (ESI) are now among the most popular techniques for identification and quantification of several volatile and non-volatile compounds simultaneously in pharmaceutical, biomedical and environmental analysis. It is also a very rapid method that requires virtually no sample preparation.

2.8.2.3.2 Principles of ionization

APCI-MS is a gas-phase ion-molecule reaction process leading to the ionization of sample molecules under atmospheric pressure condition (Bajic *et al.*, 1993). This technique is a "soft" ionization method that comes little fragmentation of the compounds and produces predominantly protonated molecular ions by addition or abstraction of proton (Taylor and Linforth, 2000).

A schematic diagram of an APCI-MS system is presented in Figure 2.10. Sampling of trace amounts of volatiles in a carrier gas may be directly from air or from a gas or liquid chromatography effluents (Mitchum and Korfmacher, 1983). Samples flow directly into the ionization chamber at atmospheric pressure, as comprised with a pressure of 1 torr of chemical ionization (CI) or 10^{-4} torr for electron impact (EI) (Watson, 1997). Sample molecules are ionized by mechanisms depend on the reagent gas (e.g. N_2, O_2, H_2O, etc.). Primary ionization is initiated either by the high energy of β–radiation emitted from a ^{63}Ni foil or by a corona discharge needle. The low energy electrons ionize a reagent gas that through a complex series of

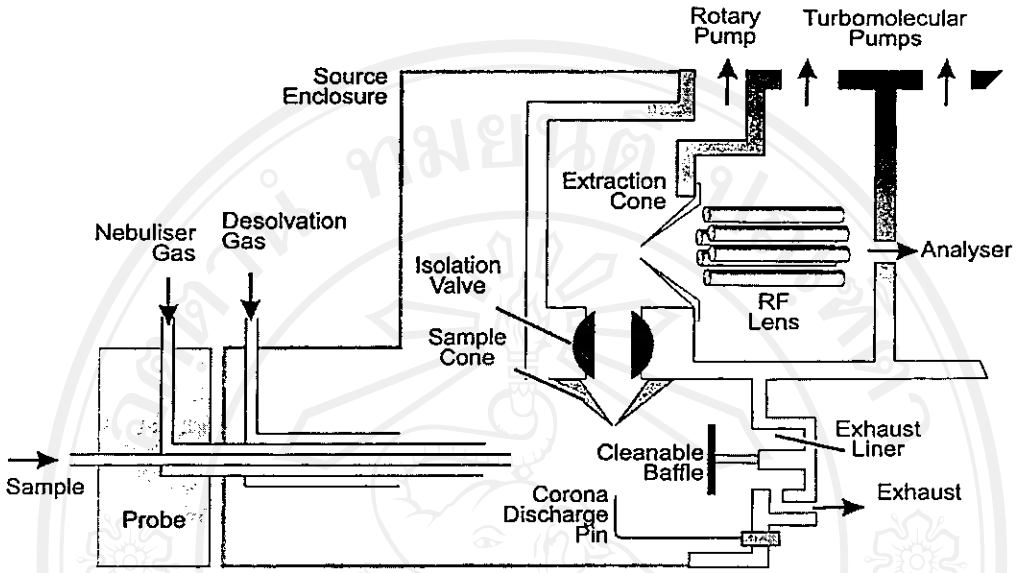


Figure 2.10 **Schematic diagram showing the principle components of APCI** (Bajic *et al.,* 1993).

In the positive ionization mode, a series of protonated water clusters $[H^+(H_2O)_n]$ are generated from the moisture in the ambient air following a series of ion-molecule reactions initiated by the corona discharge (Benoit *et al.*, 1983). A common cascade of reactions occurring in the presence of moisture in ambient air, nitrogen gas and the high voltage corona discharge is the following (Carroll *et al.*, 1981):

$$N_2 + e \rightarrow N_2^{+\cdot} + 2e \quad (1)$$

$$N_2^{+\cdot} + 2N_2 \rightarrow N_4^{+\cdot} + N_2 \quad (2)$$

$$N_4^{+\cdot} + H_2O \rightarrow H_2O^{+\cdot} + 2N_2 \quad (3)$$

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + HO^{\cdot} \quad (4)$$

$$H_3O^+ + H_2O + N_2 \rightarrow H^+(H_2O)_2 + N_2 \quad (5)$$

Ionization of a sample molecule (M) produces protonated molecule-water clusters $[M + H)^+(H_2O)_n]$ of varying size depending on "n" (reaction 7). Upon passage of a clustered target ion through a gas (N_2) curtain located between the ion chamber and the mass analyser, collisions with the neutral nitrogen molecules activate the ion sufficiently to break up (decluster) the loosely held water molecules and a protonated molecular ion $[(M + H)^+]$ is formed (reaction 8) and transmitted to the analyzer (Benoit *et al.*, 1983):

$$M + H^+(H_2O)_n \rightarrow (M + H)^+(H_2O)_n \quad (7)$$

$$(M + H)^+(H_2O)_n + N_2 \rightarrow (M + H)^+ + (n)H_2O + N_2 \quad (8)$$

Most sample molecules in positive mode are ionized by addition of a proton to give $(M + H)^+$. Conveniently, water is an excellent choice for the reagent molecule as its proton affinity lies between those of the main components of air (O_2, N_2 and CO_2) but below that of most volatile organic compounds. This means it will transfer its charge to flavour volatiles but it will not interact with the component in air. Thus, water is an essential component for ionization to occur, whereas with electron impact (EI) and chemical ionization (CI), it can interfere with the ionization process (Taylor *et al.*, 2000).

In the negative mode of operation the reagent ion O_2^- is generated by a corona discharge ionization of oxygen in ambient air. The usual observed reactions with the sample molecule are charge transfer and proton abstraction (reaction 9 and 10):

$$M + O_2^- \rightarrow M^- + O_2 \quad (9)$$

$$M + O_2^- \rightarrow (M - H)^- + HO_2 \quad (10)$$

The development of techniques utilizing APCI has pioneered the coupling of liquid chromatography (LC) with MS, particularly by mean of electrospray ionization (ESI). In liquid phase analysis with APCI, liquid is [illegible]

spray and rapidly evaporated and converted to a vapour/gas at the stainless steel capillary probe tip by a coaxial nitrogen steam and heating the nebulizer to high temperature (350-500°C). Although these temperatures may degrade the analyses, the high flow-rates and coaxial nitrogen flow prevent breakdown of the molecules. Hot gas from the probe passes between the sample cone and the corona discharge pin, which is typically maintained at 4.0 kV. The spray expands into the atmospheric pressure interface where desolvation assisted by pure nitrogen, which is introduced in concurrent flow to the spray, decreases the droplet size.

The ionization in an APCI interface consisted of the standard Z-spray source fitted with a corona discharge pin and a heated nebulizer probe. The electrospray nebulization is performed orthogonally to the sampling cone. Ions are extracted orthogonally from the spray into the sampling cone, while large droplets and nonvolatile materials are collected onto a baffle plate. Subsequently, the ions are extracted orthogonally from the expansion behind the sampling cone into the vacuum of the mass spectrometer (Niessen, 1999).

The ionization in an ESI interface is considered primarily a liquid-phase ionization technique. It consists of the standard Z-spray source fitted with an electrospray probe. Mobile phase from the LC column or infusion pump enters through the probe and is pneumatically converted to an electrostatically charged aerosol spray. The solvent is evaporated from the spray by means of the desolvation heater in the atmospheric pressure chamber assisted by a countercurrent of pure nitrogen. Charge-preserving solvent evaporation results in a decreasing droplet size, which is accompanied by an increasing electric field at the droplet surface until it explodes. Since coulomb repulsion between the surface charge exceeds the cohesive forces. These are giving smaller droplets that will undergo the same fate (Marquet and Lachatre, 1999). The resulting analyses and solvent ions are then drawn through the sample cone aperture into the ion block where they are then extracted into the mass analyzer (Figure 2.11).

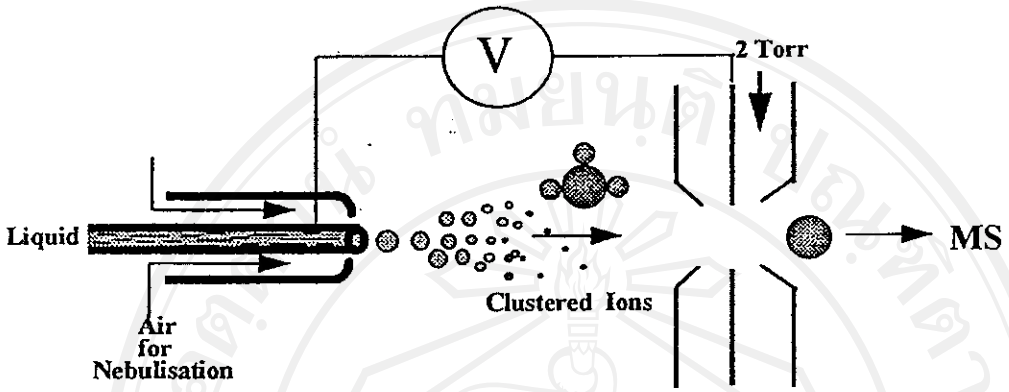


Figure 2.11 **Schematic diagram showing the workings of electrospray ionization** (Marquet and Lachatre, 1999).

2.8.2.3.3 Advantages and disadvantages

A major advantage of APCI is that ionization at atmospheric pressure eased interfacing with nose and detection limits at the parts per trillion (pptr) level can be attained (Harrison, 1992). This technique required water as part of charge transfer ionization process. None of the air components are ionized but the wide ranges of volatile organic compounds are susceptible to ionization. The capability of APCI has much more sensitivity than EI and the ionization efficiency for EI is 0.01-0.1% whereas APCI is almost 100% of the sample molecules introduced into the vacuum system (Siegel and McKeown, 1976).

As indicated in the preceding section, ionization of volatile mixtures by APCI-MS ideally produces one characteristic ion from which it can be quantified. However, some compounds may occur at the same m/z value as another compound. Thus, identification of compounds with the same mass is not possible to identify. Moreover, positional isomers like 2- and 3-methylbutanal can only be measured as

2.8.2.3.4 Applications

APCI-MS has been successfully applied in a wide range of determination of components in biological samples, air samples and environmental samples. This technique has proved useful for following volatile release *in vivo* on a breath by breath basis and flavour release on a dynamic headspace dilution method (Marin *et al.*, 1999; Taylor and Linforth, 2000). This interface has been applied for the study of a wide range of food product. For example, the quantification for differences in flavour release profile when food is reformulated (Brauss *et al.*, 1999; Brauss *et al.*, 2000) and also provided information on the link between flavour release and sensory perception (Baek *et al.*, 1999; Linforth *et al.*, 1999). Volatile flavour compounds formed by thermal treatment of skim milk powder has been studied in real time monitoring (Turner *et al.*, 2002). The rapid volatiles through the lipid oxidation pathway in tomato fruit using APCI-MS has been measured *in vivo* for individual fruit in real time (Linforth *et al.*, 1994; Brauss *et al.*, 1998; Boukobza *et al.*, 2001). It has also allowed new empirical models to predict volatiles release from foods using physicochemical parameters (Linforth *et al.*, 2000), the change in gas-liquid partition behavior in aqueous sucrose solution (Friel *et al.*, 2000) and retronasal transport of aroma compounds (Linforth *et al.*, 2002).

APCI interface is available from all major mass spectrometers. This technique has been directly interfaced to both gas chromatograph (GC) and particularly for liquid chromatograph (LC) systems for analysis of many different non-volatile compounds. Hagiwara and co-workers (1999) determined the quantities of the sweetener aspartame in chocolate, soy sauce and miso paste. The usage of LC/APCI-MS for the quantitative determination of monomeric catechins in a baking chocolate are also reported (Nelson and Sharpless, 2003). LC/APCI-MS has been used for low-level detection of various sugars by chloride attachment in negative-ion mode (Kato and Numajiri, 1991) and for dimethylhylene-dihydrothiophene (Shiea *et al.*, 1996) and contaminants in air in positive-ion mode, which indicated a detection limit down to the parts per trillion (pptr) level (Ketkar *et al.*, 1989). Another significant target for

drinks (Barnes *et al.*, 1995). Anacleto and co-workers (1991) used supercritical fluid chromatography (SFC) couple with APCI-MS as interface for analysis of polycyclic aromatic compounds (PAC) from coal tar and heavy oils.

APCI techniques have dramatically influenced recent advances in the application of mass spectrometry. APCI-MS will continue to be the most important interfacing and ionization approach for liquid and gas phase in food industry applications.

2.9 Lipoxygenase and flavour generation

2.9.1 General aspects

Lipoxygenase (LOX; linoleate:oxygen oxidoreductase, EC 1.3.11.12) is a nonheme-iron-containing dioxygenase that catalyzed the hydroperoxidation of unsaturated fatty acids containing a *(Z)-(Z)*-1,4-pentadiene producing hydroperoxide fatty acids with *(Z), (E)*-diene conjugation (Hildebrand, 1989; Gardner, 1991; Wong, 1995; Feussner and Wasternack, 2002). Enzymes of this type are formally known as oxidase, carotene oxidase and lipoxidase (Galliard and Chan, 1980). LOX is widespread in nature, including higher plants (Eriksson, 1979), animals (O'Connor and O'Brien, 1991) and eukaryotic organism (Hildebrand, 1989).

2.9.2 Lipoxygenase isoenzymes

Tomato LOX enzymes have been purified and characterized by many researchers (Zamora *et al.*, 1987; Bowsher *et al.*, 1992; Smith *et al.*, 1997). Microsomal and chloroplastic membrane-associated and soluble LOX isoforms from tomato fruit have also been characterized (Todd *et al.*, 1990; Bowsher *et al.*, 1992). A LOX purified from ripe tomato was reported to be inactive on complex membrane phospholipids (Regdel *et al.*, 1994).

According to their overall sequence 70% similarity, most plant LOX could be

possible that the difference LOX forms may have a specific function. It could contribute with temporal differentiation of action, to an orchestration of the formation of hydroperoxide fatty acids (Feussner and Wasternack, 2002). Genes of a number of LOX have been cloned and sequenced. Five tomato isozymes *TomloxA, TomloxB, TomloxC, TomloxD* and *TomloxE* are composed of 860, 859, 900, 908 and 862 amino acids, respectively (NCBI, 2002).

2.9.3 LOX pathway

LOX pathway is the metabolism of polyunsaturated fatty acids (PUFAs) via the LOX-catalyzed steps and the subsequent reactions (Hildebrand, 1989). The general pathway of lipid oxidation by LOX under aerobic and anaerobic conditions was proposed (Galliard, 1978; Galliard and Chan, 1980; Hildebrand, 1989; Gardner, 1991,1995). To date, four metabolic pathways for the metabolism of hydroperoxide fatty acids by LOX have been characterized (Feussner *et al.*, 2001; Feussner and Wasternack, 2002). The schematic metabolism of PUFAs by LOX is presented in Figure 2.12.

The first pathway involved cleavage by hydroperoxide lyase (HPL) of 13-hydroperoxide linolenic acid, leading to the formation of short chain aldehydes (C6 or C9) and the corresponding C12 compound (12-oxo-dodecenoic acid) or C9-ω-keto fatty acid (Hildebrand, 1989). The second route is the allene oxide synthase (AOS) pathway, in which AOS converts hydroperoxide fatty acids to unstable allene oxides, which either form nonenzymatically leading to α- and γ-ketols or may be metabolized to jasmonates. The third pathway is the peroxygenase (POX) pathway, in which intramolecular oxygen transfer converts fatty acid hydroperoxides to epoxy or dihydrodiol polyenoic fatty acids. Finally, the divinyl ether synthase (DES) pathway, which converts fatty acid hydroperoxides into cytotoxic divinylethers such as colnelic acid or colnelenic acid. In addition, the other reactions for hydroperoxide metabolism such as ketodiene forming pathway by LOX, epoxy alcohol synthase (EAS) pathway and reductase pathway are not as well characterized.

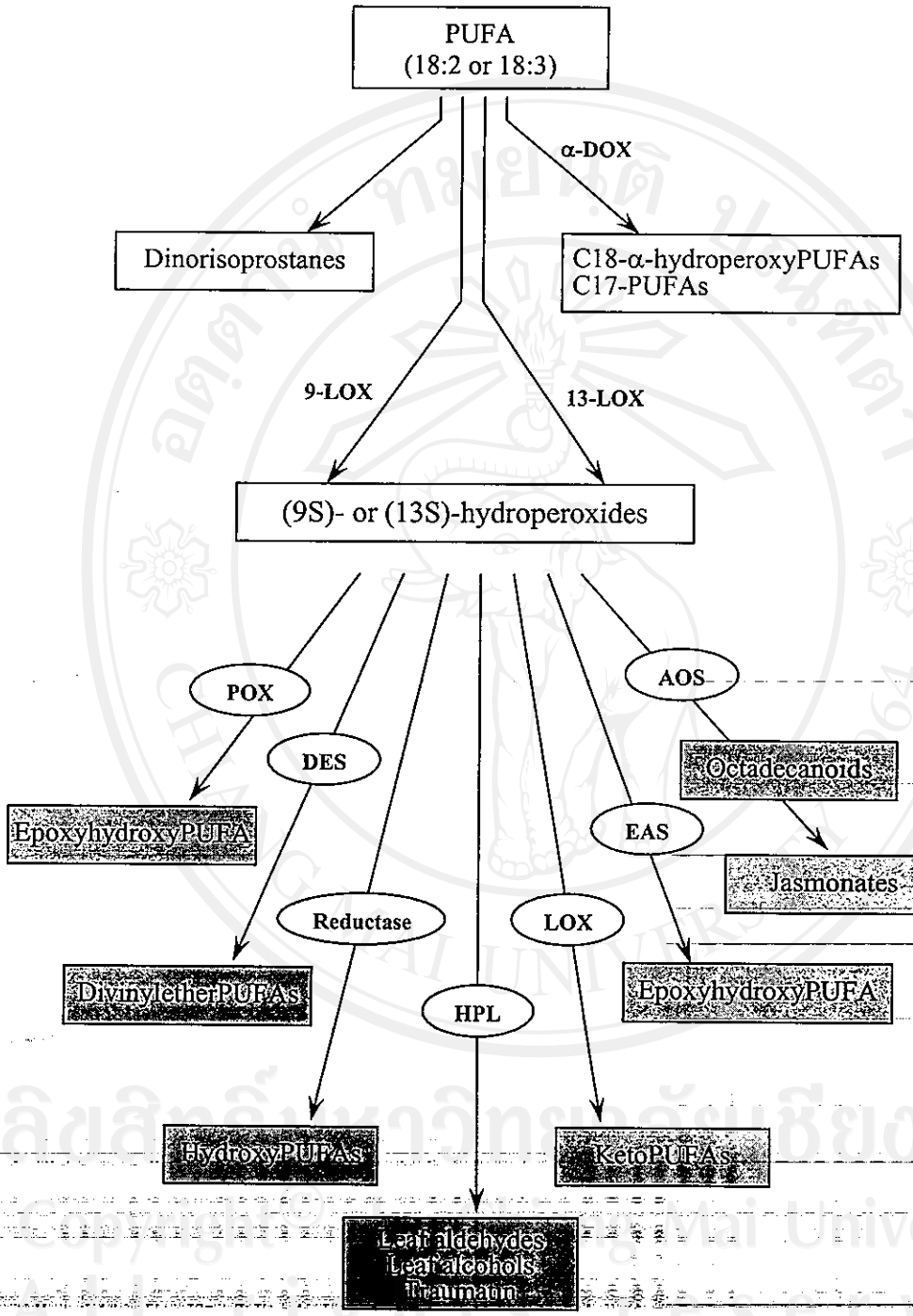


Figure 2.12 Metabolism of PUFAs leading to 9-LOX and 13-LOX derived hydroperoxide PUFAs in plants (Feussner and Wasternack, 2002)

2.9.4 Role of LOX in the biogenesis of flavour

Some aroma compounds are present in the intact fruit, being produced during the ripening stage. However, many of the volatile aroma compounds are produced only when the fruit or vegetable is subjected to chewing, cutting or processing that allow mixing of enzymes and substrates which are normally compartmentalized. Only these aroma and flavour compounds are resulted from LOX action. The LOX catalyzed the formation of a cleavage of the fatty acid hydroperoxides by a hydroperoxide lyase (HPL). The pathway involved in the production of several different volatile carbonyl compounds important in the flavour of fruits and vegetables. At least three enzymes (LOX, HPL and isomerase) are involved in the formation of volatile compounds from linoleic and linolenic acids. Several reports involved LOX and HPL in the biogenesis via the oxidative degradation of fatty acids has been studied in tomato and cucumber (Eriksson, 1979).

The differences in types and proportions of volatiles produced have an impact on the distinctive aroma of a particular fruit and vegetable.

2.9.4.1 Tomato

The principle volatiles produced from lipid oxidation in tomato are described in section 2.5.3.3. Hexanal and *(Z)*-3-hexenal are predominant flavour volatile compounds found in tomato fruit. Although the major products of tomato LOX are the 9-hydroperoxide isomers of linoleic and linolenic acids. These products are not attacked by the cleavage enzymes. However, only the minor 13-hydroperoxides are susceptible to the action of tomato hydroperoxide lyase, which converted 13-hydroperoxides into the C6-aldehydes. The fate of 9-hydroperoxide product is unknown, but about 0.1% of its may be converted to hexanal under high concentrations (Regdel *et al.*, 1994).

These cleavage activities in tomato fruit appear to differ in one important aspect from that in cucumber, for example in substrate specific. With the important difference that in cucumber, both 9- and 13-hydroperoxide isomers are cleaved

2.9.4.2 Cucumber

The principal predominant aroma compounds in cucumber are C9 aldehydes, although C6 aldehydes are also present. Linoleic and linolenic acid are the major fatty acids component of cucumber at amount of 40% and 26.3% respectively, which are the major precursor of C9 aldehydes. These volatiles are not found in intact cucumber tissue but cutting or chewing of the tissue induces the production of C9-carbonyl compounds. The most important of which is *(E)*-2, *(Z)*-6-nonadienal which provides most of the key characteristic aroma compounds of fresh cucumber (Sekiya *et al.*, 1977; Scieberle *et al.*, 1990). Galliard (1976) showed that at least three enzymes are involved in the formation of cucumber volatiles from fatty acids precursors in cucumber tissues; LOX, HPL and enal isomerase. It has been reported that cucumber peel contains twice active substrates were linoleic acid (100%), linolenic acid (77%) and arachidonic acid (23%) with no activity of oleic acid (Fleming *et al.*, 1968; Wardale and Ambert, 1980).

2.9.5 Importance of LOX in plant science and food science and technology

2.9.5.1 Role of LOX in bread making improvement

LOX in the form of soybean flour is often used in baking industry as a dough improver. LOX also increases the ability of the dough to withstand over mixing. The most accepted hypothesis is that oxidation of thiol groups of gluten by the lipid hydroperoxide formed is responsible for improving the rheological properties of the dough. This results in the production of loaves with larger volumes and retarded bread staling (Nicolas and Potus, 1994).

2.9.5.2 Role of LOX in biogenesis of flavour and aroma

While LOX generated volatile compounds generally cause off flavours in beans, the same compounds are often responsible for the typical flavour

offensive flavours in many fruits and vegetables including tomato and cucumber (see section 2.9.3). The odours of hexanal and *(Z)-3*-hexenal have been described as green beany or green/grassy, respectively, with responsible for aroma characteristic of tomato. While *(Z)*-3-nonenal and *(E)*-2,*(Z)*-6-nonadienal have been related to cucumber aroma characteristic. Moreover, the initial products of lipid oxidation by LOX are converted by other enzymes into alcohols and acids, which then forms an ester.

2.9.5.3 Off flavour production

Off flavour generation is a potential problem in legumes such as soybean and green peas. Volatile degradation products from lipid oxidation by LOX have been associated with grassy-beany and rancid off-flavours, which affect the consumer acceptability of soybean products. The principle compounds to the reduction of off-flavour development in soybean products are inactivated and inhibition of LOX and the development of cultivars deficient in LOX isozyme. Recently, the soybean seed with lacking some of LOX isozyme has been studied (Satouchi *et al.*, 2002).

2.9.5.4 Degradation of pigments and vitamins

LOX enzymes are particularly interest to the food scientist due to their ability to form free radicals, which can then attack other constituents such as vitamins, pigments and proteins. LOX generally causes the bleaching of chlorophyll at a slower rate than that of carotenoids in plant extract and tissue (O'Connor and O'Brien, 1991). LOX is probably responsible for the degradation of chlorophyll in underblanched green beans and other leguminous foods (Whitaker, 1991). Degradation of fat-soluble vitamins by LOX is the problem in food industry. Vitamin A, D2, D3 and E are all susceptible to rapid co-oxidation of neutral pH in the presence of soybean LOX (O'Connor and O'Brien, 1991).

2.9.5 Physiological functions of LOX in plants

abscisic acid biosynthesis and ethylene generation have been suggested (Gardner, 1991). The hydrolytic HPL product, traumatic acid was detected as the plant wound hormone and flowering initiation factors. In plants volatile aldehydes play an important role in antimicrobial defense including the inhibitor of the growth of pathogenic fungi (Grechkin, 1998).