CHAPTER 4 : RESULTS AND DISCUSSION

Since volatile compounds are directly responsible for the characteristic of impact flavours of fresh fruits and vegetables. Sample preparation techniques are logically and influentially considered in order to investigate for flavour volatile sampling during plant tissue disruption. To successfully conduct this measurement, the method that is simple, efficient and artifact-free is required for this research. In addition, the rapid method with real time analysis for monitoring the dynamic of volatile release from samples in a short time was needed to invest for analysis of large numbers of fruit samples. The significant variations due to the rapidity of postharvest metabolic changes in the fruits were also precautionary, because these changes could affect on the alteration of flavour quality.

The technique, using the special maceration device connected to the Atmospheric Pressure Chemical Ionization (APCI) inlet, was developed for real time monitoring of the volatile release during the maceration of cucumber and tomato fruits. This technique is intensive with a typical running time within 3 minutes for each sample and less than one-minute delay for changeover. The total running time interval per sample is around 5 minutes for examining many volatiles simultaneously. It is, therefore, possible to be able to analyze a large amounts of samples per day and reduces fruit to fruit variation.

Apart from the qualitative and quantitative analysis of volatile compounds, generated in intact fruits, the technique is allowed to investigate the volatiles secondarily generated through the lipid oxidation pathway after tissue disruption in cucumber and tomato fruits.

4.1 Biochemical study of volatile generation on cucumber model

Cucumber is a non-climacteric fruit that the respiration gradually declines with

generation by enzymatic lipid oxidation is quite similar in some aspects. The characteristics of flavour volatile compounds of cucumber and tomato are due mainly to oxygenation of unsaturated carbonyls after fruit maceration by chewing or cutting. Thus, this technique offers the possibility to study the biochemical pathway and the effect of some factors involved in volatiles generation such as anaerobic conditions and some substrates. Cucumber is a good sample to achieve the understanding what comprises flavour and how it is generated. It was used as a model to confirm the hypothetical mechanism of the volatile production in particular from lipid oxidation. This study was primarily led to clearly understand in the alteration of volatile composition in both wild-type and transgenic tomato fruits.

4.1.1 Identification of main flavour volatiles of cucumber

Figure 4.1 shows a gas chromatogram of volatile compounds from cucumber fruit. The key components were hexanal, *(E)*-2-hexenal, nonanal, *(Z)*-3-nonenal, *(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal eluting from a range of 10.18 to 26.07 minutes, respectively. The main characteristic volatile compounds of cucumber were *(E)*-2,*(Z)*-6-nonadienal, *(E)*-2-nonenal which had been investigated by several researchers (Forss *et al.*, 1962; Fleming *et al.*, 1968; Hatanaka *et al.*, 1975; Scieberle *et al.*, 1990; Palma-Harris *et al.*, 2001). The principle aroma compounds in cucumber are C9 aldehydes. However, C6 aldehydes are also present, mainly hexanal and *(E)*-2-hexenal. These volatiles are not found in intact cucumber tissues but cutting or chewing of the tissue induces the production of these carbonyl compounds (O'Connor and O'Brien, 1991). Cucumber contains acyl hydrolase and lipoxygenase. The latter having a specificity that favors the formation of 9-hydroperoxide from linoleic acid like the tomato lipoxygenase. Nevertheless, the hydroperoxide cleavage system in cucumber, in contrast to that in tomato, cleaves both 9- and 13-hydroperoxides of linoleic and linolenic acids (Eriksson, 1979). The ability of cucumber homogenates to form C9 aldehydes (*(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal from linolenic and linoleic acids, respectively) was found to increase stepwise during the ripening of

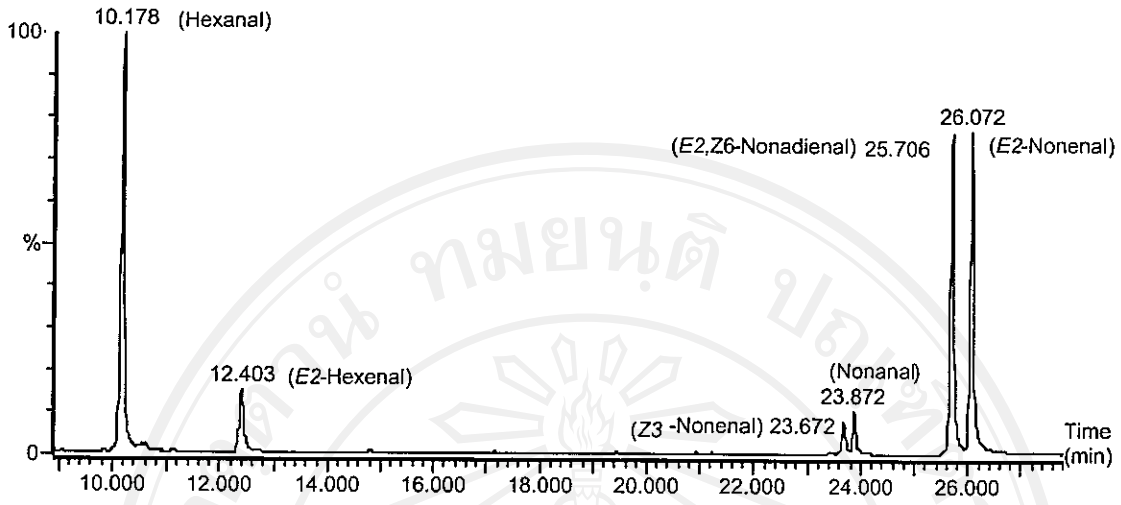


Figure 4.1 Chromatogram of volatile compounds from cucumber homogenates.

4.1.2 Quantification of the main flavour volatile compounds

The typical release profile for the headspace concentration of five key volatile compounds in the headspace above the cucumber homogenates during 10 minutes after maceration using APCI-MS is demonstrated in Figure 4.2. Hexanal was the first volatile compound released from headspace samples, followed by nonadienal, nonenal, nonanal and hexenal, respectively. However, both of C6 and C9 aldehydes were produced when cucumber cells were broken by maceration to allow lipid oxidation enzymes contacting with released lipid substrates, particularly linoleic and linolenic acids.

Table 4.1 I_{max} and T_{max} of cucumber volatiles in headspace during maceration.

Volatile	I_{max}, ppbv* (% CV)	T_{max}, min* (% CV)
Hexanal	1874 (18)	4.55 (13)
Nonadienal	1018 (37)	4.33 (13)
Nonenal	730 (36)	3.92 (15)
Nonanal	70 (34)	4.95 (14)
Hexenal	66 (34)	4.75 (14)

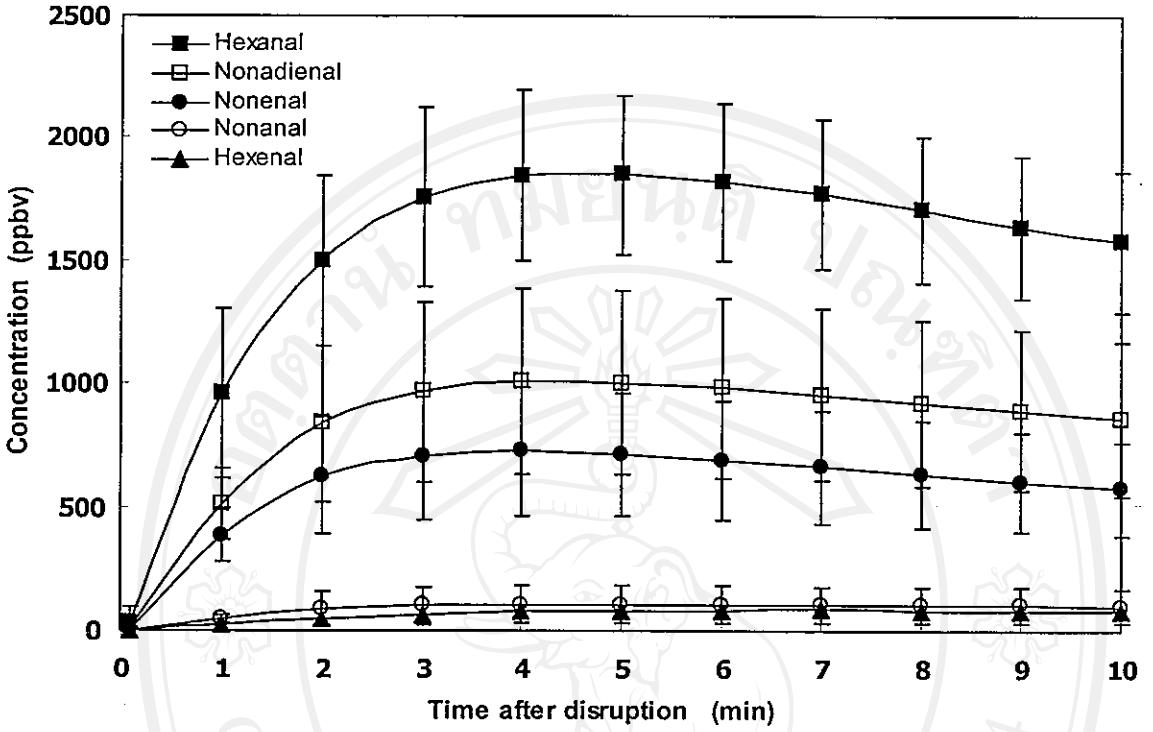


Figure 4.2 **Release profiles of main volatiles from cucumber homogenates.**
(Data are the mean of 5 replications; bars are ± SD)

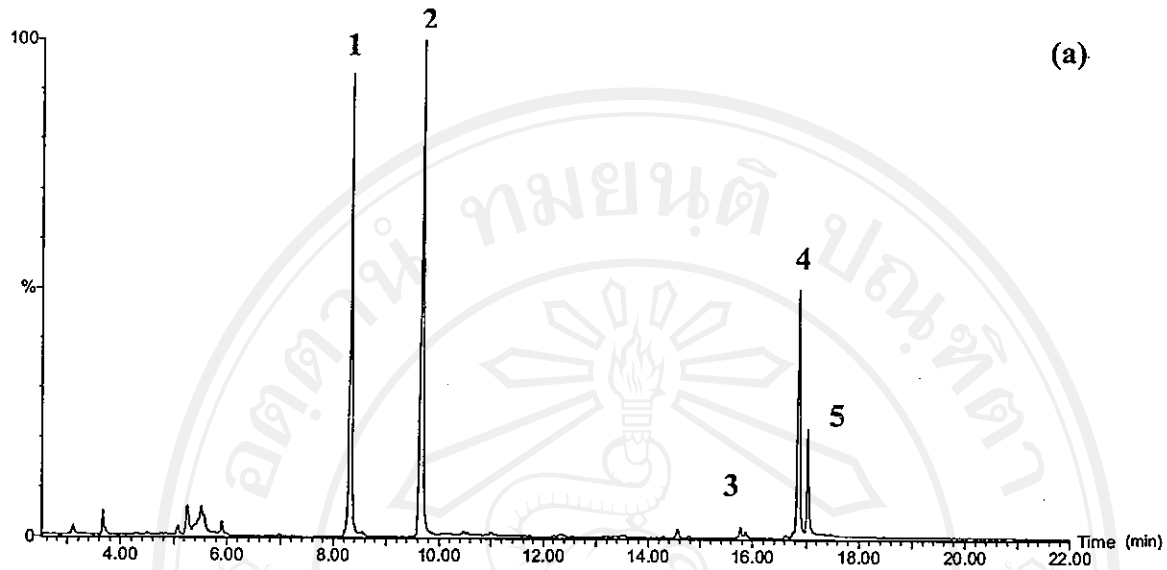
Table 4.1 shows the maximum intensity (I_{max}), the time to maximum intensity (T_{max}) and the percentage coefficient of variation (% CV) of each cucumber volatile compound during maceration, calculated from the time-release curves. The T_{max} of main volatiles in cucumber was around 4-5 minutes. The most important odorants of cucumber flavour was nonadienal, followed by nonenal. The concentration of nonadienal in cucumber homogenates was approximately 2 times higher than that of nonenal and 2 times less than that of hexanal. However, the odour units of nonadienal were 50 times higher than those of nonenal (Scieberle *et al.*, 1990).

were very similar qualitatively, indicating the same sensitivity to compounds between the two systems. Mass spectra from the EI-MS were represented to identify of each peak, so that the ion mass of corresponding peak by the APCI-MS could be matched to that compound.

The chromatograms of cucumber volatile are shown in Figure 4.3. Only five key volatile compounds were detected by EI and APCI-MS. The C6 and C9 aldehydes generated by the lipid oxidation pathway were the predominant volatile compounds in cucumber. Generally, the APCI ion mass is equal to the molecular weight (MW) plus one, due to this technique produces the protronated molecular ion $[M+H]^+$ for almost volatile compounds. However, some alcohols can be dehydrated, resulting a $[M-H_2O+H]^+$ (Taylor *et al.,* 2000). The major ion mass obtained from the full scan data of APCI-MS was a single compound. The ion mass of 139 and 141 was represented of *(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal, respectively, corresponding the identification by matching with library mass spectra by EI-MS (Table 4.2).

Table 4.2 Identification GC/APCI-EI/MS peaks of cucumber volatile compounds.

Peak No.	RT (min)	Identification compound	MW	APCI-MS mass	GC/EI-MS mass (es)
1	8.36	Hexanal	100	101	56
2	9.72	*(E)*-2-Hexenal	98	99	83
3	15.78	Nonanal	142	143	98 (124)
4	17.02	*(E)*-2, *(Z)*-6-Nonadienal	138	139	69 (136)
5	17.16	*(E)*-2-Nonenal	140	141	70 (138)



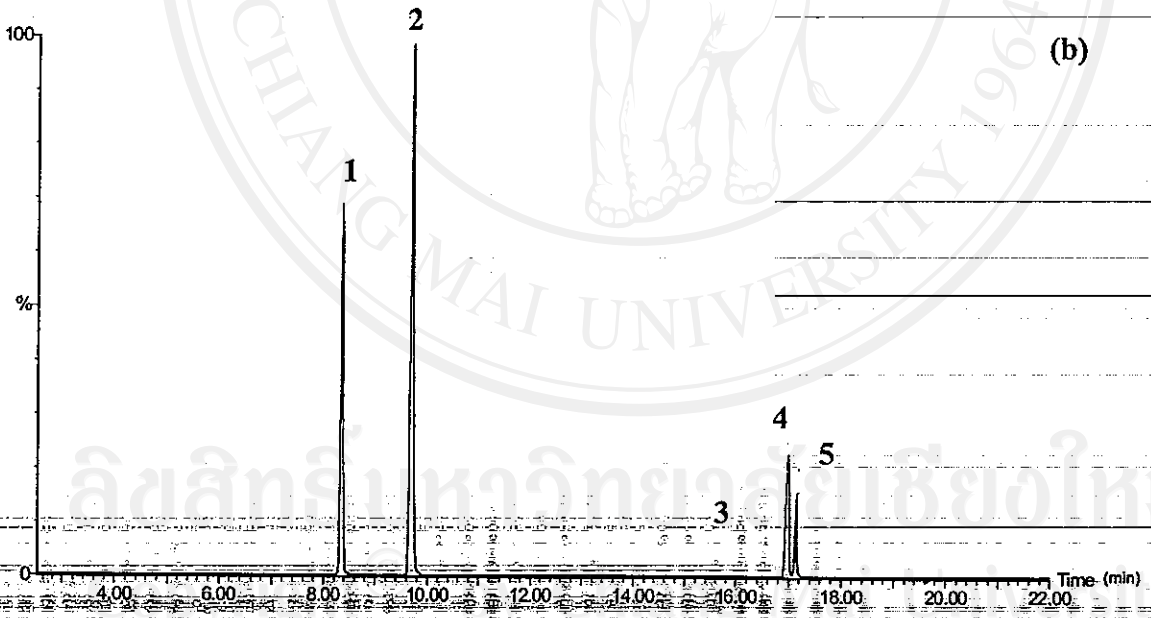
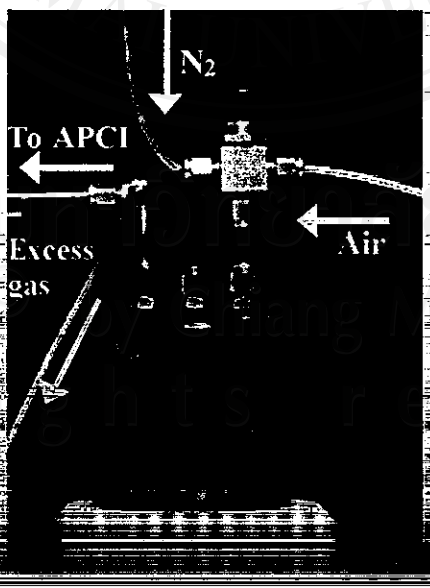


Figure 4.3 Chromatograms of volatile compounds from cucumber [illegible]

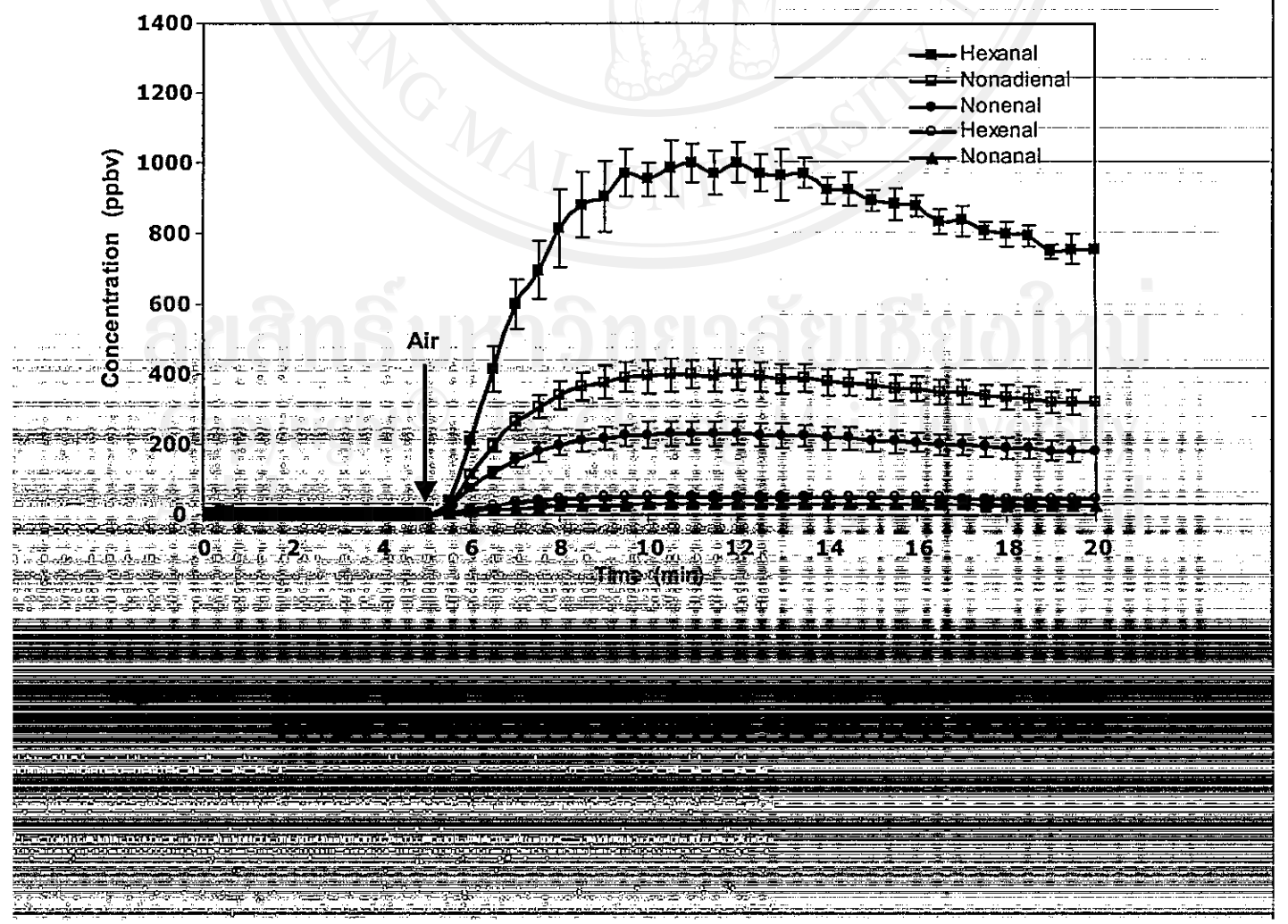
4.1.4 Study on lipid oxidation pathway for volatile generation

4.1.4.1 Effect of oxygen on a cucumber flavour generation

The effect of anaerobic condition influencing the biochemical pathway for volatile generation was studied using APCI-MS with a modified maceration device. The modified maceration device was composed to three outer fittings as described in section 3.2.3. One of the outer fittings was connected to two PTFE lines (4 mm o.d.) for flushing either air or nitrogen gas through the blender (Figure 4.4). The blender containing the fruit was first purged with nitrogen gas for 20 minutes at a flow rate of 170 ml.min^{-1} prior maceration to remove all traces of oxygen in the blender. The fruit was then blended for 20 seconds and held under nitrogen for a further 5 minutes or longer time periods for monitoring volatiles release in the headspace above the macerated fruit. Air was introduced into the blender at the same flow rate (170 ml.min^{-1}) after 5 minutes under anaerobic conditions. Volatile concentrations released in the headspace above the macerated cucumber were monitored for another 5 or 20 minutes after air was introduced into a blender. A control composed of macerated cucumber under aerobic condition with air at the same flow rate (170 ml.min^{-1}) was also monitored.



There was a rapid formation of aldehydes such as *(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal which were responsible for the fresh cucumber flavour characteristic produced during cutting or mechanically rupture in the presence of oxygen. The C6 and C9 aldehydes generation were prevented by holding under an atmosphere of nitrogen for first 5 minutes before blending (Figure 4.5). As soon as air was introduced into the maceration device from 5 minute onwards, all selected volatiles were immediately produced from the lipid oxidation pathways. Linoleic and linolenic acids were degraded by lipid oxidative enzymes. However, the concentrations of all cucumber volatiles generated after introduction of air were decreased by half compared to that of the control (under air) (Figure 4.6). During the maceration of cucumber under the atmosphere of nitrogen, enzymes could either be deactivated because of the decrease in pH of cucumber homogenate or be used in the other pathways for some new compounds generation.



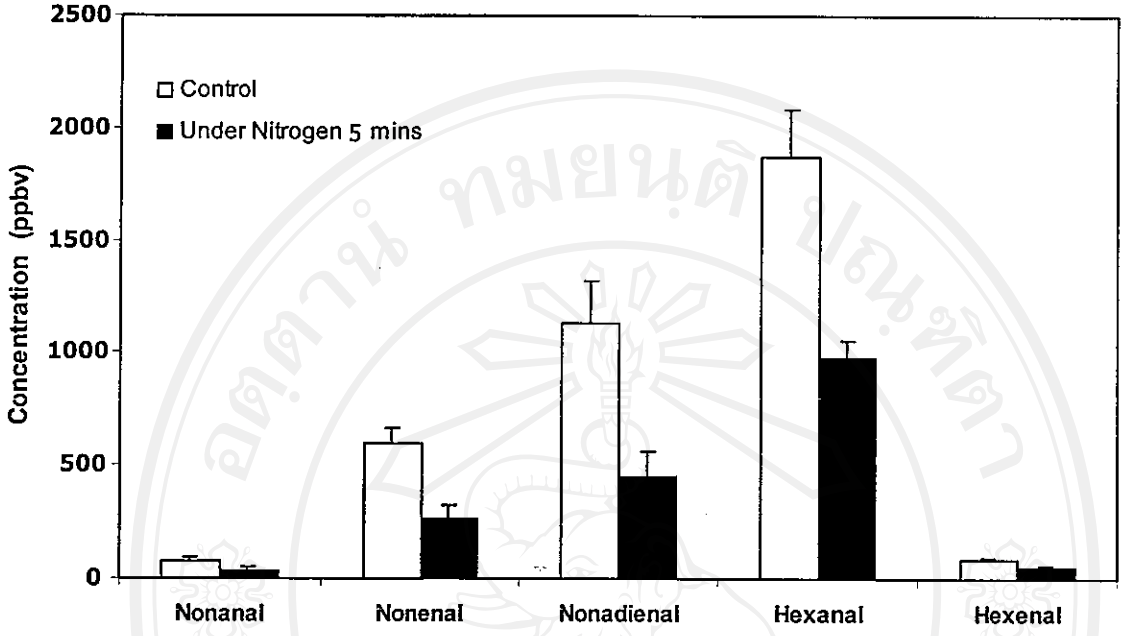


Figure 4.6 **Effect of oxygen on the cucumber volatiles concentration.** *(Data are the mean of 5 replications; bars are ± SD)*

Furthermore, to confirm that there were no new compounds generation in headspace during maceration under nitrogen. Samples were analyzed using a full scan mode for a mass range of 40-200. The result showed that no new volatile compound was released during the maceration under nitrogen gas (data not shown).

These results clearly indicated that the volatile compounds of cucumber were enzymatically produced especially both of lipoxygenase and hydroperoxide lyase. These enzymes converted linoleic and linolenic acids via hydroperoxide intermediates to C6 and C9 carbonyl fragments by lipid oxidation pathway when the cucumber tissue was disrupted in the presence of oxygen (Friksson, 1979).

4.1.4.2 Effect of addition of substrates on flavour generation

C6 and C9 aldehyde volatile compounds are derived from linoleic and

cucumber tissue in the formation of lipid degradation products, which are responsible for the characteristic flavour of cucumber (Galliard *et al.*, 1976).

The effect of exogenous free fatty acids in the emulsion substrates on cucumber flavour volatile generation is shown in Figure 4.7. The control was used to compare by macerating cucumber tissues with the same volume of sodium phosphate buffer mixed with Tween 20 without the substrate addition.

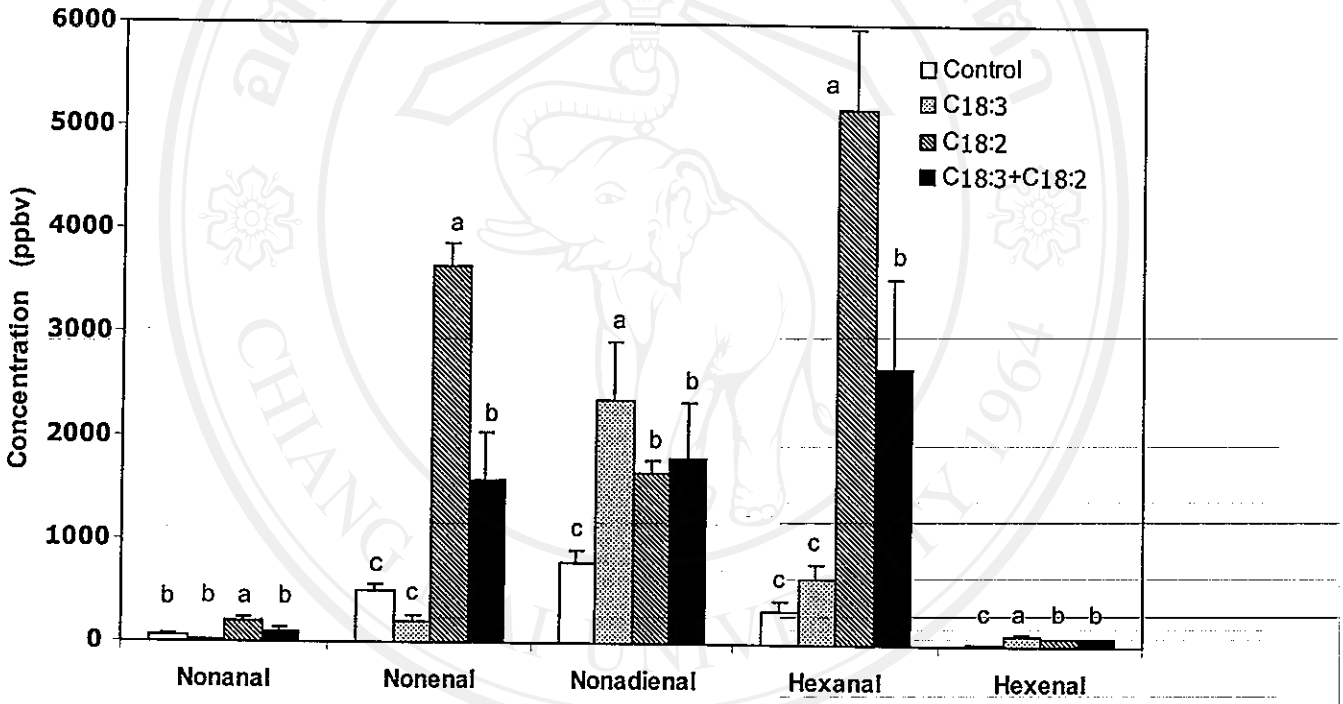


Figure 4.7 Effect of addition of free fatty acids on cucumber flavour volatiles generation. *(Data are the mean of 5 replications; column and its bar is mean ± SD. Means with the same letter on the bar of each volatile are not significantly different by LSD procedure, $p>0.05$)*

Nonadienal and hexenal concentrations were significantly increased by the

nonenal, but the quantity of each was less than in samples which had fatty acids added individually. The reduction in the combined sample may have been due to competitive inhibition between the two precursors or their products (Buescher and Buescher, 2001). These results confirmed that the formation of nonadienal and nonenal in cucumber resulted from the addition of C18:3 and C18:2, respectively. It has been suggested that the precursors of *(E)*-2,*(Z)*-6-nonadienal and *(E)*-2-nonenal in cucumber are linolenic and linoleic acids, respectively (Hatanaka *et al.*, 1975; Wardale and Ambert, 1980). Although, the addition of C18:3 suppressed the formation of nonenal to one third of the control, the production of hexanal was not suppressed. The quantity of hexanal was not significantly different from that of control. These results may have been involved in the specificity of hydrolytic enzymes and substrates.

When C18:2 was added, hexenal levels increased more than that of the control. This is due to the presence of double-bond reducing enzyme, which converts hexanal to hexenal (Eriksson, 1979). The contribution of hexanal derivative resulted in the elevated level of hexenal when C18:2 was added. Interestingly, the addition of C18:2 increased the level of nonadienal. The cause of increase in nonadienal with addition of C18:2 is unknown. One possibility is that the minor fragment ions from some compounds, which may occur at the same ion mass, contribute to the ion intensity of main ions (Taylor *et al.*, 2000). The reduction of nonenal and hexanal generation by the addition of linolenic acid suggests that lipoxygenase or hydroperoxide lyase show higher specification to linolenic acid or its hydroperoxide, respectively than to linoleic acid. The amount of nonanal was significantly increased by addition of linoleic acid, but was not affected by adding linolenic acid or the combination of both fatty acids. This volatile might be come from the C9 carbonyl fragments by 13-hydroperoxide of linoleic acid.

The volatile release profiles of nonadienal, nonenal, hexanal, hexenal and nonanal when cucumber tissues were macerated with buffer alone (control) or blended with buffer containing the linoleic or linolenic acid

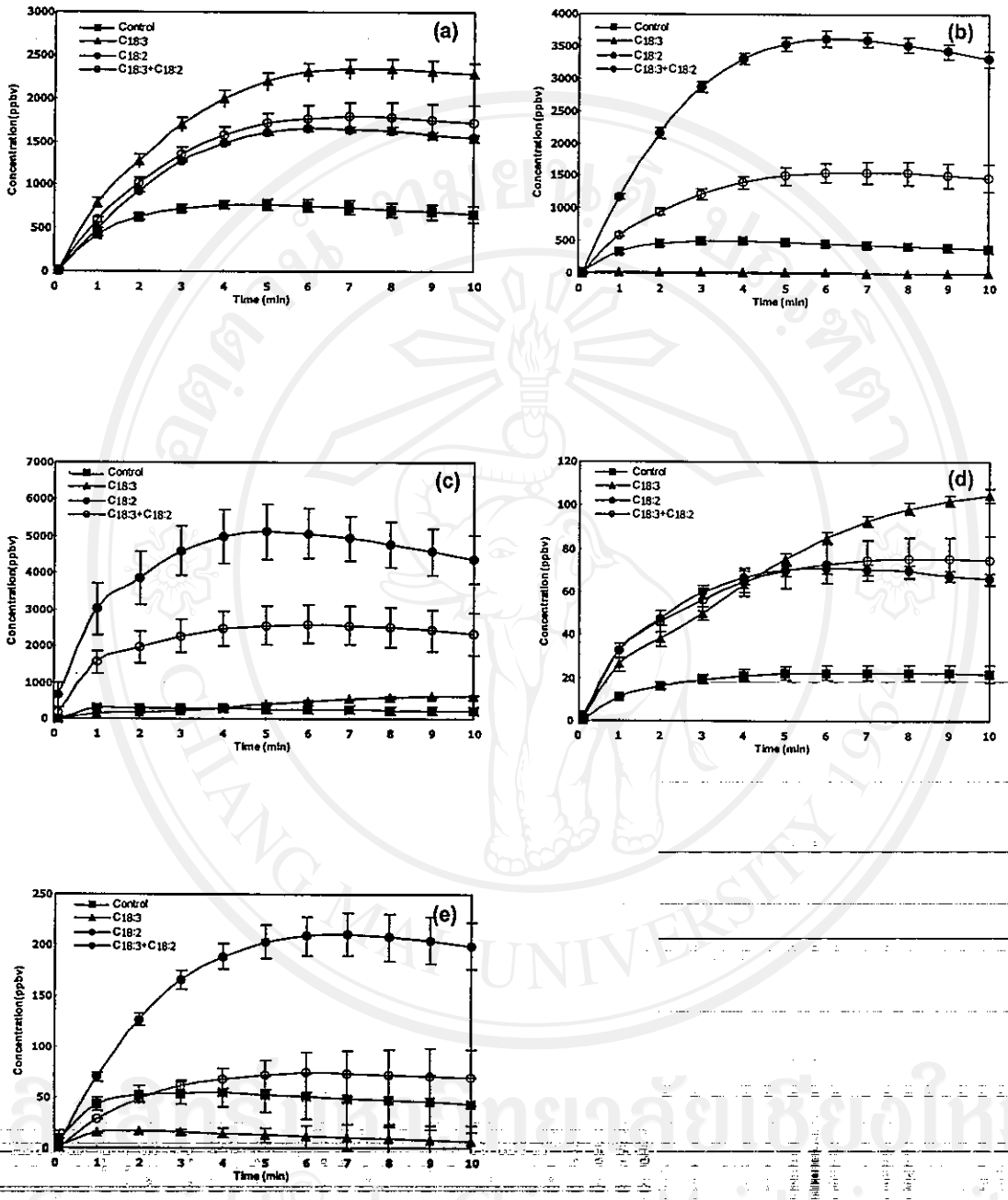
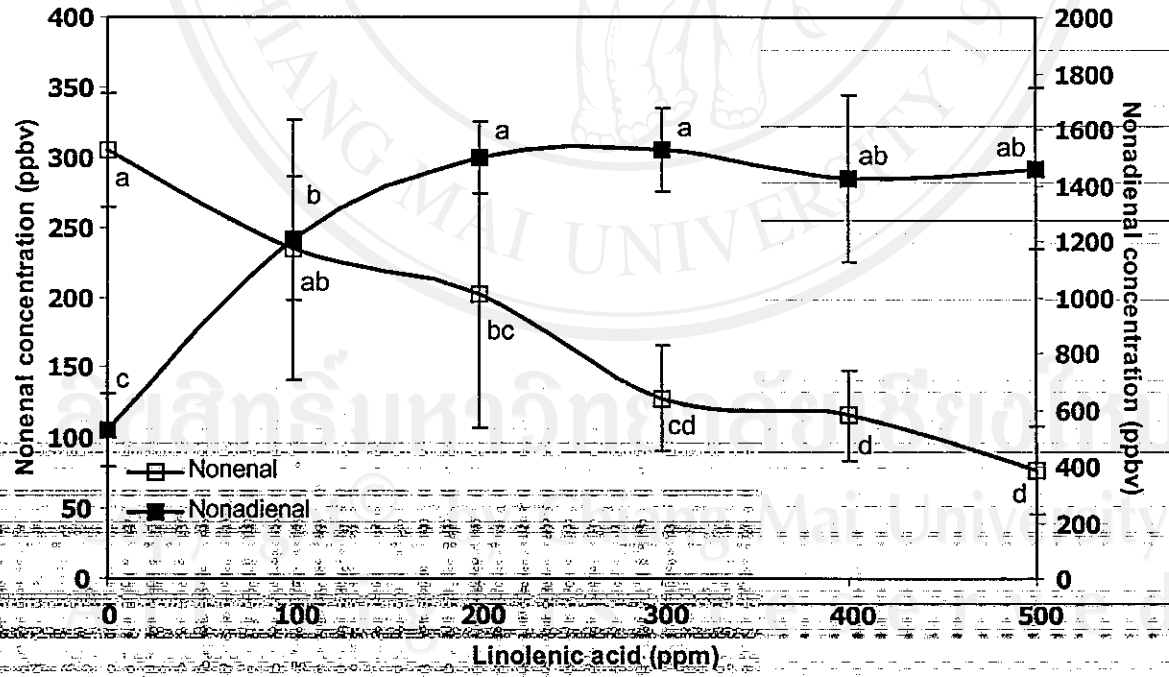


Figure 4.8 Release profile of nonadienal (a), nonenal (b), hexanal (c), hexenal (d) and nonanal (e) after cucumber tissue disruption in the control

4.1.4.3 Effect of linolenic acid concentration on nonenal and nonadienal generation

Linolenic acid is the most prominent feature of fatty acid composition and the most active substrate for lipoxygenase in cucumber. The high content of linolenic acid can account for the observed volatile formation, derived from the polyunsaturated fatty acids when cucumber tissue is disrupted (Galliard *et al.*, 1976; Fishwick *et al.*, 1977; Wardale and Ambert, 1980). The correlation between an increase in nonadienal and decrease in nonenal concentrations by adding of linolenic acid is shown in Figure 4.9. The addition of linolenic acid from 100 to 500 ppm resulted in a large enhancement of nonadienal but a decrease in nonenal production. The maximum formation of nonadienal was achieved by 300 ppm linolenic acid and no significantly different from 200-500 ppm (Figure 4.9). This result may have been involved the efficient volatile production relative to the amount of substrates added and enzyme activities.

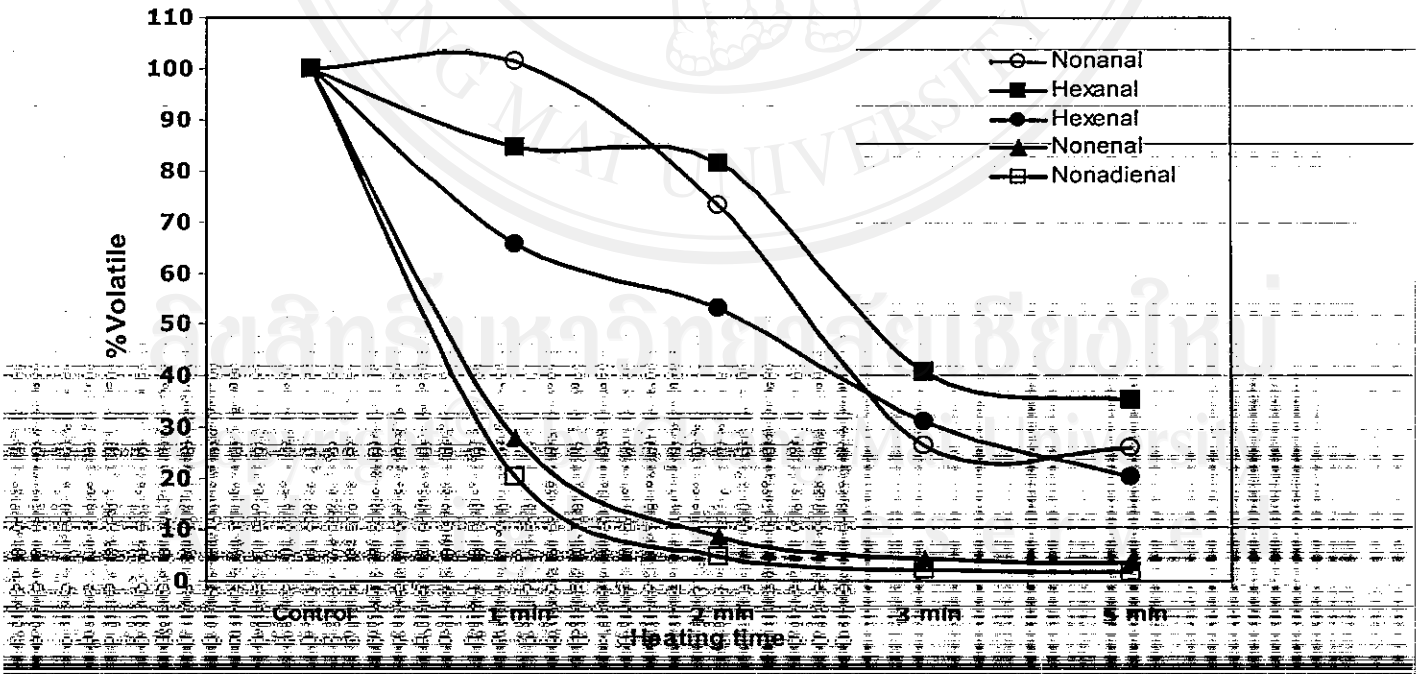


4.1.5 Quantification of volatile compounds in the fruit

Generally, the amount of volatile compounds generated in fresh fruits and vegetables are expressed in a unit of ppm or $mg.kg^{-1}$ of fresh weight. However, using APCI-MS, an interface has been developed that allows real time measurement of volatile release in the headspace above the homogenates at low concentration (Linforth and Taylor, 1998). The amount of these volatile compounds was calculated as mg volatile compounds per m^3 air in the headspace or ppbv.

4.1.5.1 Effect of heat inactivation on the formation of cucumber volatiles

The formation of cucumber volatiles was prevented by microwave heating before blending. The result of heat inactivation of volatiles formation in cucumber homogenates by microwave heating at high power of 800W for 1, 2, 3 and 5 minutes is shown in Figure 4.10. The heating time of 3 minutes was sufficient for inactivation enzyme activities, generated the cucumber volatiles mainly nonadienal and nonenal. Some volatiles such as hexanal was still present at a high concentration. However, it was less than the control about 60%.



4.1.5.2 Measurement the concentration of volatiles in gas and liquid phases

The experimental conditions involved a liquid phase containing 5 authentic compounds in a closed maceration device were dissolved in distilled water or heated cucumber homogenates at concentrations of 1, 3, 5 and 10 ppm. Samples were blended for 20 seconds and left for the next 20 minutes to reach an equilibrium condition. After equilibrium between the liquid and air at atmospheric pressure, the gas phase was diluted by introducing fresh air at a fixed flow rate (170 $ml.min^{-1}$). All volatiles released in the headspace of the maceration device were directly sampled into the APCI-MS and then measured. The calibration procedure was preformed by introduction of hexane solution of five standard volatile mixtures into the air stream by syringe calibration method described in the section 3.2.5. The concentration of each volatile in the gas phase as a function of time was determined as a maximum concentration in $mg.m^{-3}$ or ppbv of gas in the headspace above the sample. Therefore, the amount of volatile in fresh fruit sample could be calculated in unit of $mg.g^{-1}$ fresh weight by comparing known authentic concentration in gas and liquid phases. The values obtained from this method for nonenal and nonadienal estimated as 5 and 8 $mg.kg^{-1}$ fresh tissue, respectively. These values compared well with the published values of 8 $mg.kg^{-1}$ for nonenal and 4 to 13 $mg.kg^{-1}$ for nonadienal (Buescher and Buescher, 2001).

The concentration in gas phase of each volatile released from distilled water and heated cucumber homogenates containing a mixture of five added standards at concentrations of 1, 3, 5 and 10 ppm is shown in Figure 4.11. These results showed that at the low concentration of added standards (1-5 ppm) in heated cucumber homogenates or distilled water as a liquid medium, the amount of each volatile released from liquid phase to gas phase was not shown much differences. However, high concentration of added standards (10 ppm) in the liquid phase showed larger differences in amounts of volatile released between cucumber homogenates and distilled water. Therefore, it would be expected that the measured volatile amount at

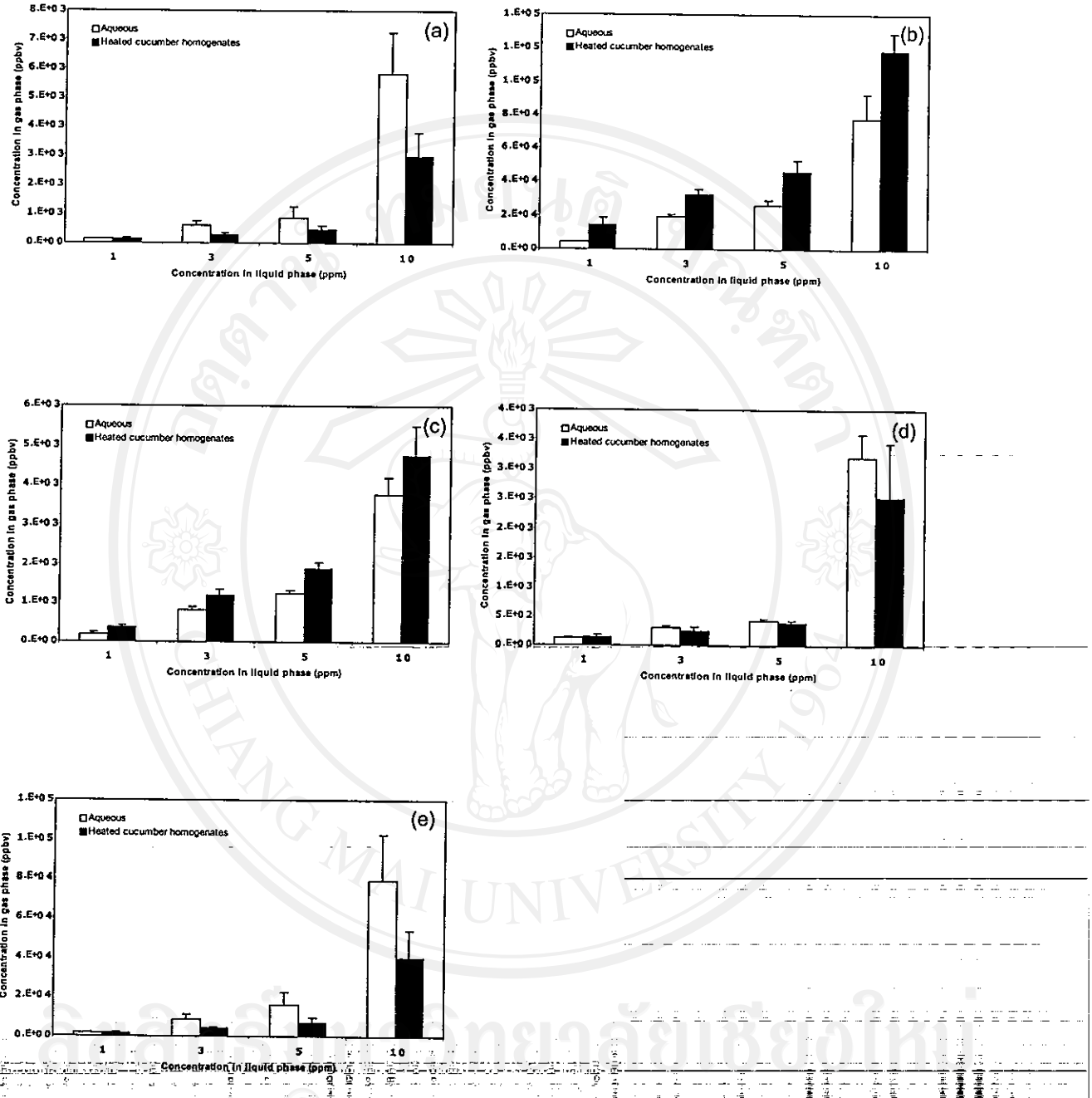


Figure 4.11 The concentration in gas phase of nonenal (a), hexanal (b), hexenal (c), nonadienal (d) and nonanal (e) released from aqueous medium and heated cucumber homogenates containing a mixture of five added standards at concentration of 1, 3, 5 and 10 ppm. *(Data are the mean of 5 replications, bars are ± SD)*

4.2 Tomato flavour analysis

4.2.1 Analysis of tomato volatiles

4.2.1.1 Identification of main flavour volatiles of tomato fruit

From the preliminary experiments using a dynamic headspace sampling to concentrate volatiles released from tomato homogenates at room temperature with thermal desorption at the interior of a GC injector led to the isolation and qualification of approximately 13 volatiles. A representative chromatogram of volatiles in fresh tomato homogenates from a wild-type, cv. Ailsa Craig is shown in Figure 4.12.

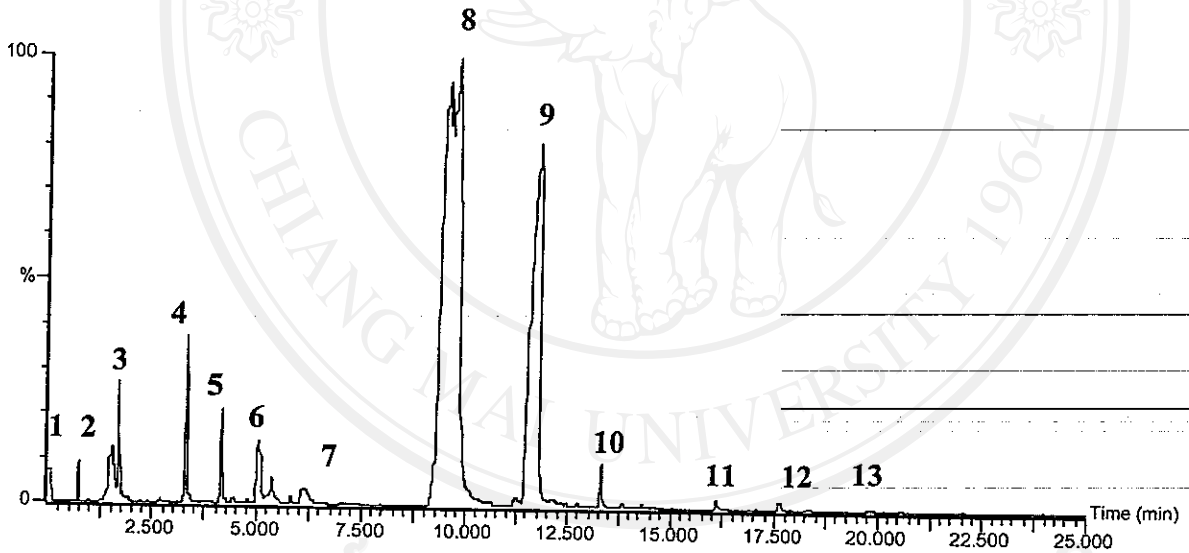


Figure 4.12 Chromatogram of volatile compounds from fresh tomato homogenates.

Aldehydes were the most abundant volatile compounds in fresh tomatoes and

volatile contributing a green leafy odour. (Buttery *et al.,* 1987; Baldwin *et al.,* 1991b). 2-Isobutylthiazole (Peak No. 13) is the only alkylthiazole that often found in tomato fruit, and was previously reported to be formed during the tomato ripening process. (Petro-Turza, 1987; Buttery *et al.*, 1987).

4.2.1.2 Confirmation of main flavour volatiles by GC-EI/APCI-MS

In order to confirm the identified volatile compounds in tomato fruit homogenates using dynamic analysis on GC, the combination of GC-EI and APCI-MS was used to determine which compounds correspond to which ion mass in APCI-MS. Thirteen volatile compounds found in tomato homogenates were obtained by comprising their mass spectra from EI-detector with mass spectra and linear retention indices (LRI) of authentic compounds and mass spectra held in library database with two systems. The chromatogram of volatile compounds from tomato homogenates by GC-EI and APCI-MS is presented in Figure 4.13. The identified peaks from GC/APCI-EI/MS of tomato volatiles are shown in Table 4.3.

Volatile compounds monitored in tomato sample were identified and confirmed by the combination of GC-EI/APCI-MS. The chromatograms from both GC-EI and APCI-MS were very similar qualitatively in term of both the compounds detected and the relative intensities of the compounds, indicating same sensitivity to compounds between the two systems. Mass spectra from the EI-MS were represented to identify of each peak so that the ion mass of corresponding peak by the APCI-MS could be matched to that compound. Generally, the APCI ion mass is equal to the molecular weight (M) plus one, due to this technique produces the protonated molecular ion $[M+H]^+$ for almost volatile compounds. However, some alcohols can be dehydrated, resulting a $[M-H_2O^+H]^+$ (Taylor *et al.*, 2000). The major ion mass obtained from the full scan data of APCI-MS was a single compound. The ion mass of 101 and 142 from APCI-MS was represented of hexanal and 2-isobutylthiazole, respectively, corresponding the identification by matching with library mass spectra

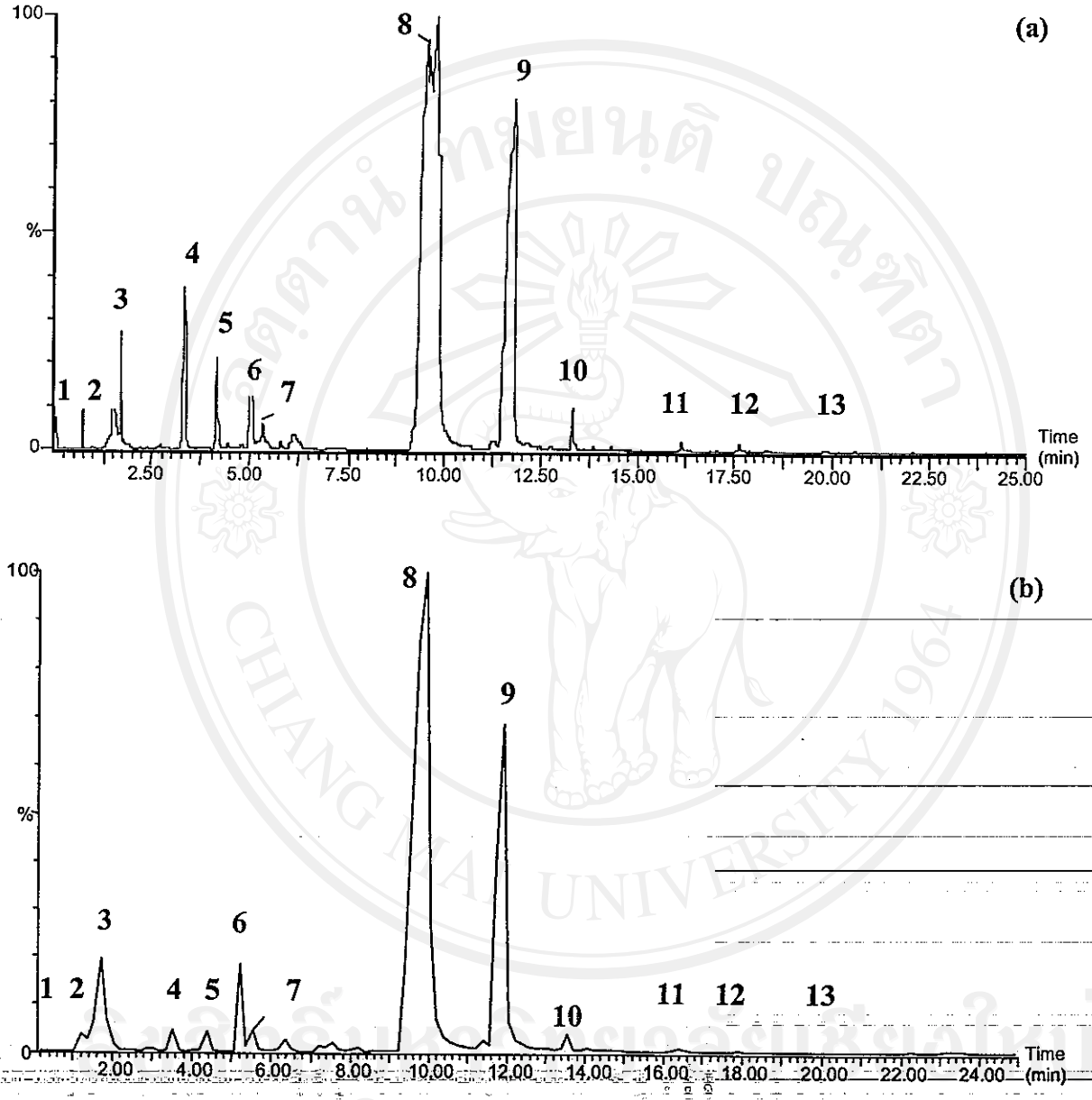


Figure 4.13 Chromatograms of volatile compounds from tomato homogenates by GC-FID (a) and APCI-MS (b). Peak numbers 1-13 were

Table 4.3 Identification GC/APCI-EI/MS peaks of tomato volatile compounds.

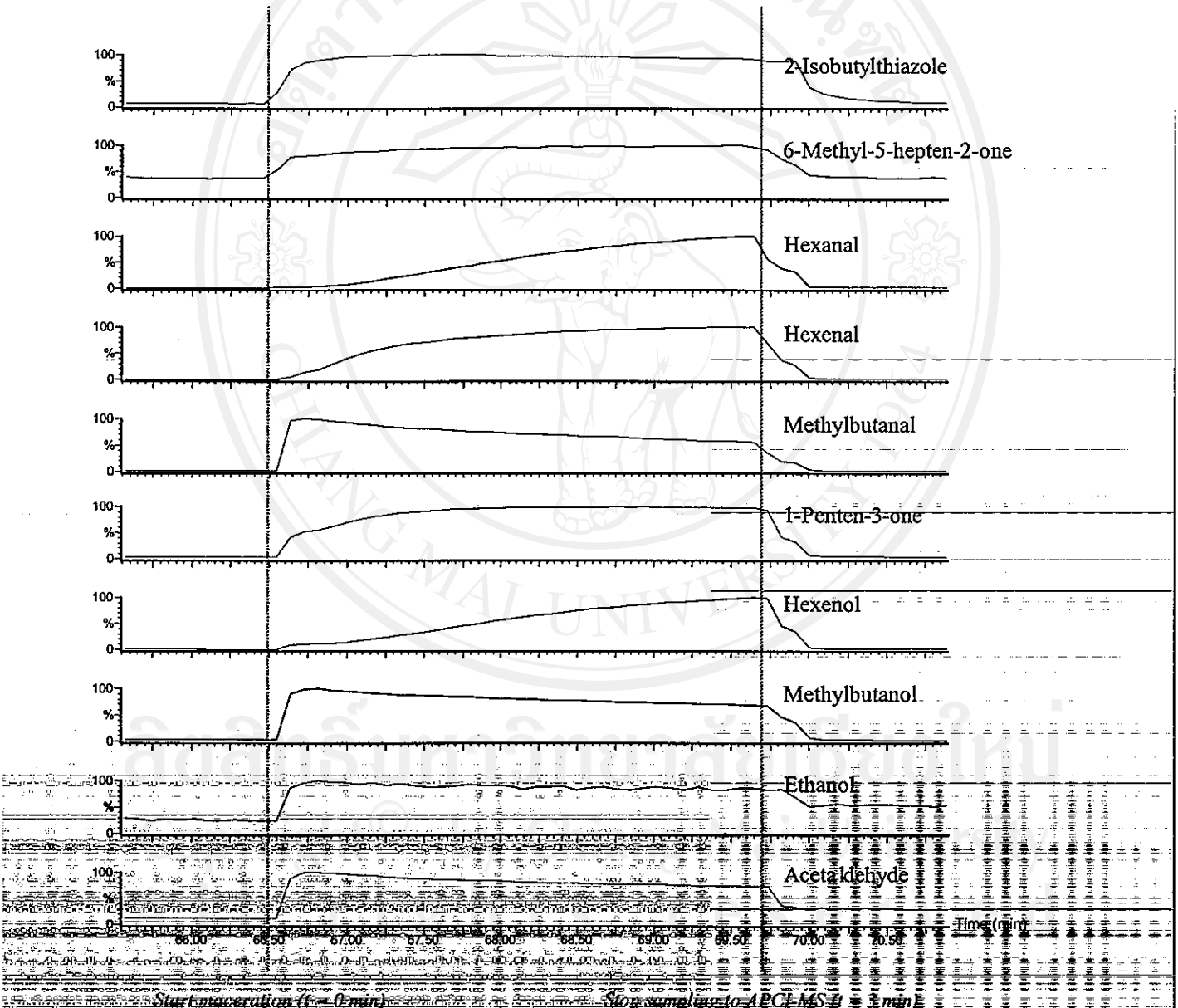
Peak No.	RT (min)	Identification compound	LRI	MW	APCI-MS mass	Confirmation[a]
1	0.96	Acetaldehyde	-	44	45	*
2	1.52	Acetone	-	58	59	*
3	1.70	Pentane	-	72	73	*
4	3.33	Ethyl acetate	602	88	89	**
5	4.15	3-Methylbutanal	633	86	87	**
6	5.04	1-Penten-3-one	667	84	85	**
7	6.73	3-Methylbutanol	722	88	71	**
8	9.88	Hexanal	803	100	101	**
9	11.23	*(E)*-2-Hexenal	834	98	99	**
10	12.14	Hexenol	855	100	83	**
11	16.12	*(Z)*-2-Heptenal	945	112	113	**
12	17.61	6-Methyl-5-hepten-2-one	978	126	127	**
13	19.80	2-Isobutylthiazole	1036	141	142	**

[a] *Symbols are as follows: *confirmation by library mass spectra, **confirmation by mass spectra and LRI of authentic standard.*

4.2.1.3 Quantification of main flavour volatiles of tomato fruit

The release profiles of main volatiles from tomato homogenates using APCI-MS are shown in Figure 4.14. Tomato was blended at t=0 minute and the release of its volatiles were monitored for 3 minutes after maceration. Ten key volatiles of tomato above the headspace were observed during this period. The shape of each volatile release was similar, rising up to the maximum intensity then slightly declined to the end of the time after maceration. Some compounds were released rapidly within 10-20 seconds after blending such as methylbutanal, methylbutanol and 6-methyl-5-hepten-2-one. It has been suggested that these compounds are formed in the intact

(such as nonenal and nonadienal) were detected in tomato fruit because the activities of some specific enzymes on the cleavage of hydroperoxide fatty acids were not favored. This result confirmed the work of Galliard and co-workers (1977) who found that only C6 aldehydes were formed by the enzymatic degradation of acyl lipid in disrupted tomato fruit.



Five ripening stages of tomato fruits from each variety were collected and analyzed the amount of the main volatile compounds. The volatile components in wild-type and two transgenic tomato fruits at various stages of ripening are shown in Figures 4.15 to 4.19.

The results were clearly shown that the amounts of 2-isobutylthiazole, 6-methyl-5-hepten-2-one and 1-penten-3-one increased as ripening proceeded from the green stage to the mature red stage (Figures 4.15, 4.16). In mature green fruits, 2-isobutylthiazole was undetectable both in wild-type and transgenic tomato fruits (Figure 4.15a). This compound occurs only in ripe tomato fruits and has not been detected in leaves or other parts of the tomato plant (Buttery and Ling, 1993). Wild-type tomato and *PG* sense suppression tomato had significantly higher levels of this compound compared to *ACO1* antisense fruit at 21 days post-breaker stage of ripening (Figure. 4.15a). In contrast, 6-methyl-5-hepten-2-one and 1-penten-3-one were detected in fruits at the mature green stage and the amounts increased with ripening. Again the amounts were significantly higher in wild-type and *PG* sense suppression fruits compared to *ACO1* antisense fruit at the ripening stage (Figures 4.15b, 4.16). The former volatile, a lycopene-derived volatile, was found at lower levels in mature green fruit with a dramatic increase during ripening, presumably due to the increased synthesis of lycopene as ripening progress. Carotenoids and lycopene were both synthesized during fruit ripening and 6-methyl-5-hepten-2-one was the main volatile from lycopene degradation (Buttery *et al.,* 1988). Since ethylene production often correlated with synthesis of pigment, it was possible that these volatiles might be regulated by the ethylene either directly or indirectly via lycopene accumulation. This result indicated that the reduction of lycopene accumulation and volatile formation is effected from low ethylene production by *ACO1* antisense fruit. In contrast lycopene accumulation was unaffected in *PG* sense suppression fruit (Smith *et al.,* 1990b). Therefore, the generation of 6-methyl-5-hepeten-2-one, the lycopene degraded compound were within the range normally found in wild-type fruit at the 21 days

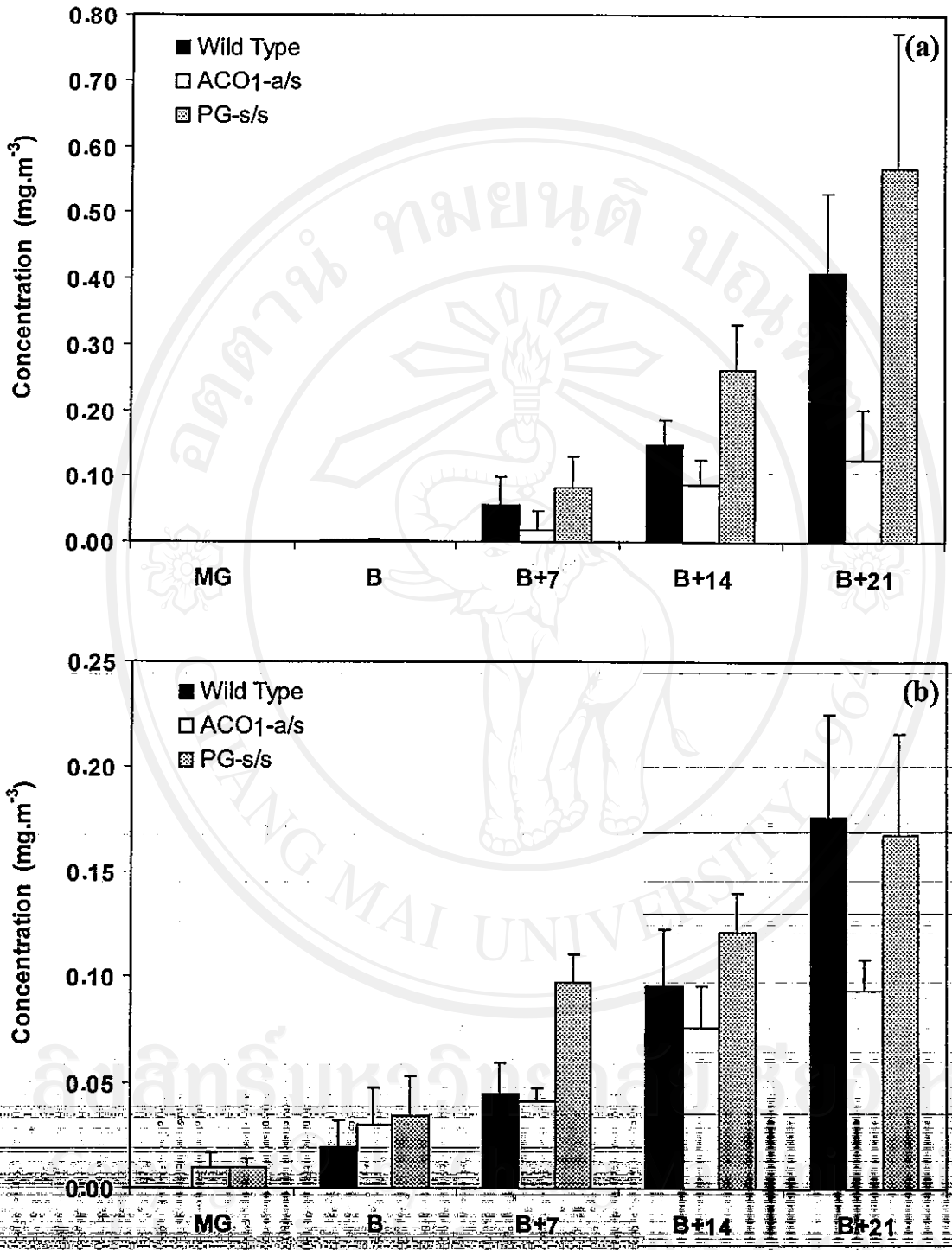


Figure 4.15 The production of 2-isobutylthiazole (a) and 6-methyl-5-hepten-2-one (b) in wild-type, *ACO1* antisense (ACO1 a/s) and *PG* sense

The origins of 1-penten-3-one were not so clear but it is reported to be an oxidative product of metabolism (Figure 4.16). Although variation was quite high (as shown by the error bars), there was a clear trend in that the fruit with decreased ethylene production showed a reduced production of all three of these compounds, over the whole ripening period.

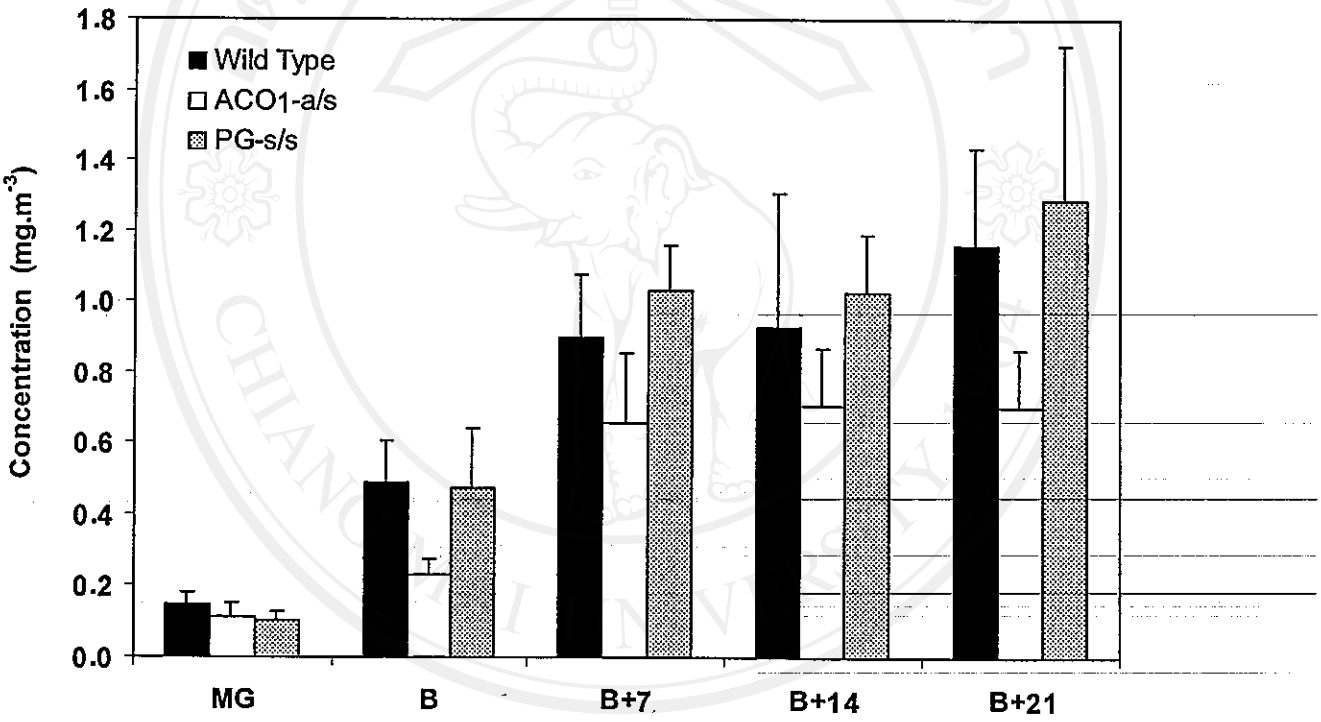


Figure 4.16 The production of 1-penten-3-one in wild-type, *ACO1*- antisense (ACO1 a/s) and *PG* sense suppression (PG s/s) fruits at the mature green (MG), breaker (B), 7 days post-breaker (B+7), 14 days post-breaker (B+14) and 21 days post-breaker (B+21) stages. *(Data are the mean of five replications, bars are ±SD).*

Figure 4.17 and Figure 4.18 show the amounts of aldehydes present in the headspace above fruit during ripening. Hexanal and hexenal are produced by enzymatic oxidation of linoleic and linolenic acids, respectively, only when the fruit is macerated (Galliard *et al.,* 1977). Hexanal and hexenal were reported to be the important flavour volatiles of tomato with high odour unit values (Buttery *et al.,* 1987; Petro-Turza, 1987; Buttery *et al.,* 1988b).

The amount of hexenal showed a steady increase with ripening while hexenal levels remained steady after the mature green stage (Figure 4.17). There were no significant differences between fruit types. This suggested that neither the amount of fatty acid substrate, nor the relevant enzyme activities in the lipoxygenase pathway are affected by the genetic changes to the low *ACO1* and *PG* fruits.

In contrast, the other two aldehydes, methylbutanal and acetaldehyde, which were formed during metabolism prior to maceration, showed significant differences between the down regulated *PG* fruit and the wild-type and ethylene suppressed fruit (Figure 4.18). Acetaldehyde concentrations from the down regulated *PG* fruit showed the greatest difference from the wild-type and *ACO1* fruits. The presence of acetaldehyde in fruits and vegetables was often associated with anaerobic metabolism. One potential explanation was that the transportation of oxygen into the fruits was reduced because the cell walls in the *PG* down regulated fruit maintain their integrity for a longer period or have altered hydration compared to the wild-type or *ACO1* antisense fruits (Smith *et al.,* 1990a). This might be decreased the amount of oxygen available and the cell metabolism compensates by shifting to biochemical pathways that generate $NADH^+$. This is a well-known phenomenon in other temporary anaerobic situations such as lactic acid formation in muscle and ethanol production in yeast. The production of 3-methylbutanal showed a similar increase but to lesser degree. Methylbutanal was significantly higher in *PG* sense suppression fruit than in wild-type and *ACO1* antisense fruits through the ripening period. The levels of these aldehydes were lower in *ACO1* antisense fruit than in wild-type and *PG* sense

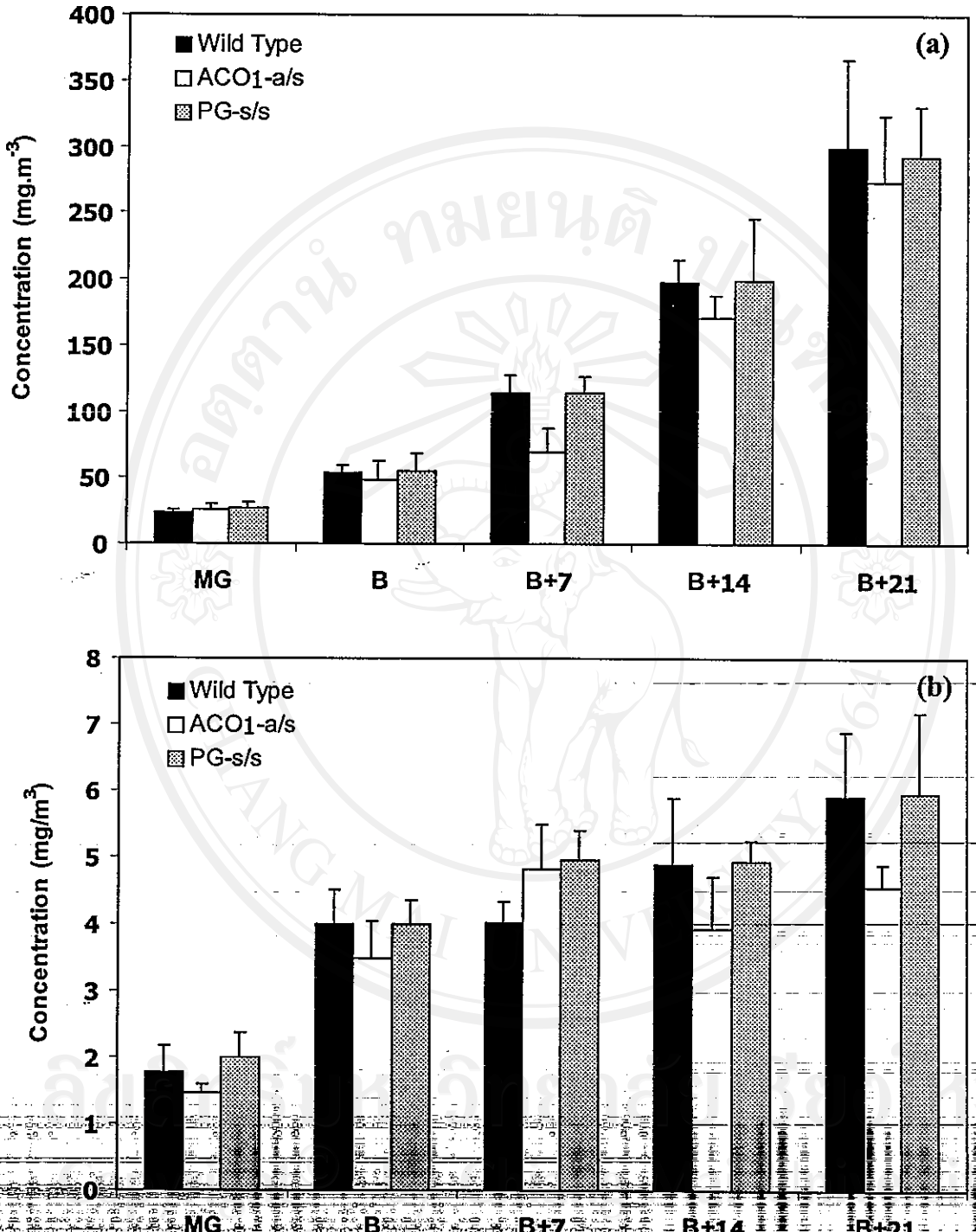


Figure 4.17 The production of aldehyde compounds; hexanal (a) and hexenal (b) in wild type, *ACO1* antisense (ACO1 a/s) and *PG* sense

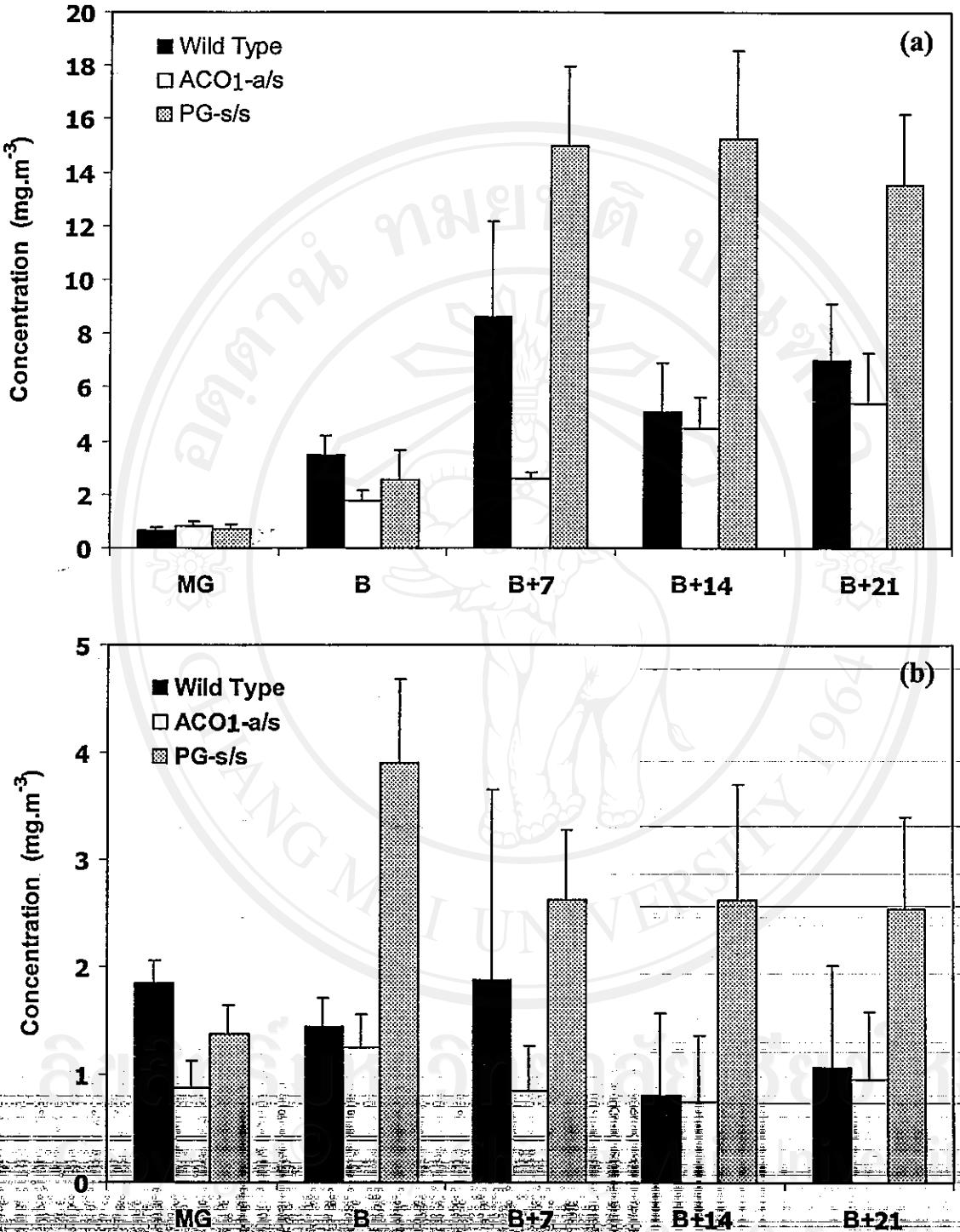


Figure 4.15 The production of aldehyde compounds; methylbutanal (a), and [illegible] (b) in wild type (cv. [illegible]), antisense (ACO1-a/s) and PG

If anaerobic condition was present in the down-regulated *PG* fruit, it might be expected that the levels of alcohols from the corresponding aldehydes might be increased by the aldehyde to alcohol conversion by the action of alcohol dehydrogenase (Eriksson, 1979). Measurements of hexenol (from hexenal) and methylbutanol (from methylbutanal) during the ripening period are shown in Figure 4.19.

Although there were minor fluctuations in hexenol concentrations in Figure 4.19a, they did not show a consistent trend or a significant difference and this reflects the trends in hexenal content (Figure 4.17b). For methylbutanol, there were again clear and significant differences between the *PG* sense suppression fruit and the other two fruits. This supports the notion that conditions in the *PG* fruit during the breaker stage were anaerobic. It was interesting to note that differences in acetaldehyde concentration could be seen at the breaker stage onwards. For methylbutanal and methylbutanol, the differences were only clear at the B+7 stage onwards.

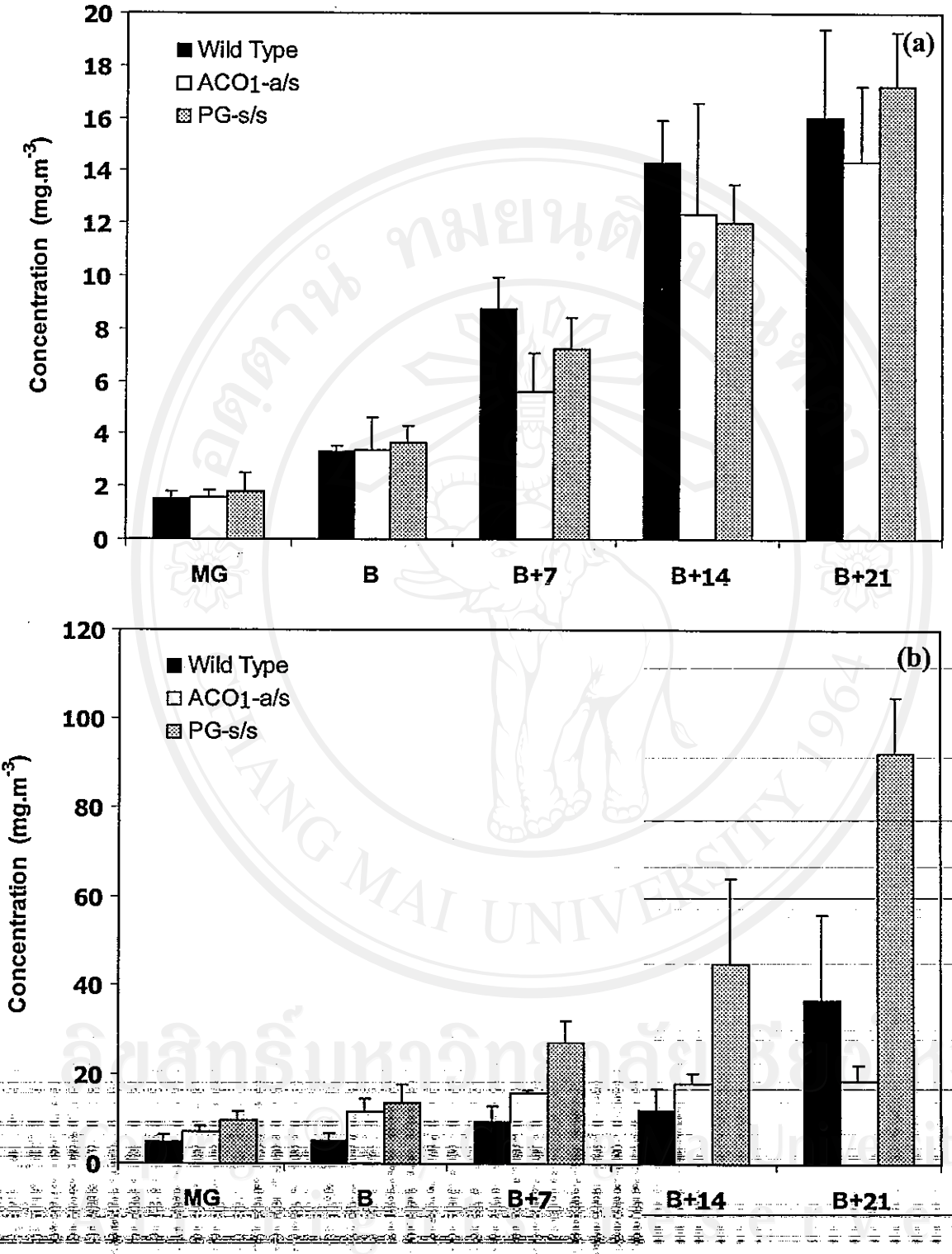


Figure 4.19 The production of alcohols; hexenol (a) and methylbutanol (b) in wild-type, *ACO1* antisense (ACO1 a/s) and *PG* sense suppression (PG-s/s) fruits at the mature green (MG), breaker (B), 7 days after breaker (B+7), 14 days after breaker (B+14) and 21 days after breaker (B+21) stages.

4.2.2 Analysis of tomato non-volatile compounds

The only difference was due to changes associated with normal ripening, such as colour development (Figure 4.20). 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 as chloroplasts are transformed to chromoplasts (Alexander and Grierson, 2002). The colour development of red colour was delayed in the onset of ripening in transgenic lines. Fruits from line *ACO1* antisense tomato showed some orange-red patches at B+14, while wild-type and *PG* sense suppression fruits were fully red at this stage (Figure 4.20).

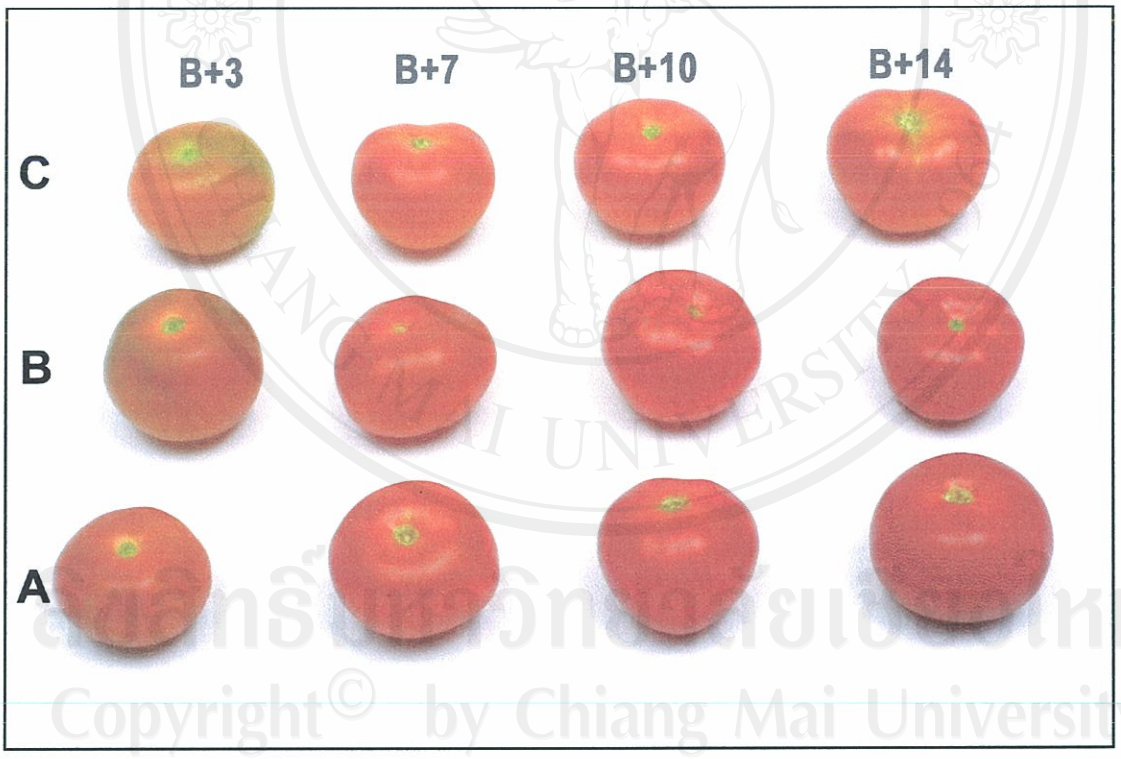


Figure 4.20 **Colour development in wild-type (A), *PG* sense suppression (B) and *ACO1* antisense transgenic tomato fruits (C); 3 days (B+3), 7 days (B+7), 10 days (B+10) and 14 days (B+14) post-breaker stages.**

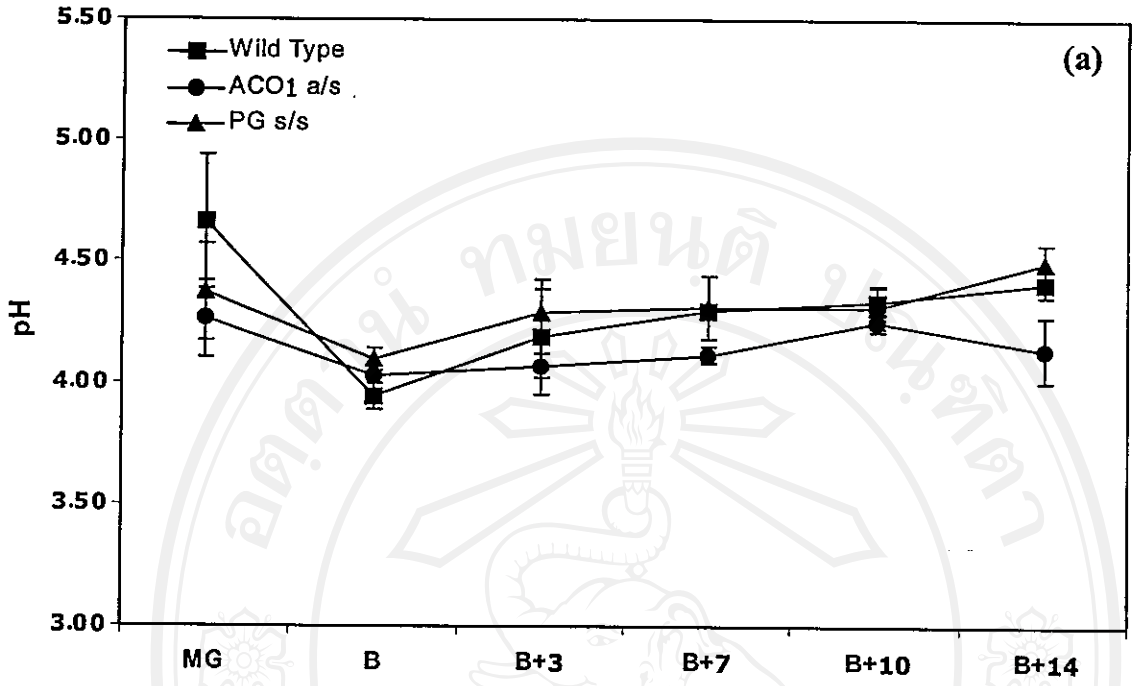
Non-volatile compositions, affected tomato taste and quality of tomato fruit, for example, sugar content (sucrose and glucose), acid content (citric and malic acids) potassium, calcium and free amino acid content (glutamine, glutamic and aspartic acids) between the different genotypes were also measured. (Figures 4.21 to 4.27).

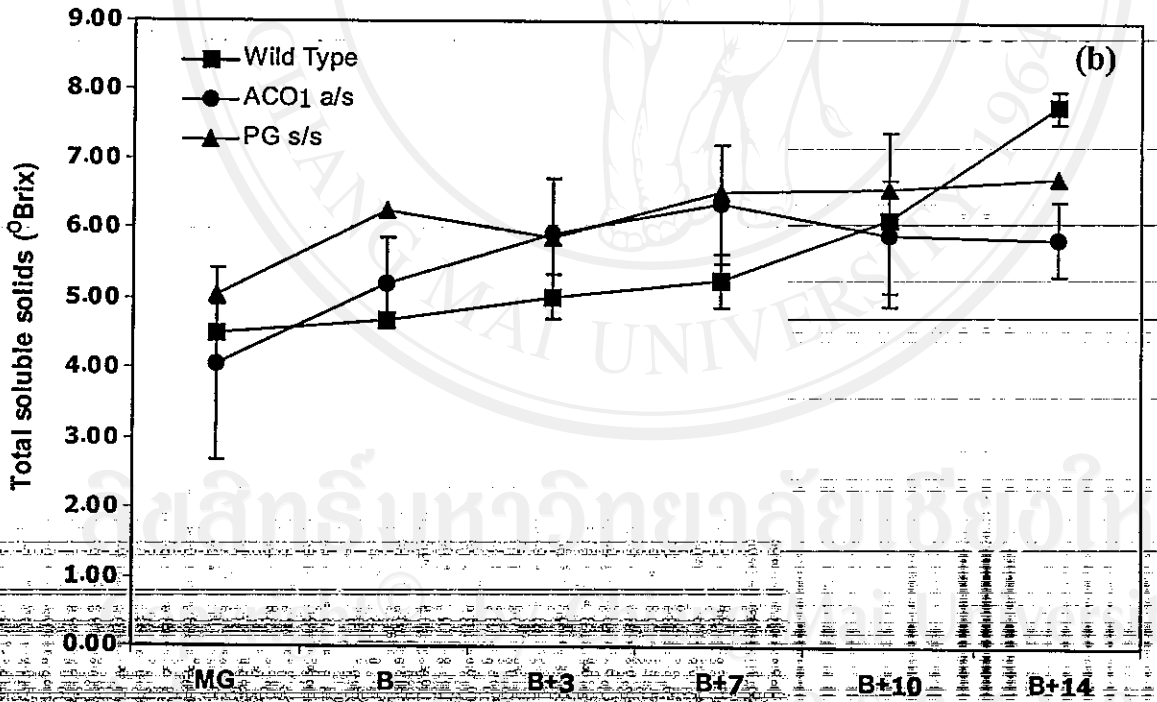
4.2.2.1 Total soluble solids and pH

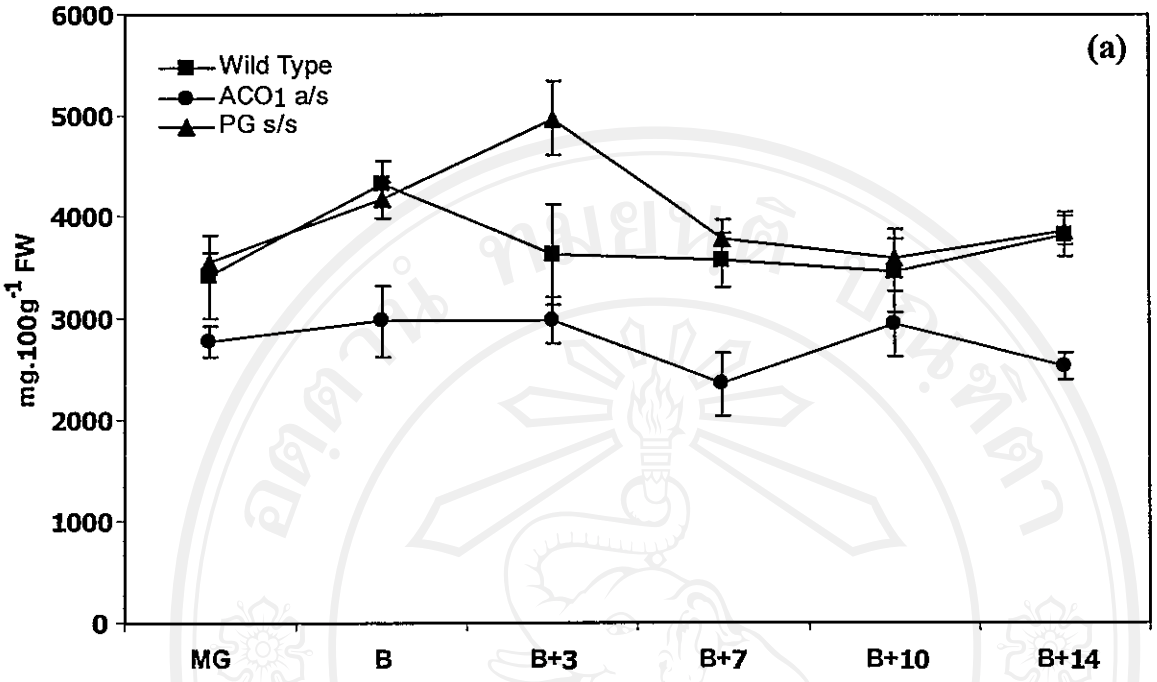
There were small differences in total soluble solids (4.0-7.5) and pH values (3.9-4.7) between the wild-type and transgenic tomato fruits (Figure 4.21). The overall range of pH for different tomato cultivars is widely from 3.9 to 4.9 (Petro-Turza, 1987). The effects of quality and quantity of organic acids composed in tomato fruit were concerned with the pH of tomato fruit. The lowest pH in both wild-type and transgenic tomato fruits were observed at the breaker stage, which were corresponding the increase of acid contents. The total soluble solids depend to a large extent on the rate of starch accumulation during the rapid growth phase of development (Hobson and Grierson, 1993). The total soluble solids showed a steady increase with ripening period in both wild-type and transgenic tomato fruits (Figure 4.21b).

4.2.2.2 Sugars and organic acids

Wild-type and *PG* sense suppression fruits had higher levels of both glucose and sucrose compared to *ACO1* antisense tomato fruit (Figure 4.22). Sugar content of tomato fruit tend to increase due either to increased sugar importation from the plant or to the mobilization of starch reserves within the fruit during ripening. As tomato ripening, sucrose concentration decreased while glucose and fructose concentrations increased, resulting from the action of enzyme invertase. This enzyme is distributed in fruit and often increases its activity during tomato fruit ripening (Hobson and Grierson, 1993). The highest content of sucrose was founded at the breaker stage and it remained low throughout the ripening period. Both sucrose and glucose levels


Wild Type
ACO1 a/s
PG s/s
(a)
pH
5.50
5.00
4.50
4.00
3.50
3.00
MG
B
B+3
B+7
B+10
B+14


Wild Type
ACO1 a/s
PG s/s
(b)
Total soluble solids (°Brix)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
MG
B
B+3
B+7
B+10
B+14



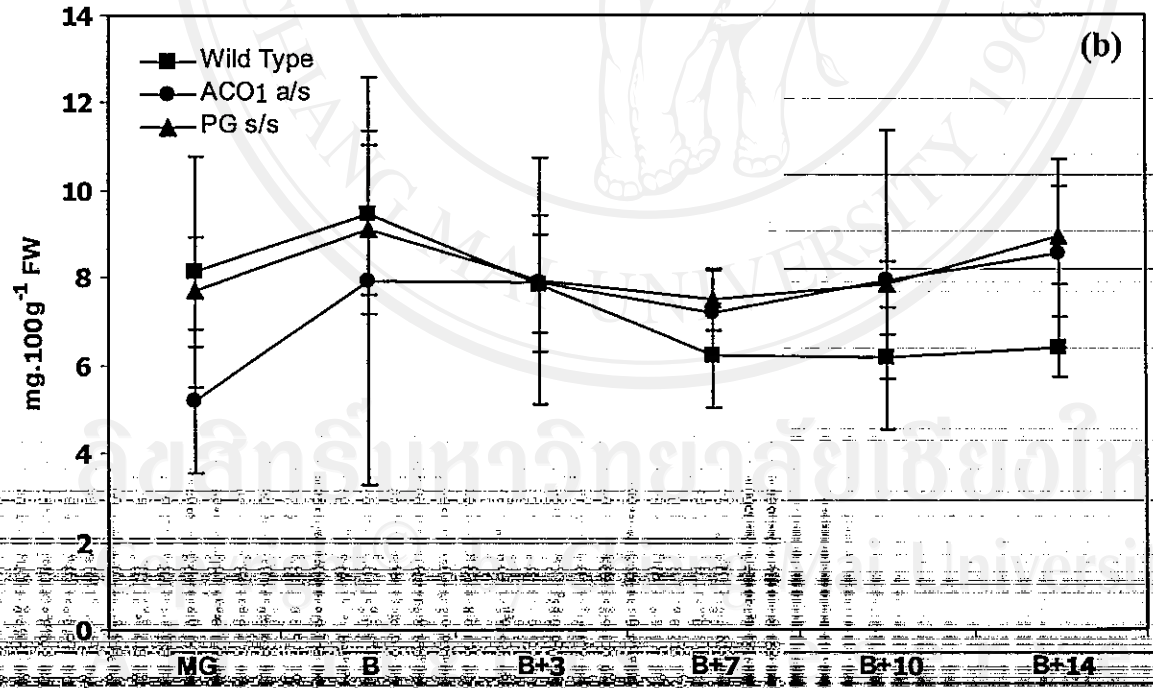
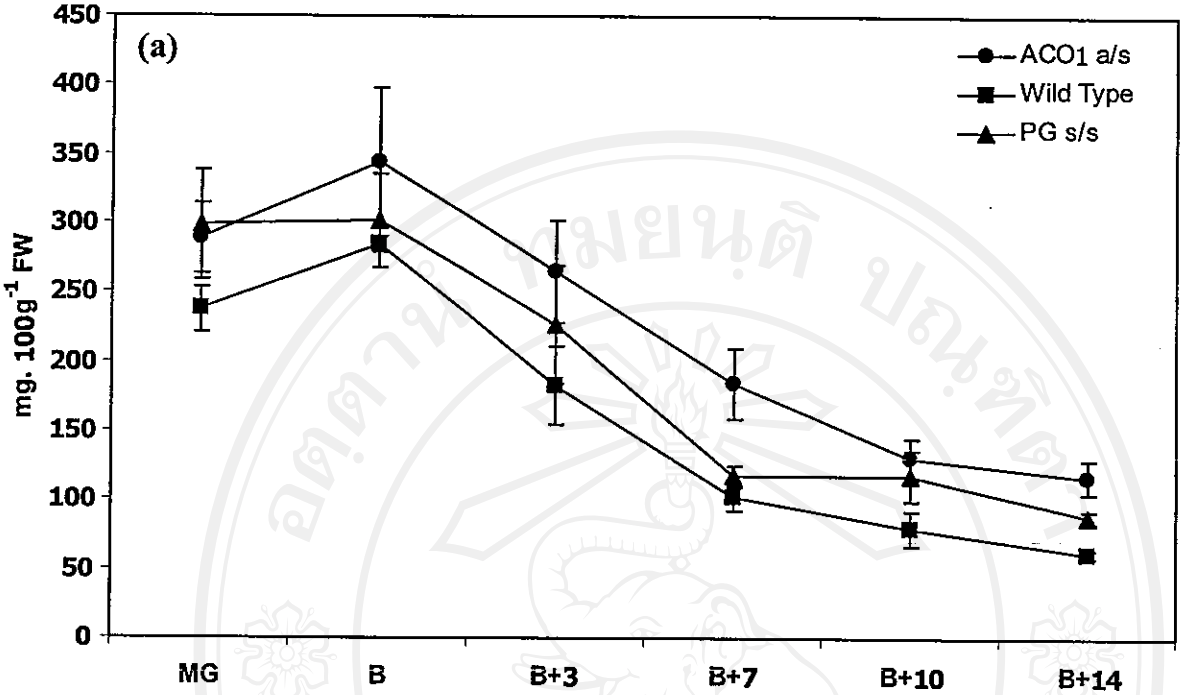
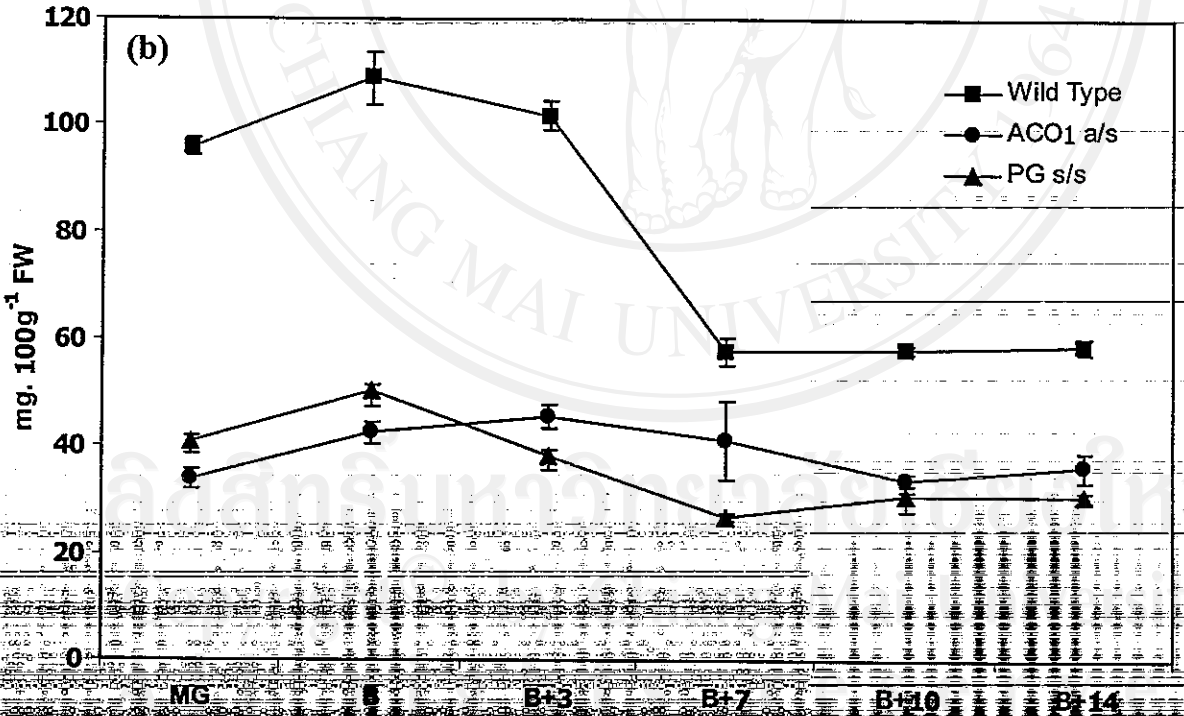


Figure 43 Chicago (a) and crossed cultivars (b) in Wild Type, PG sense

Acid contents in the three lines showed a dramatically decreased during ripening period with peaking at the breaker stage of fruit ripening (Figure 4.23), while pH values dropped to the lowest value in the range of 3.9-4.1 (Figure 4.21a). Citric acid is the most abundant organic acid in tomato fruit (Davies and Hobson, 1981; Stevens, 1986). Citric acid content of wild-type tomato decreased from 280 $mg.100g^{-1}$ fresh weight at the breaker stage to 60 $mg.100g^{-1}$ fresh weight at 14 days post breaker stage. It has been suggested that the reduction of acid content during fruit ripening involves mainly malate utilization as a respiratory substrate most likely by NADP-dependent malic enzyme and by respiration of the pyruvate (Hobson and Grierson, 1993). There were small differences but significant between the wild-type and transgenic tomato for citric acid content, with the exception of high levels of malic acid in wild-type tomato. It is interesting to note that the levels of malic acid in both transgenic tomato fruits were significantly lower than in wild-type tomato fruit. Malic acid levels in fruit types remained steady after 7 days post breaker stage of ripening.

The ratio of sugar to acid was almost equal at mature green and breaker stages, but progressive increase at final stage of ripening. The ratio of sugar to acid plays a major role in determining the taste of a tomato fruit, with high sugar-acid ratio being flavoured (Stevens *et al.*, 1977a; Hobson, 1981). In the later stage of ripening, the wild-type and *PG* sense suppression tomato fruits had more sugar-acid ratio than *ACO1* antisense tomato fruit (Figure 4.24). From this result, it would appear that the wild-type and *PG* sense suppression fruits were favoured than *ACO1* antisense fruit.


(a)
ACO1 a/s
Wild Type
PG s/s
mg. 100g-1 FW
450
400
350
300
250
200
150
100
50
0
MG
B
B+3
B+7
B+10
B+14


(b)
Wild Type
ACO1 a/s
PG s/s
mg. 100g-1 FW
120
100
80
60
40
20
0
MG
B
B+3
B+7
B+10
B+14

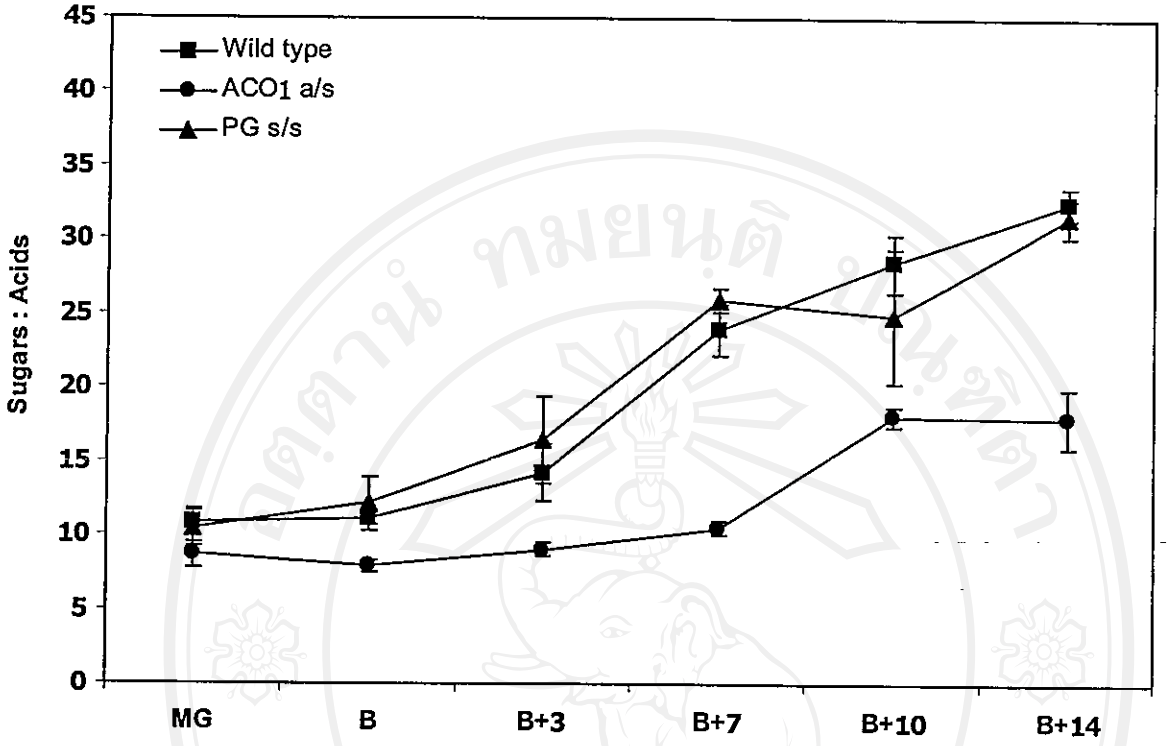
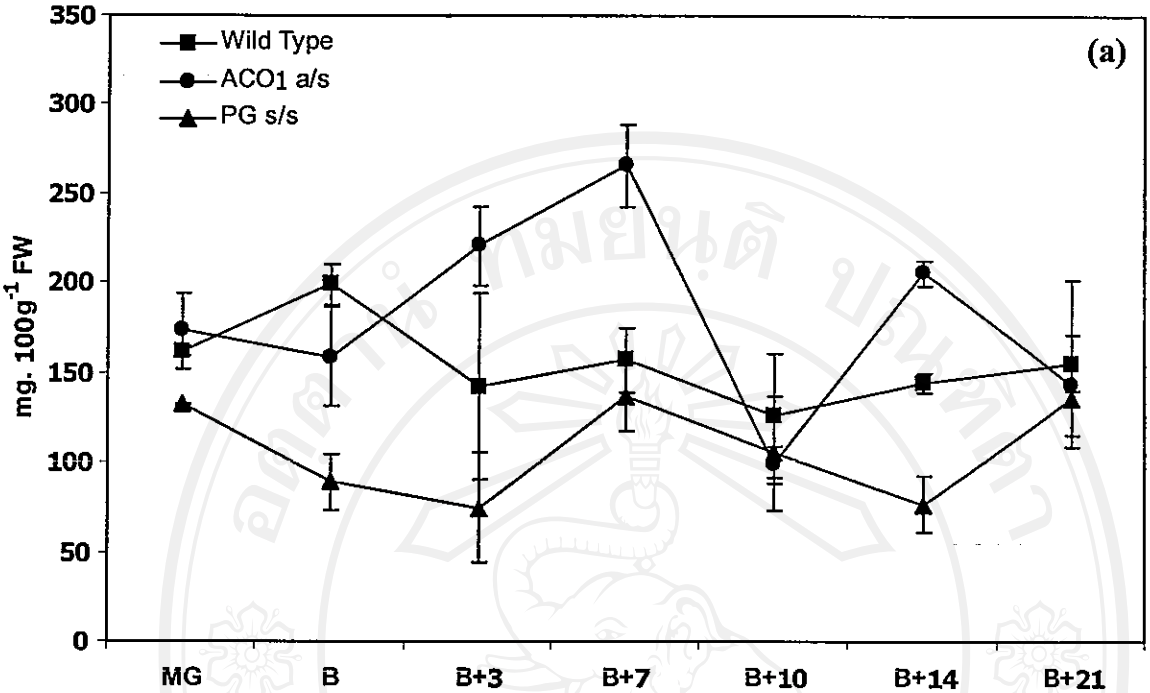
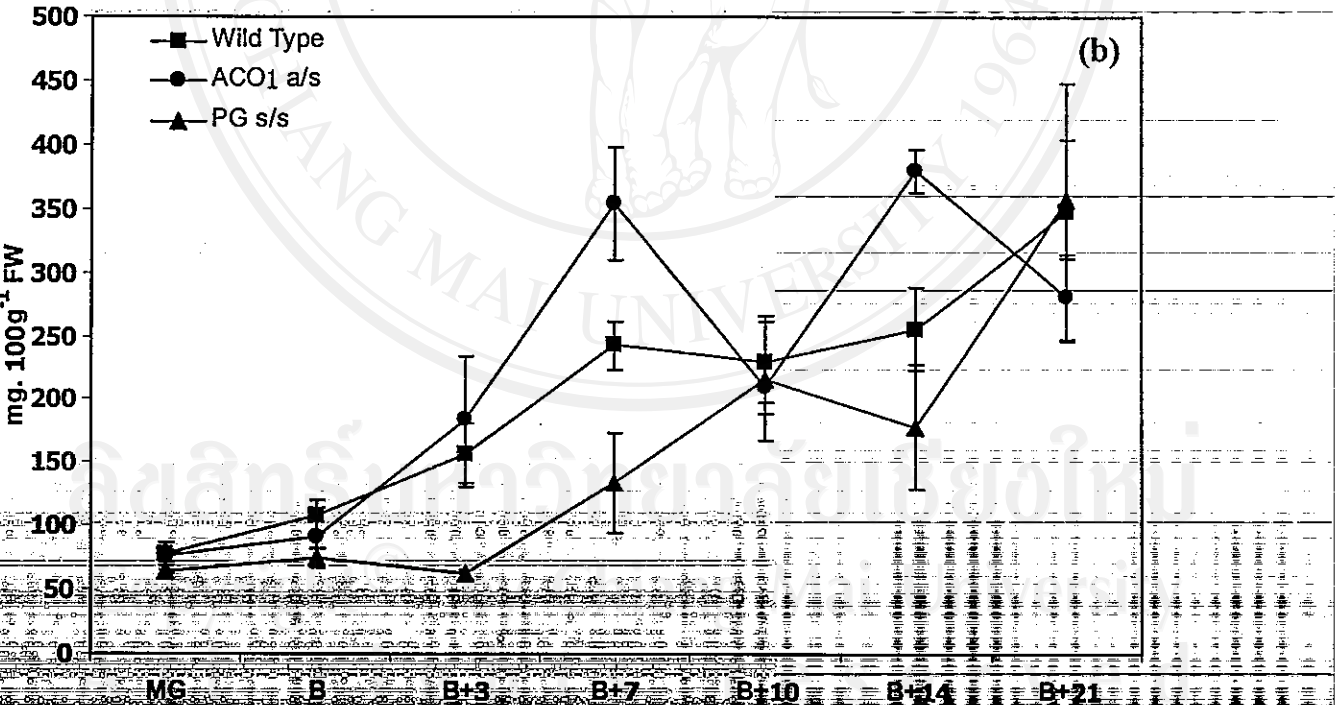


Figure 4.24 **Sugar-acid ratios in wild-type, *PG* sense suppression and *ACO1* antisense tomato fruits harvested at different stages of ripening; mature green (MG), breaker (B), 3 days (B+3), 7 days (B+7), 10 days (B+10) and 14 days (B+14) post-breaker stages.** (*Values are the mean of 5 replications; bars are ± SD)*

4.2.2.3 Free amino acids

The free amino acids are affected to characteristic taste of tomato by their own taste. Glutamic, aspartic and γ-aminobutyric acids, together with glutamine, are mainly free amino acids in fruit ripened on or off the plant, with glutamic acid making the greatest contribution (Davies and Hobson, 1981). The levels of free amino acids which were small difference between wild type and transgenic tomato fruits


Wild Type
ACO1 a/s
PG s/s
(a)
mg. 100g-1 FW
350
300
250
200
150
100
50
0
MG
B
B+3
B+7
B+10
B+14
B+21


Wild Type
ACO1 a/s
PG s/s
(b)
mg. 100g-1 FW
500
450
400
350
300
250
200
150
100
50
0
MG
B
B+3
B+7
B+10
B+14
B+21

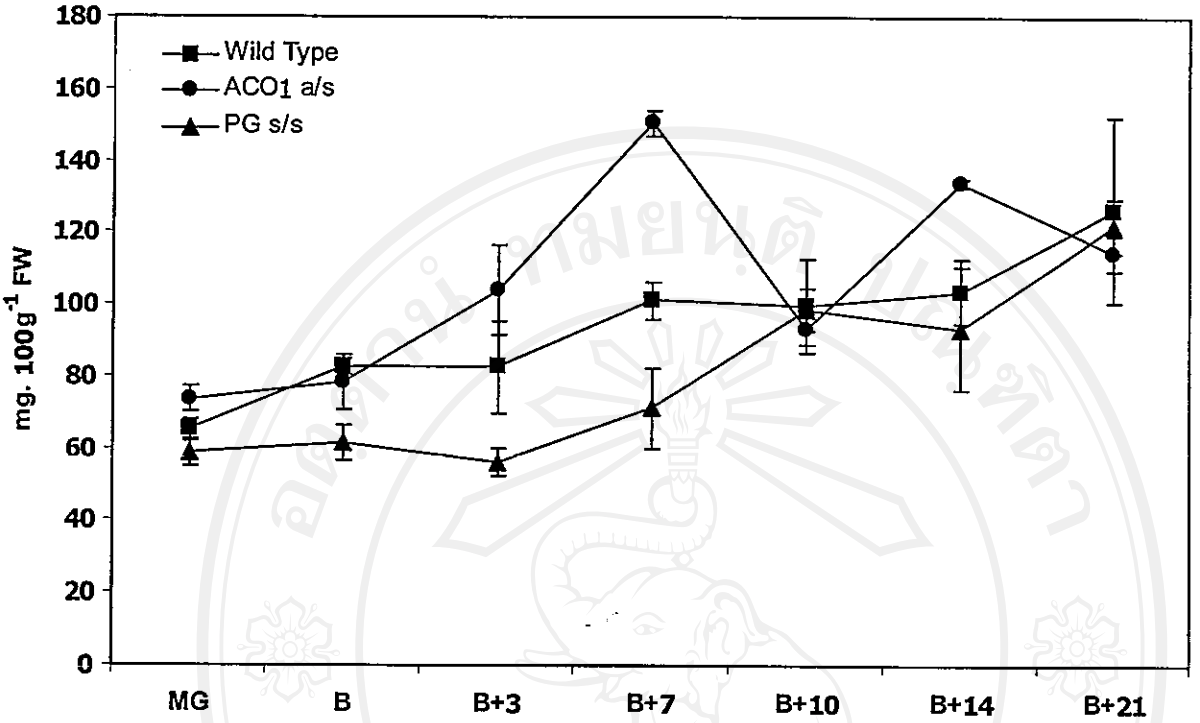
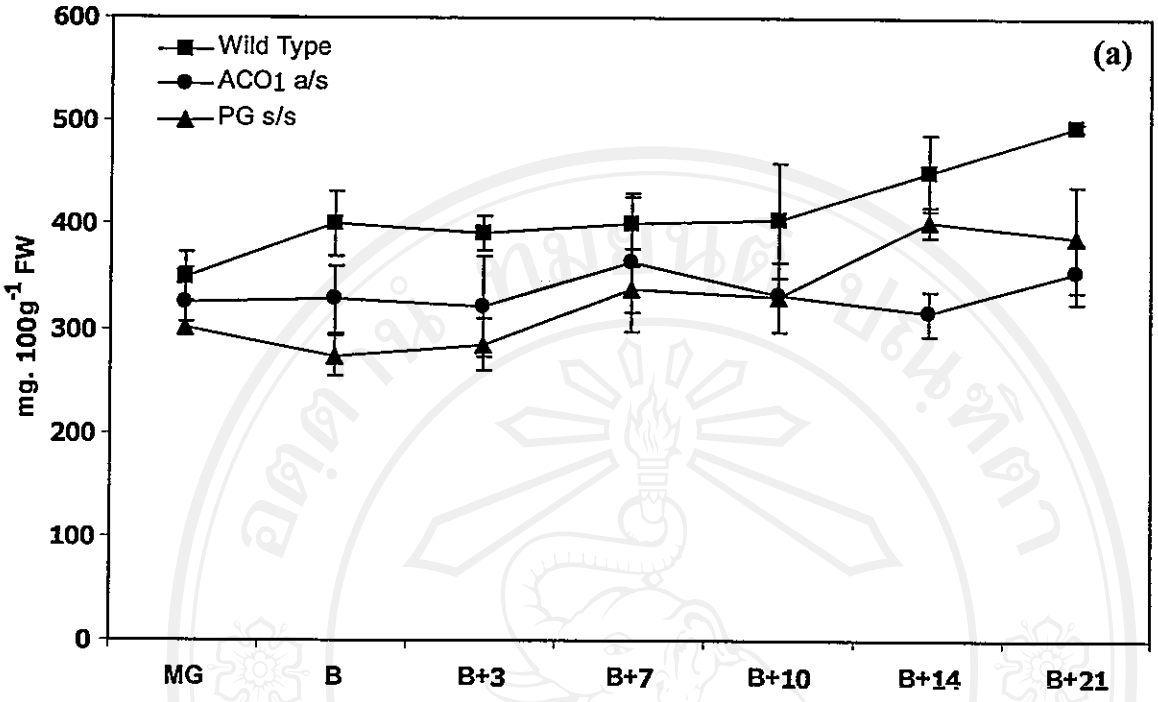


Figure 4.26 **Aspartic acid content in wild-type, *PG* sense suppression and *ACO1* antisense tomato fruits harvested at different stages of ripening; mature green (MG), breaker (B), 3 days (B+3), 7 days (B+7), 10 days (B+10), 14 days (B+14) and 21 days (B+21) post-breaker stages.** (*Values are the mean of 5 replications; bars are ± SD)*

4.2.2.4 Potassium and calcium

The levels of potassium and calcium of wild-type and transgenic fruits generally remained after the mature green stage with a slightly fluctuation throughout the fruit ripening. There were no significant differences between fruit types. Potassium and calcium contents were particular variable and values range between 300-500 and 150-300 mg 100g^{-1} fresh weight, respectively (Figure 4.27). The similar



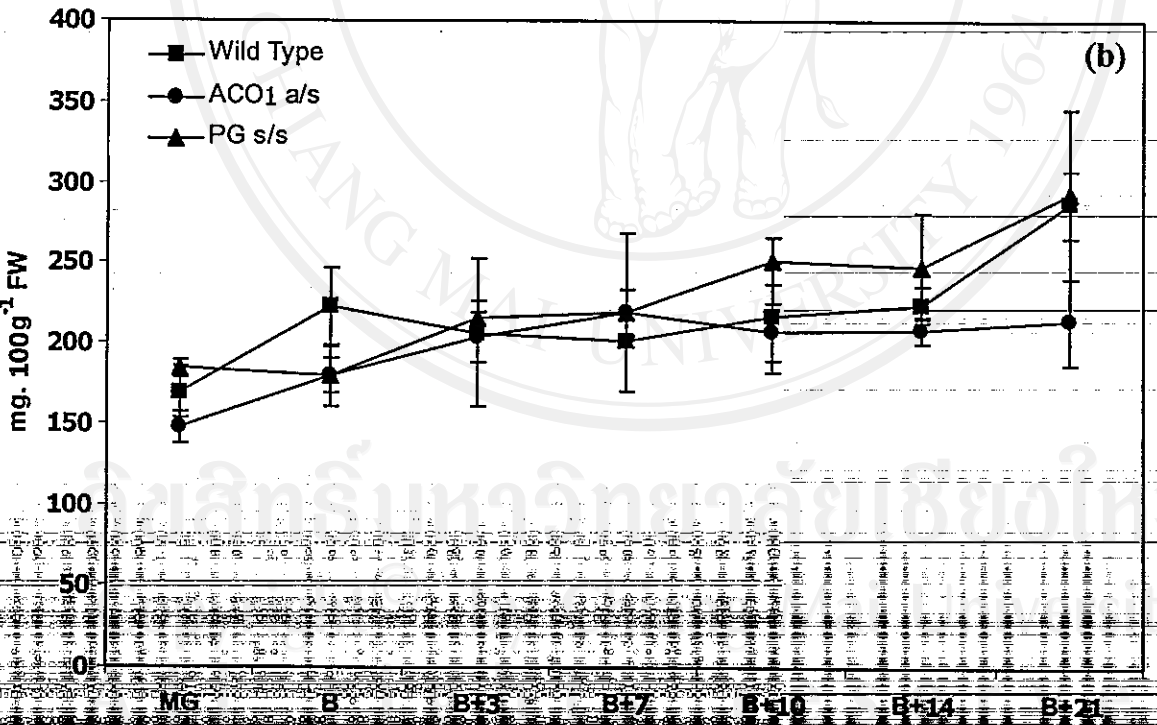


Figure 4.27 Potassium (a) calcium content (b) in wild type, *PG* sense

4.2.3 Molecular analysis

4.2.3.1 Analysis of ripening-related genes

Tomato fruit ripening involves complex biochemical changes, which finally result in alterations to the physiological changes in colour, texture, flavour and aroma (Gray *et al.,* 1994; Griffiths *et al.*, 1999a). These alterations are derived from co-ordinated expressions of many associated genes. Therefore, the study of correlation between volatile analysis and genetic molecular biology, which was focused on the expression of ripening-related genes, was carried out to obtain the information about function and regulation of aromatic generation in tomato during ripening. Links between volatile formation and its gene expression were also considered.

The expressions of ACC-oxidase 1 (*ACO1*), polygalacturonase (*PG*), alcohol-dehydrogenase (*ADH*) and lipoxygenase (*TomloxA, TomloxB, TomloxC, TomloxD* and *TomloxE*) genes in wild-type and transgenic fruits were investigated by Northern analysis due to consideration of genes. They are apparently involved in the flavour and aroma generation during ripening process. The corresponding probes, used in Northern analysis, were synthesized by *in vitro* transcription in order to generate single stranded RNA with high specific hybridisation (see section 3.6.3) that were kindly provided by the Plant Science's staffs (Don's group, Lab A49, the University of Nottingham).

Wild-type, cv. Ailsa Craig (Ac^{++}), *ACO1* antisense and *PG* sense suppression transgenic tomato fruits were used for comparing the gene expressions. The fruits at six ripening stages were collected: mature green (MG), breaker (B), 3, 7, 14 and 21 days after breaker (B+3, B+7, B+14, and B+21). The results are shown in Figures 4.28 to 4.31.

4.2.3.2 ACO1 mRNA accumulation

Ethylene, a gaseous plant hormone, is known to regulate many plant developmental processes and expressions of specific genes involved in the changes of

during ripening are presented in Figure 4.28. The expression of the *ACO1* mRNA in the *ACO1*-antisense fruit was greatly inhibited and undetectable throughout the ripening stages. This result is in agreement with Hamilton *et al.* (1990) who found that *ACO1* antisense plant correlates with the action of the expressed transgene leading to a dramatic reduction in the level of endogenous *ACO1* mRNA. In wild-type and *PG* sense suppression fruits, the gene was barely detectable in mature green fruit, but maximum accumulation was observed at the 3 days post breaker before declining thereafter. The expression of *ACO1* mRNA in *PG* sense suppression fruit was shown that there was no effect on ethylene production in low-*PG* expression fruit. In the other word, the absence of *PG* expression did not influence the ethylene production including lycopene accumulation, invertase and pectinesterase activities during ripening (Smith *et al.*, 1990b). The pattern of ethylene synthesis with a peak at between 3 and 4 days post-breaker is typical of ripening in wild-type cv. Ailsa Craig (Picton *et al.*, 1993).

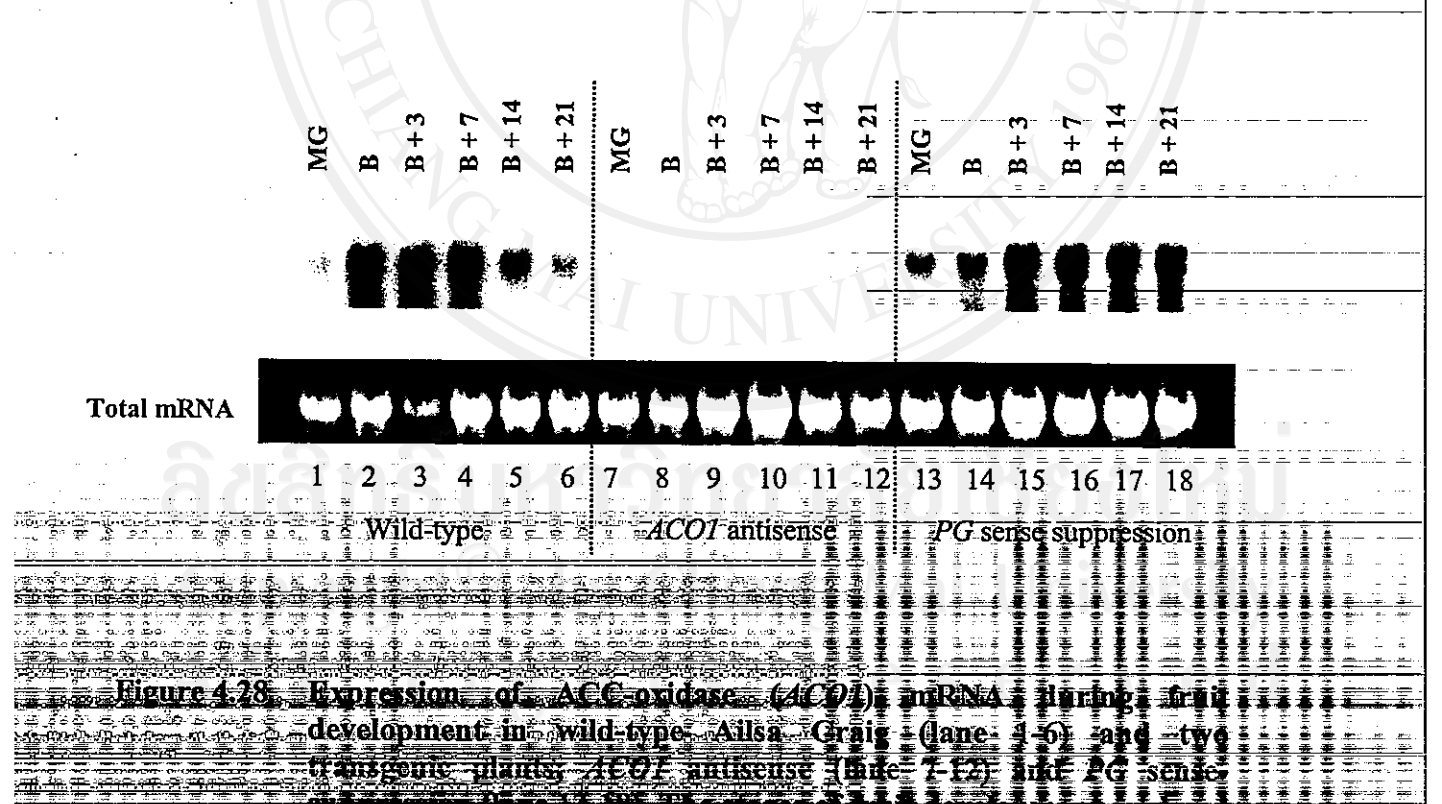


Figure 4.28. Expression of ACC oxidase (*ACO1*) mRNA during fruit development in wild-type Ailsa Craig (lane 1-6) and two transgenic plants *ACO1* antisense (lane 7-12) and *PG* sense

4.2.3.3 PG mRNA accumulation

The *PG* sense suppression fruit did not show *PG* mRNA transcript accumulation throughout fruit ripening (Figure 4.29). From the result, it is suggested that a homology dependent gene-silencing (HDGS) phenomenon occurs (Meyer and Saedler, 1996). The endogenous *PG* gene was inhibited, resulting in a substantial reduction in *PG* mRNA during ripening. The inhibition of *PG* gene expression by sense gene may result from *PG* mRNA degradation (Smith *et al.*, 1990b). The expression of *PG* mRNA in the wild-type fruit was not detected in mature green fruit but progressively increased during ripening, and reached the peak at 7 days post breaker stage before declining. The same pattern was similar to the *ACO1* antisense fruit. It has been suggested that the rate of transcription of the *PG* gene was unaltered in plants in which another antisense construct was successfully used to control *PG* gene expression (Smith *et al,* 1990a). These observations were in agreement that inhibiting ethylene production did not affect *PG* mRNA accumulation (Oeller *et al.*, 1991).

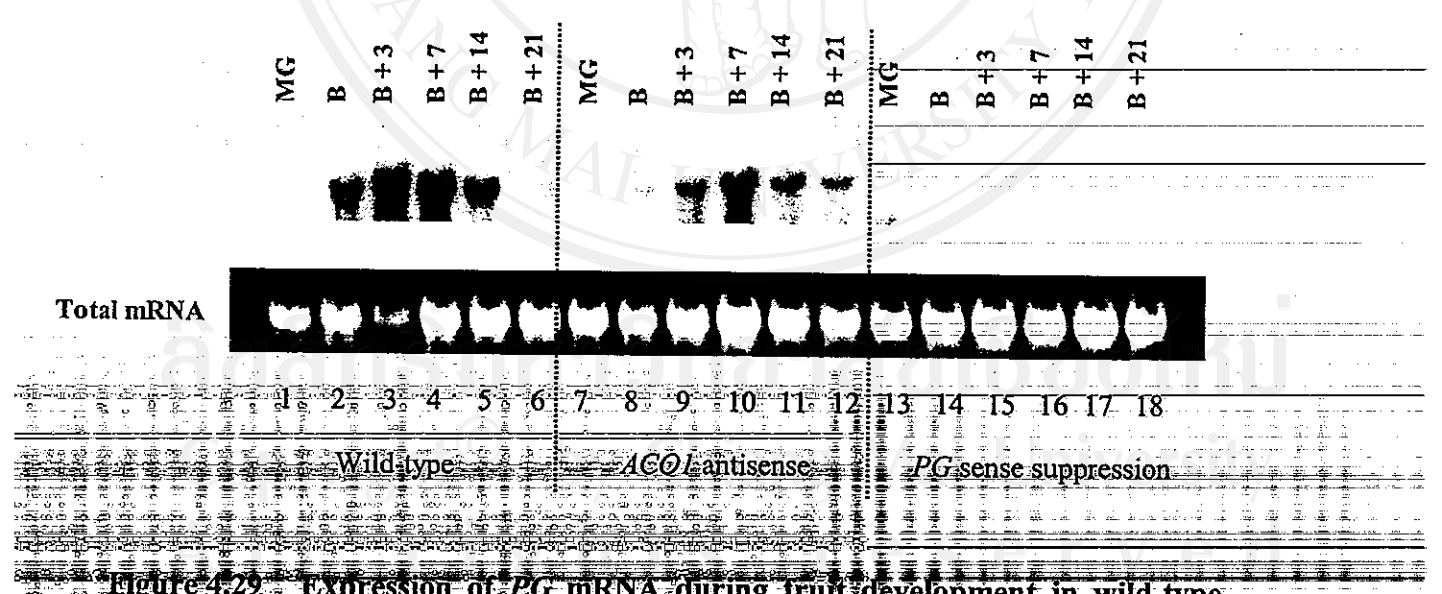


Figure 4.29 Expression of *PG* mRNA during fruit development in wild-type Ailsa Craig (lane 1-6) and two transgenic plants; *ACO1* antisense (lane 7-12) and *PG* sense suppression (lane 13-18). The stages of

However, the *PG* mRNA levels were lower in *ACO1* antisense fruit than in wild-type fruit. The low levels of endogenous ethylene in *ACO1* antisense fruit may be sufficient to induce *PG* mRNA expression.

4.2.3.4 ADH mRNA accumulation

The accumulation of *ADH* mRNA in wild-type, *ACO1* antisense and *PG* sense suppression fruits are presented in Figure 4.30. Transcripts of *ADH* showed a significant increase in both wild-type and transgenic fruits, until at the 14 days post breaker stage and then slightly declined as ripening progressed. Little difference was observed in the accumulation of *ADH* mRNA between wild-type and two transgenic fruits. *ADH* has been shown to accumulate during the later stages in ripening concomitant with the accumulation of flavour volatiles. *ADH* is involved in the inter-conversion of a board range of aldehydes to alcohols including the key aroma volatiles in tomato, hexanal, *(Z)*-3-hexenal and *(E)*-2-hexenal (Speirs *et al.*, 1998; Prestage *et al.*, 1999).

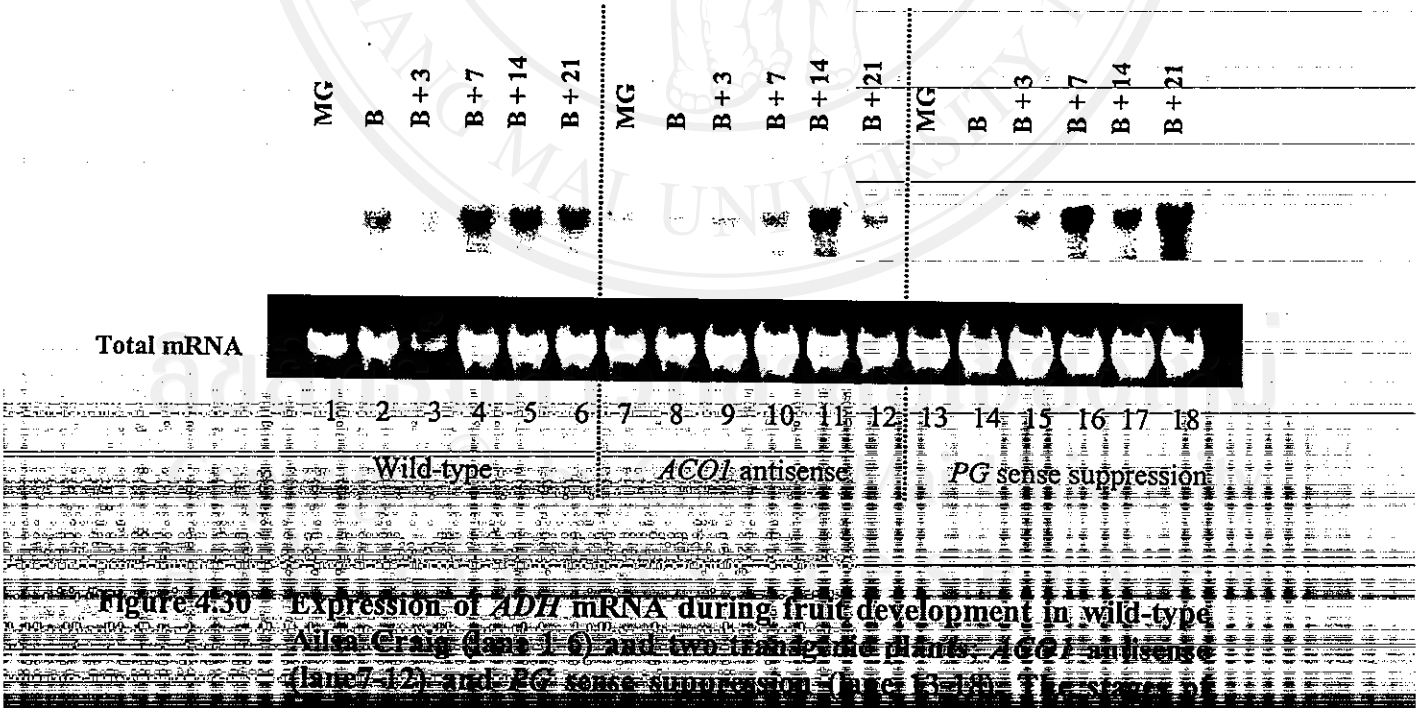


Figure 4.30 Expression of *ADH* mRNA during fruit development in wild-type Ailsa Craig (lane 1-6) and two transgenic plants, *ACO1* antisense (lane 7-12) and *PG* sense suppression (lane 13-18). The stages of

4.2.3.5 Tomlox mRNA accumulation

The expressions of the five tomato LOX clones during fruit ripening in wild-type and two transgenic fruits are shown in Figure 4.31. It has been reported that lipoxygenases are involved in stress responses, wounding and pathogen attack and flavour biogenesis via lipid oxidation pathway (Griffiths *et al.*, 1999a).

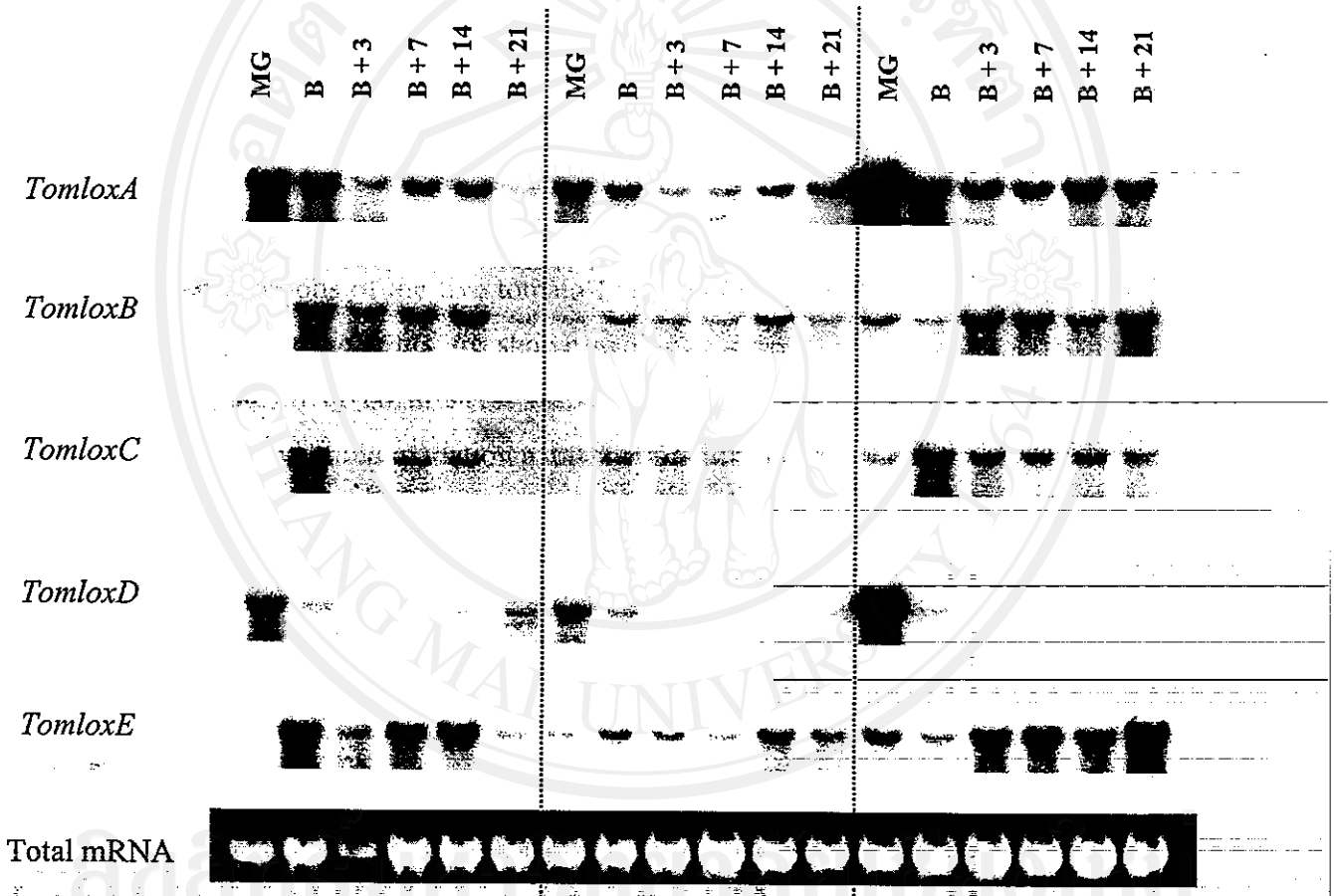


Figure 4.31 Expression of *TomLoxA*, *TomLoxB*, *TomLoxC*, *TomLoxD*, and *TomLoxE* mRNA during fruit development in wild-type Alisa Craig (lane 1-6) and two transgenic lines, *ACO1* antisense line

The expression of *TomloxA* mRNA was the most abundant in mature green fruit and then declined gradually afterward. *TomloxA*, which may provide the essential defense components, involved in a defense signaling for pathogen attack in unripe fruit, shows to be expressed during fruit ripening, with maximum expression at the breaker stage of fruit development and also in germinating seeds (Ferrie *et al.*, 1994). *TomloxA*, like *TomloxC*, may be regulated by an ethylene-independent pathway (Griffiths *et al.*, 1999a).

TomloxB mRNA was detectable only in ripening fruits. While the accumulation was presented at low levels in mature green fruit, the expression was broken through reach the highest-levels at the breaker stage and then declined thereafter. A similar transcription pattern was observed in wild-type and *ACO1* antisense fruits. It has been reported that *TomloxB* appeared to be fruit-specific with maximum expression in the ripe fruit (Ferrie *et al.*, 1994).

TomloxC is a fruit ripening-specific gene, and the expression was detected at the breaker and red ripe stages. The same expression was found in *TomloxE*. It has been suggested that *TomloxC* may encode the major fruit lipoxygenase involved in flavour generation (Griffiths *et al.*, 1999b). Down regulation of *TomloxC* shows a great reduction of total volatiles in tomato in particular C6 derivatives (Dr. Guoping Chen, the Don's group, personal communication).

TomloxD, involved in the octadecanoid defence signaling pathways in response to herbivore and pathogen attack (Heitz *et al.*, 1997), mainly expressed in leaves. *TomloxD* mRNA accumulation, like transcripts of *TomloxA,* reached a maximum level at the mature green fruit.

These results indicate that flavour generation in tomato fruit may be involved in collaborated performing of LOX isoforms throughout fruit developing stages. The *TomloxA* expression declines whereas *TomloxB* expression increases during fruit ripening. The *TomloxD* is expressed mainly in leaves and expressed at a very low

LOX genes, especially *TomloxC* and *TomloxE*. These results led to the reduction of volatile formation in the intact of *ACO1* antisense fruit.

4.2.3.6 Tomato lipoxygenase activity

Table 4.4 shows the LOX activity, protein content and specific activity of LOX, extracted and partially purified from both wild-type and two transgenic fruits. There was no statically different between the specific LOX activity of wild-type and *PG* sense suppression transgenic fruits during ripening. *ACO1* antisense fruit showed higher levels of specific LOX activity at the late stages of ripening. The maximal activity was observed at the breaker stage of ripening and then slightly declined as ripening progressed in consistent with the expressions of *TomloxB* and *TomloxC* in Figure 4.31. However, all tomato LOX isoforms started to express at the early stages of ripening, resulting in the highest level of LOX activity at the breaker stage. These results support an observed peak of LOX activity at the breaker stage of tomato ripening (Riley *et al.*, 1996). However, the biogenesis of volatiles is continually produced (see section 4.2.1) that might probably be generated from the other isoforms of LOX, especially *TomloxC* and *TomloxE*, which directly involved for the volatile generation. The maximum expressions of these isoforms were found in ripening fruit and trend to be continued increase throughout the ripening process.

As the ripening process development, thylakoid membranes break down as the chloroplasts are transformed to chromoplasts. It has been reported that LOX might be the trigger for chloroplast to chromoplast transition (Ferrie *et al.*, 1994). The release of fatty acids from the thylakoid membrane might be a source of LOX precursor, which may require the function of a specifically targeted LOX activity.

At the early stages of ripening, *TomloxA* and *TomloxD* accumulations are dominant. However, their accounts of the total LOX activity were declined in the fully ripe fruit. Only *TomloxE* and *TomloxC* are continued to increase up to 21 days post breaker stage and then inclined to increase throughout the ripening process.

LOX activity, protein content and specific LOX activity of wild-type, *ACO1* antisense and *PG* sense suppression tomato fruits harvested at various stages of ripening.

ments	Varieties	MG	B	B+3	B+7	B+14	B+21
	Wild-type	14.81 ± 2.29b	20.66 ± 3.56b	25.70 ± 1.62	10.23 ± 0.53b	4.59 ± 0.36c	7.46 ± 2.00
...n)	*ACO1 a/s*	26.36 ± 0.15a	10.28 ± 1.24b	18.93 ± 12.2	10.66 ± 0.12b	12.68 ± 2.19a	11.85 ± 1.89
	PG s/s	28.00 ± 1.59a	36.24 ± 8.23a	28.99 ± 1.06	16.40 ± 0.66a	8.10 ± 2.61b	8.89 ± 4.34
	Wild-type	38.72 ± 11.77b	14.00 ± 2.00b	17.37 ± 4.54b	20.03 ± 4.63b	26.47 ± 7.55a	28.05 ± 1.46b
	ACO1 a/s	21.62 ± 12.82c	19.37 ± 11.8ab	12.10 ± 6.30b	8.30 ± 1.76c	13.06 ± 4.46b	10.20 ± 2.12c
	PG s/s	65.82 ± 4.46a	31.79 ± 8.96a	30.34 ± 3.82a	28.51 ± 0.23a	28.61 ± 2.11a	45.85 ± 3.04a
activity	*Wild-type*	0.42 ± 0.38b	1.53 ± 1.48	1.62 ± 0.76	0.53 ± 0.19b	0.19 ± 0.06b	0.27 ± 0.08b
...ug^{-1}	*ACO1 a/s*	1.67 ± 1.17a	1.57 ± 1.35	1.47 ± 0.25	1.33 ± 0.29a	1.08 ± 0.52a	1.22 ± 0.39a
	PG s/s	0.43 ± 0.02b	1.21 ± 0.42	0.97 ± 0.14	0.59 ± 0.03b	0.28 ± 0.09b	0.20 ± 0.10b

...mean of 4 replications.

...same letter in the same column within each measurement are not significantly different by LSD procedure (p>0.05).