CHAPTER 3 : MATERIALS AND METHODS

3.1 The study of lipid oxidation pathway for volatile generation in the model of cucumber

3.1.1 Cucumber samples

Fresh cucumbers *(Cucumis sativus*, Spain origin) were purchased from Sainsbury's supermarket and washed with tap water prior to use. Cross-sectional slices (average weight 70 g, 1 cm thick/4 pieces) taken at least 3 cm from the ends of cucumber were provided for identification by GC-MS and quantitative analysis by dynamic headspace method via APCI-MS.

3.1.2 Emulsion preparation

3.1.2.1 Lipid substrates

Linoleic and linolenic acids were 99% pure (free amino acid) and used without further purification. Substrates were stored under nitrogen, protected from light and stored at –20°C after first opening.

3.1.2.2 Buffer

0.1 M Sodium phosphate buffer pH 6.5 was freshly prepared from stock solutions (1.0 M Na_2HPO_4 and NaH_2PO_4, prepared weekly and stored at 4°C).

3.1.2.3 Emulsifying agents

Tween 20 (Polyoxyethylene sorbitan monostearate) was stored under nitrogen, protected from light and kept at room temperature for up to 6 months. Lecithin (Lec

3.1.2.4 Substrate emulsion stock solutions

Substrate emulsion stock solutions, corresponding to substrate:emulsifier volume ratio of 1:1 were obtained by adding deionized water to a mixture of substrate and emulsifying agent. In this method, Tween 20 was first dissolved in 100 ml deionized water by vortexing for 3 minutes. The surfactant solution was then progressively added to the fatty acid (linoleic acid or linolenic acid) and mixed thoroughly. Substrate emulsion stock solutions were prepared on daily basis and stored at 4°C and protected from light until required.

3.1.3 Standard stock solutions

A solution of cyclohexane containing the 5 standard compounds in hexane was prepared following the routinely calibration method specifically developed for a gas phase calibration with APCI-interface (Taylor *et al.*, 2000).

A stock solution (10 ml) contained 5 volatile compounds was prepared in a solution of hexane by adding the corresponding volume for each compound as given in Table 3.1. (for preparation method see section 3.3.5).

Table 3.1 The composition of stock solutions of cucumber volatile compounds for calibration.

Compound	Mass (mg)	Density ($mg.ml^{-1}$)	Volume (µl)	Final concentration (ppbv)
Nonanal	31.679	0.825	38.4	100
(*E*)-2-Nonenal	31.201	0.846	36.9	100
(*E*)-2-(*Z*)-6-Nonadienal	30.818	0.860	33.1	100
Hexanal	22.300	0.815	137.0	500
(*E*)-2-Hexenal	21.821	0.844	51.6	200

3.1.4 GC-MS operation

Volatile compounds of cucumber were performed by purged and trapped method. The volatiles were identified by GC-MS (for methodology see section 3.3.1) on the capillary column (*DB-5, 60 m. x 0.25 mm. i.d., 1μm film thickness; J&W Scientific, USA*). The analyses were transported along the column using helium as the carrier gas, and were separated using a temperature gradient setting of 30°C for 2 minutes, then temperature programmed from 30°C to 106°C at 8°C.min^{-1} and subsequently at 6°C.min^{-1} to 200°C and held at the final temperature for 10 minutes, resulting in a 37 minutes total run time. The separated analyses were subsequently ionised via electron impact within the mass spectrometer region. Analyses identification was determined by comparing the sample mass spectra with mass spectra from authentic compounds and mass spectra in the database which were searched against the National Institute of Standards and Technology (NIST) mass spectral reference collections.

3.1.5 APCI-MS operation

Cucumber samples cross-sectional sliced of 4 pieces (70 g) were immediately placed into modified blender, which contained three outlet ports (for methodology see section 3.3.3).

Cucumber slices were blended for 30 seconds prior the headspace above the cucumber homogenates were sampled into the APCI-MS at flow rate of 1.1 ml.min^{-1} through a heated transfer line (*0.53 mm. i.d. fused silica tube "SGE" held at 160°C*) under dynamic condition of air at a flow rate of 170 ml.min^{-1}. The excess air was vented to atmosphere via the outlet port.

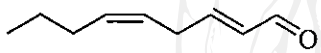
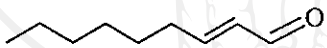
The mass spectrometer was operated both in full scan mode (mass range 40-200, scan time 1.0 sec and inter-scan delay 0.02 sec) and in selected ion recording

characteristic, ion mass (m/z) values and cone voltages used for the analysis of each compounds of interest were given in Table 3.2.

3.1.6 Data processing and calibration procedure

For the methodology see section 3.3.4 and 3.3.5.

Table 3.2 Monitored volatile compounds of cucumber with corresponding structure, molecular weight, m/z value and cone voltage.

Compound	Structure	MW	Ion mass (m/z)	Cone voltage (V)
(E)-2- Hexenal		98	99	18.0
Hexanal		100	101	12.0
(E)-2,*(Z)*-6-Nonadienal		138	139	16.0
(E)-2-Nonenal		140	141	16.0
Nonanal		142	143	16.0

3.2 The study of volatile and non-volatile compounds in tomato

3.2.1 Tomato fruits

1) Wild-type tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig)

2) Transgenic tomato transformed with an aminocyclopropane-1-carboxylic acid oxidase (*ACO1*) antisense gene construct, as described by Hamilton *et al.* (1990).

3) Transgenic tomato transformed with an expression polygalacturonase (*PG*)

3.2.2 Plant materials and growing conditions

Both wild-type and transgenic tomato plants were grown in a glasshouse at Sutton Bonington Campus, The University of Nottingham during the winter season (October 2002–March 2003) and followed the university handbook procedure for tomato plant (BBSRC Glasshouse User Guide, The University of Nottingham, 1998).

3.2.2.1 Plants of wild-type tomato

Wild-type tomato seeds were sown in compartmentalised plastic containers (*Plantpak P24*) filled with Levington peat based F2S compost (*Levington Horticulture, Ipswick, Suffolk, UK*) for two weeks in a propagation room until the first two cotyledons were appeared and large enough for handle. The seedlings were gently transferred into a 3-inch pots containing Levington F2 compost. When outgrowing, the plants were again transferred into 5-inch pots until the first truss flowers were fully formed. After the forth or fifth truss stage, the tomato plants were transferred into 10-inch pots containing Levington M2 compost mixed together with small vessels of volcanic rock (*Silvaperl, Willium Sinclair Horticulture Ltd.*) for aeration and drainage improvement in the compost. The plants were moved for their growth in the controlled-compartment room with minimum day temperature of 22°C and minimum night temperature of 18°C and 16 hours per day light supplement.

Jute garden string was used to tie bases of the stems and twisted around the stems of the plants before being attached and suspended to overhead wire supports above the plants. Because tomato plants had weak stems and were incapable of supporting any weight of fruits vertically. The string was continuously twisting around the stems as the growth progress. High potash fertiliser was continuously fed daily with a balanced 1:1:1 NPK fertiliser (*Sangral*) when the lowest truss began developing. In order to keep tomato plants growing on a single stem (cordon) to avoid a disadvantageous large vegetative growth, all side-shoots were regularly

control. In order to control the height of tomato plants, the growing tips beyond the top truss of the plants were cut after the forth or fifth truss started to flower.

3.2.2.2 Plants of transgenic tomato

Transgenic tomato plants both *ACO1* antisense construct and *PG* sense suppression construct were also grown for fruit production. Seeds of transgenic tomatoes were surface-sterilised by soaking in 50% sodium hypochlorite solution (*Smartprice, Thin Bleach, ASDA Stores Limited, Leeds, UK*) for 10 minutes and rinsed 5-6 times in sterile distilled water. Seeds were sown in MS-medium (4.4 g of Murashige and Skoog Basal Salt Mixture (1962), $30g.L^{-1}$ sucrose, adjust pH to 5.8, $8g.L^{-1}$ bacteriological agar) containing 50 ppm of kanamycin sulphate to allow seeds containing the antibiotic resistance gene germinating around 2 weeks in a tissue culture room in cycles of 16 hours of light, 24°C and 8 hours of dark.

After present of roots and the large-enough first two cotyledons, the seedlings were gently transferred to Levington F2S compost (*Levington Horticulture, Ipswick, Suffolk, UK*) and were covered with plastic bag for humidity control for 2-3 days. The plants were grown in the propagation room in the glasshouse and transferred to bigger pots following the wild-type tomato plants growing procedure as above.

3.2.3 Stages of development analysis

Tomato fruits were harvested from wild-type plants and from transgenic tomato plants for their analysis. Seven different stages of fruit ripening for flavour and biochemical analysis were picked and defined as follows. Mature-green fruits (MG), having a shiny surface but with no visible signs of colour change, were picked after approximately 50-55 days post anthesis. The fruits at this stage were shown a full size with the calyx hemisphere remaining dark green and competent to ripen. Breaker fruits (B) were classified as fruits that were just beginning to ripen and the first visible

All tomato fruits from each variety were analyzed at the same stage of ripening both volatile and non-volatile components. Five replications were usually performed for each analysis.

3.3 Analysis of volatile compounds

3.3.1 Gas Chromatograph/Electron Impact-Mass Spectrometry (GC/EI-MS)

Tomato fruits were macerated using a commercial food blender (*Philip, HR-2914*) for 1 minute and then 100 g of the tomato homogenates were placed into a 250 ml glass bottle (*Schott, Fisher Scientific, Loughborough, UK*). All volatile compounds of the tomato samples were trapped onto a stainless steel Tenax™ TA (*105 mm x 3 mm i.d.; SGE (UK) Ltd.*) by purging nitrogen gas for 20 minutes at flow rate of 30ml.min^{-1}.

The volatiles were thermally desorbed from the Tenax traps at 240°C for 10 minutes in the GC injector (*Unijector, SGE, Milton Keynes, UK*) by purging helium gas at column head pressure 18 psi as carrier gas. The compounds were cryofocused onto a 400 mm region of capillary column (*BP-1, 25 m x 0.22 mm i.d., 1μm film thickness; SGE*) with liquid nitrogen. The compounds were then chromatographed *(Hewlett Packard, HP5890 Series II Gas Chromatograph)* after holding for 1.50 minutes. After desorption, the column was held at 35°C for 2 minutes, then temperature programmed from 35°C to 106°C at 4°C.min^{-1} and subsequently at 15°C.min^{-1} to 145°C and held at this final temperature for 8 minutes, resulting in a 30 minutes total run time.

The volatile compounds were detected using a MD-800 mass spectrometer (*VG Masslab, Fisons Scientific, Manchester, UK*). Mass spectra were recorded in a full scan mode and an electron impact mode at an ionization voltage of 70 eV. A scan range of m/z 35-200 with a scan time of 0.4 second and inter-scan delay time of 0.01

the National Institute of Standards and Technology (NIST) mass spectral reference collections. Lab–Base software (*version 2.22, LAB-BASE system*) was used for data acquisition analysis and controlled GC-MS system.

3.3.1.1 Kovarts linear retention indices (LRI)

The identifications of compounds were confirmed in most cases by comparing the Kovarts linear retention indices (LRI) of an alkane series (C5-C11), which run under the same operating condition and on the same stationary phase with those of the sample components. LRI could be calculated as follows:

$$LRI = 100 \times \frac{(RT_x - RT_n)}{(RT_{n+1} - RT_n)} + 100n \quad (1)$$

where, RT_x = retention time of compound of interest;

RT_n = retention time of alkane eluting immediately before compound;

RT_{n+1} = retention time of alkane eluting immediately after compound;

n = number of carbon atoms in alkane eluting immediately before compound.

Some compounds, such as acetaldehyde and acetone, eluted before C5, so it was not possible to calculate their LRI.

3.3.2 APCI-MS (Atmospheric Chemical Ionization-Mass Spectrometry) operation

The Micromass Platform II quadrupole mass analyser based detector *(Micromass, Manchester, UK)* operating in a positive ion mode for gas phase (a negative ion mode for liquid phase) was fitted with a specifically designed air-

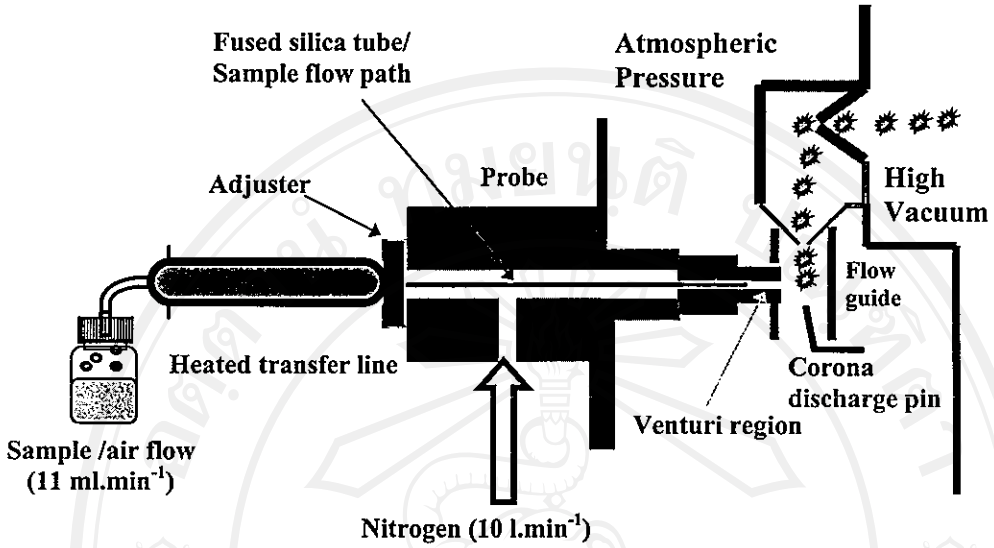


Figure 3.1 Schematic diagram of the APCI system.
(Adapted from Micromass, Manchester UK.)

Gas was continuously pulled into the APCI-MS source by mean of a Venturi effect caused by a high flow rate of nitrogen (10 l.min^{-1}). The analyses were sampled to APCI at flow rate of 11.0 ml.min^{-1} through a heat transfer line *(0.53 mm i.d. deactivated fused silica tube, SGE)* held at 160°C to prevent water and volatile condensation. For headspace analyses, fused silica was placed directly into the headspace above the sample. Volatile compounds were ionized by a 4 kV corona discharge pin at cone voltage range 12-37 volts in the ionization chamber before they were extracted orthogonally from the sample cone into the high vacuum region of the hexapole transfer lens by the extraction cone where they were then extracted into the quadrupole mass analyzer. Because resolution was entirely relied on a mass basis, compounds producing ions with the same mass could not be differentiated, including sterioisomer and positional isomer. Therefore a signal for methylbutanal represented the sum of two isomers of 2-methylbutanal and 3-methylbutanal; similarly to the

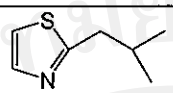
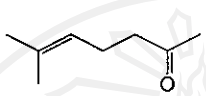
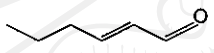
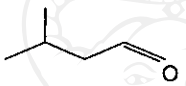
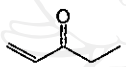
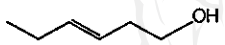
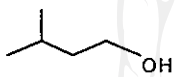
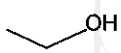
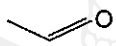
The mass spectrometer was operated either in a full scan mode (mass range 40-200, scan time 1.0 second and inter-scan delay 0.02 second) or in a selected ion-recording mode (dwell time 0.50 second, inter-channel delay 0.02 second). This technique allows real-time monitoring of several compounds simultaneously. Ten different volatile compounds with different chemical and biochemical origins as well as their contribution in tomato volatiles were monitored. The characteristics, ion mass (m/z) values and cone voltages used for the analysis of each compound of interest were given in Table 3.3.

3.3.3 Maceration device

Tomato fruit was blended in the maceration device modified from a commercial blender *(Philips, HR2810/A)* developed by Boukobza *et al.* (2001). This technique is a rapid method and high reproducibility for measurement of volatiles from tomato fruit and monitoring of volatile release in real time during maceration. The maceration device has 355 ml of total volume with three Swagelok bulkhead fittings located on the plastic blender jar. The left and right fitting were connected to 4 mm o.d. of PTFE lines for flushing air through the blender. The center aperture was fitted with a septum for addition of substrate or enzyme solution via a syringe (Figure 3.2).

The small metal box inside was constructed around the outlet aperture to prevent any juice being sucked into the APCI, which could result in block in the fused silica tube and damage to the ionization source (Figure 3.2).

Table 3.3 Monitored volatile compounds of tomato with corresponding structure, molecular weight, m/z value and cone voltage.

Compound	Structure	Molecular Weight	Ion mass m/z	Cone voltage (V)
2-Isobutylthiazole		141	142	37
6-Methyl-5-hepten-2-one		126	127	20
Hexanal		100	101	12
(*E*)-2-Hexenal		98	99	18
3-Methylbutanal		86	87	18
1-Penten-3-one		84	85	18
(*Z*)-3-Hexenol		100	83	18
3-Methylbutanol		88	71	26
Ethanol		46	47	18
Acetaldehyde		44	45	15

The tomato sample was placed inside the blender and then properly sealed. The headspace within the blender was continually flushed with air (170 ml.min^{-1}) through the inlet-side, in order to rapidly remove the volatiles formed. The airflow, which carried all volatiles formed, was continuously sampled into the APCI-MS at a flow rate of 11.0 ml.min^{-1} through a heated transfer line (0.53 mm i.d. fused silica tube) held at 160°C. The excess airflow was vented to atmosphere. The headspace was initially monitored for about 30 seconds to obtain a baseline measure of volatiles above the intact fruit. After the baseline was a steady line, the fruit was blended for

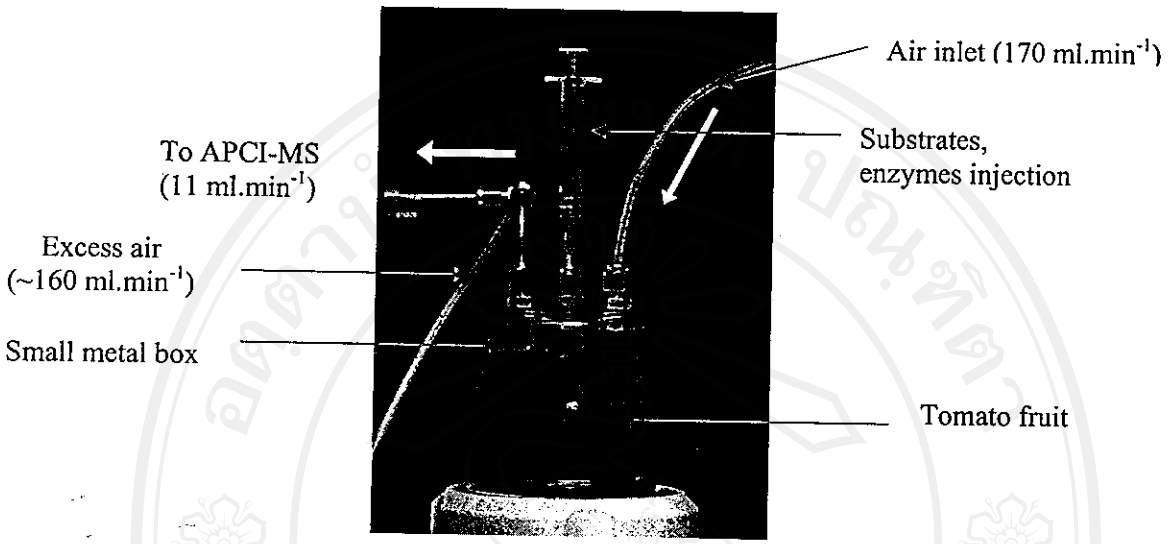


Figure 3.2 The maceration device.

3.3.4 Data processing

Data from APCI-MS analysis was collected using MassLynx software *(MassLynx 3.2, Micromass Ltd., UK, 1998)*. The intensity of ion current for each ion of interest collected by MassLynx software was copied to Excel spreadsheet *(Microsoft, USA)* and all data were processed using CDC-2000 (Cut, Delete and Calibration 2000) software developed in this laboratory. Although data collection occurred on a millisecond scale, data points were extracted every 0.2 minute in order for statistical variation to be more easily calculated at regular time points and to reduce the data file size for easier handling. The ion current intensities for each ion of interest were converted to headspace concentration ($mg.m^{-3}$ or ppbv) and were operated of 5 points smoothing algorithm and background signal subtracted at the specific time of each ion current intensity.

3.3.5 Calibration procedure

The calibration of the APCI analytical system required the generation of a gas phase calibration standard of known concentration for each chemical species. Two calibration methods based on the different polarities of the compound were used not only to calculate the concentration of each ion but also to check and correct for any change in response of the instrument.

3.3.5.1 Cyclohexane solution injection method

A solution of cyclohexane containing the 6 standard compounds, which were non-polar compounds and easily dissolved in cyclohexane were prepared following the routinely calibration method specifically developed for a gas phase calibration with APCI-interface (Taylor *et al.*, 2000).

A stock solution (10 ml) contained 6 volatile compounds was prepared in a solution of cyclohexane by adding the corresponding volume for each compound as

Table 3.4 The composition of stock solutions of tomato volatile compounds for calibration.

Compound	Mass (mg)	Density ($mg.ml^{-1}$)	Volume (µl)	Final concentration (ppbv)
2-Isobutylthiazole	31.488	0.995	31.6	100
6-Methyl-5-hepten-2-one	28.138	0.849	33.1	100
Hexanal	22.300	0.815	82.1	300
(*E*)-2-Hexenal	21.821	0.844	25.9	100
3-Methylbutanal	19.142	0.803	47.7	200
1-Penten-3-one	18.759	0.845	22.2	100

this solution was shown in Table 3.4. Both stock and working solutions were freshly prepared for each analysis.

A working solution was injected at a flow rate of 1.5 $\mu l.min^{-1}$ using syringe pump (*Harvard Apparatus 22 "55-5920", Holliston, MA, USA*) with a 10 µl syringe into the nitrogen make-up gas between the nosepiece end of the Venturi and the mass spectrometer through a septum in a "T" piece. A blank of cyclohexane was run to minimize the interference of ions from contaminants present in the solvent.

3.3.5.2 Headspace method

An aqueous solution of the standard compounds was prepared such that the headspace above the solution with concentration range of 10-100 ppm based on the air water partition coefficients of each compound. Volumes of pure standard compounds were dissolved in 100 ml of distilled water in a 250 ml glass bottle (*Schott Fisher Scienctific, Loughborough, UK*) using a flask shaker SF1 (*Stuart Scientific UK*) at 400 osc.min^{-1} for 20 minutes. Calibration was carried out by vacuum sampling the headspace above each solution into the APCI created by the

Table 3.5 The preparation of standard solutions for headspace calibration.

Compound	Final concentration in distilled water (ppm)
(*Z*)-3-Hexen-1-ol	100
3-Methylbutanol	100
Ethanol	100
Acetaldehyde	50

3.3.6 APCI/EI-MS

The combination GC with simultaneous electron impact (EI) and APCI-MS was used to confirm the identification compounds found in tomato volatiles between two systems. Mass spectra from the EI-MS were used to identify each peak, so that the ion mass of corresponding peak by the APCI-MS could be matched to that compound.

Analysis was conducted using GC with simultaneous EI-MS and APCI-MS detection (Figure 3.4). The chromatographic conditions were similar to the section 3.3.1 except that part of GC eluent was splitted using a Y piece *(SGE)* and conducted to the APCI-MS and the EI-MS sources with a deactivated fused silica tube *(0.53 mm i.d., SGE)*. Gas phase APCI-MS conditions were conducted in the same conditions as described in section 3.3.2 and were operated in a positive ion full scan mode for the mass range of 40-200 with scan time 1.0 second and inter-scan delay time 0.02 second. The path lengths led to the APCI-MS and to the EI-MS were approximately equal so that compound would arrive at about the same time both the EI-MS and the APCI-MS. Compounds were identified by LRI where authentic standards were available or mass spectral matching with NIST library.

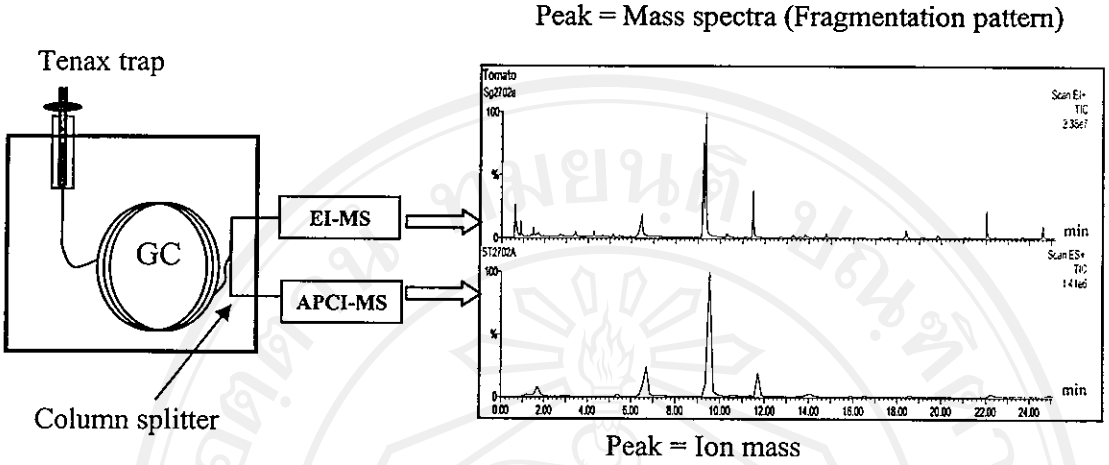


Figure 3.4 Schematic diagram of the GC with simultaneous EI-MS and APCI-MS detection.

3.4 Analysis of non-volatile compounds

A mass spectrometer equipped with a liquid chromatograph (LC-MS) interface (*Platform, LCZ, Micromass, Manchester, UK*) was used for the quantitative analysis of many different non-volatile compounds. The mass spectrometer can be run in either positive mode by adding a proton to compound of interest or negative mode that removed a proton. The monitoring and quantitative analysis of non-volatile compounds in tomato fruit were carried out according to the method developed by Davidson (2000).

3.4.1 Optimization of mass spectrometer

The optimization of LC-MS ionization was necessary performed for each individual compound, before the analysis of the non-volatile compounds composed in the tomato fruit samples took place. There were several parameters involved the [illegible]

optimize the signal for a particular compound, these parameters were adjusted and optimized maximizing the signal to noise and achieving a good analyses peak shape (sharp peak with no tailing and maximum beam stability).

The optimization process involved continuously pumping mobile phase of methanol: water (50:50, v/v) using LC-XPD pump *("PYE" Unicam Ltd, Cambridge, UK*) into the interface at an initial rate of 0.4 $ml.min^{-1}$. The standard solution was not continuously introduced into the source, but was injected in aliquots (10 µl) via a Rheodyne injection loop (*Rheodyne "7010 Cotati" CA, USA*). These compounds of interest that enter the source, were ionized and subsequently pass to the detector and the signal was recorded as a peak height or peak area on the chromatogram which proportional to the amount of compound. After this option has been tested, the sample introduction probe position was then optimized in order to obtain the maximum signal and best desirable peak shape by moving the probe from left to right and backward to forward. The optimized conditions and characteristics for all compounds used in this research were shown in Table 3.6.

In all cases of analysis, the optimum of mobile phase flow rate was 0.4 $ml.min^{-1}$, the gas flow rate was 700 $l.hr^{-1}$ and the source block and desolvation temperatures were 150°C and 400°C, respectively.

3.4.2 Standard solution

Unless otherwise stated, all chemical reagents used in this experiment were obtained from Fisher Scientific *(Leicestershire, UK).*

Table 3.6 Optimization mass spectrometry parameters for different compounds.

Compound	MW	Ionization type	Ion mass (m/z)	Dwell time (sec)	Cone voltage (V)	Capillary voltage (kV)
Sucrose	342	APCI-negative	341	0.05	18	3.00
Glucose	180	APCI-negative	179	0.05	18	3.00
Citric acid	192	APCI-negative	191	0.05	18	3.00
Malic acid	134	APCI-negative	133	0.05	18	3.00
Glutamic acid	147	APCI-negative	146	0.10	25	3.00
Glutamine	146	APCI-negative	145	0.10	25	3.00
Aspartic acid	133	APCI-negative	132	0.10	25	3.00
Potassium	39	ESI-positive	39	0.10	85	4.50
Calcium	40	ESI-positive	40	0.10	85	4.50

3.4.2.1 Standard stock solutions

Standard stock solutions were made by adding the corresponding amount for each compound as given in Table 3.7 with methanol:water (50:50, v/v). Standard mixture solutions were prepared by diluting 4 compounds (for sucrose, glucose, malic and citric acids) or 3 compounds (for glutamine, glutamic and aspartic acids) or 2 compounds (for KCl and $CaCl_2$) with methanol:water (50:50, v/v) to give five dilutions (1:2, 1:4, 1:8, 1:16 and 1:32). A 10 µl aliquot of those five dilutions of standard mixtures was injected via Rheodyne injection loop before and after tomato sample analysis took place. Standard stock solutions and mixtures were freshly prepared for each analysis.

3.4.3 Sample preparation

Ten grams of tomato fruit from seven ripening stages were blended with 90 ml of methanol:water (50:50, v/v) using Waring Blender at high speed for 3 minutes. The homogenate was filtered through Whatman No. 1 filter paper and kept refrigerated

Table 3.7 Standard stock solutions used for providence of calibration curves.

Compound	Concentration ($g.100ml^{-1}$ methanol:water, 50:50, v/v)
Sucrose	0.02
Glucose	0.20
Citric acid	0.04
Malic acid	0.01
Glutamic acid	0.20
Glutamine	0.10
Aspartic acid	0.10
Potassium chloride	0.30
Calcium chloride	0.05

An aliquot (10 µl) of methanol:water extract was injected to LC-MS (depend on type and mode of ionization) via Rheodyne injector loop with a mobile phase of methanol:water (50:50, v/v) at an initial flow rate of 0.4 $ml.min^{-1}$. The operating parameters (probe adjust, cone voltage and capillary voltage) were then altered to get the maximum signal. A dilution of extract had to make if a sample has high content of non-volatile compounds.

3.4.4 Data processing

Non-volatile contents in tomato sample were estimated by comparing the peak areas obtained from tomato sample injection with those obtained for a set of series dilution of each standard mixture solution on their calibration curves and then converting for tomato sample weight differences (see Appendix 3). Calibration curves were plotted between the peak area and the concentration of each standard compound using linear regression analysis and over the established concentration range gave good fits ($r^2 > 0.9500$). The result of non-volatile compounds analysis was expressed in mg per 100 g of fresh weight basis ($mg.100g^{-1}$ FW).

3.5 Determination of lipoxygenase activity

Lipoxygenase (LOX) activity in tomato extracts was measured using a polarographic method or an oxygen electrode, which measure the same parameter as the manometric method (Grossman and Zakut, 1979). A Clark type of oxygen sensor is a complete polarographic system in itself with the development of membrane-covered by Clark (1956) and become commercially available for a wide range of applications.

3.5.1 The Rank Brothers Clark-type oxygen electrode

3.5.1.1 Operation

Rank Brothers Ltd. *(Bottisham, Cambridge, UK)* manufactured an oxygen electrode, which is sealed into the base of the incubation chamber. Normally, it consists of a small central platinum cathode and a surrounding silver ring counter and reference anode. Conduction between the two electrodes is achieved through a saturated potassium chloride (KCl) solution used to wet the paper tissue with a 2 mm hole in its centre covering the electrodes and float this tissue paper on the KCl in the well. In order to separate the test solution from electrolyte, a Teflon membrane *(25 μm, thickness)* is placed on the top of those electrodes and fixed into place by a silicone rubber "O" ring. The incubation chamber is water-jacketed and accommodates a magnetic flea. The atmosphere can be shut off by means of a sleeve or stopper through which injection of small volumes of sample can be made. The thermostated incubation chamber is mounted on the top of a magnetic stirrer base, attached to a polarizing box. The polarizing box contains a simple circuit carrying out the two functions of maintaining the sensor at a constant negative potential and providing suitable sensitivity for electrode output to a data logger *(PICO ADC16)*.

3.5.1.2 Calibration

The [illegible] (O2) element [illegible]

$$S = K\,P(O_2) \qquad (2)$$

The proportionality constant (K) can be determined by adjusting the detector to know levels of oxygen concentration. The calibration of the 100% oxygen level can be set with a gain sensitivity control using distilled water, which has stood in air at the temperature of the experiment for several hours. Zero percent (0%) oxygen level can be achieved by adding a few crystals of sodium dithionite ($Na_2S_2O_4$), which removes oxygen from the solution. The signal should be changed very rapidly (typically giving a 90% response within 15 seconds) with a sudden changed in $P(O_2)$. After calibration, experimental samples can be pipetted into the reaction vessel. The stopper was put in position so that oxygen from the outside air cannot enter.

3.5.2 Lipoxygenases from tomato fruit

3.5.2.1 Crude extract

Lipoxygenase isozymes were extracted from both wild-type and transgenic tomato fruits. Tomato fruits of six ripening stages, MG, B, B+3, B+7, B+14, and B+21, in each variety were harvested and measured the activity of lipoxygenases. Observations of the crude extraction and partial purification of the soluble isoforms were carried out in a cold room (4°C) according to the protocols developed by Smith *et al.* (1997) and Prima-Hartley (1998). About 10 grams of frozen pericarp tissues were ground to fine powder with a mortar and pestle under liquid nitrogen and homogenized with 1.0 volume of cold extraction buffer (0.1 M phosphate buffer, pH 6.5 containing 0.1% Tritron X-100 and 1.0 mM EDTA, prepared weekly and kept at 4°C). The homogenates were centrifuged at 10,000g for 30 minutes. The supernatants were collected and filtered through four layers of cheesecloth to give the crude extracts.

3.5.2.2 Partial purification of crude extract

sulfate was added to the crude lipoxygenase extracts (320 mg salt.ml^{-1} of crude extract) and mixed properly by continuous stirring on a magnetic stirrer over 20 minutes and left the mixtures for a further 50 minutes to become equilibration. The suspension was centrifuged at 15,000g for 50 minutes and the pellets, containing the precipitated isoforms, were resuspended in 1.0 volume of 0.1 M phosphate buffer pH 6.5 containing 30% (v/v) glycerol and stored at –20°C until further use up to two weeks. The partial purified solutions were further diluted to double volumes with 0.1 M phosphate buffer pH 6.5 before determining the lipoxygenase activity (Prima-Hartley, 1998).

3.5.3 Substrate emulsions

3.5.3.1 Lipid substrate

Linolenic acid with 99% purification was used as a substrate for lipoxygenase activity assay without a further purification. The substrate was stored under nitrogen, protected from light and kept at –20°C after first opening.

3.5.3.2 Buffer

0.1 M Sodium phosphate buffer pH 6.5 was freshly prepared from stock solutions (1.0 M Na_2HPO_4 and NaH_2PO_4, prepared weekly and stored at 4°C).

3.5.3.3 Emulsifier

Tween 20 (polyoxyethylene sorbitan monosterate) was stored under nitrogen, protected from light and kept at room temperature for up to 6 months.

3.5.3.4 Substrate emulsion stock solution

Substrate emulsion stock solution, corresponding to substrate:emulsifier volume ratio of 1:1, was obtained by addition of deionized water to a mixture of substrate and emulsifier. In this method, Tween 20 (630 µl) was first dissolved in

Substrate stock solution needed for assaying lipoxygenase activity was prepared on daily basis and divided into small aliquots (1.5 ml). They were stored at 4°C under nitrogen and protected from light.

3.5.4 Lipoxygenase activity assay

The activity of lipoxygenase enzyme was measured at 25°C in a Clark-type oxygen electrode sealed into the base of a water-jacketed incubation chamber (*Clark-type oxygen electrode, Digital Model 10: Rank Brothers Ltd., Cambridge, UK*), and water circulation by water bath *("Grant" LTD6, Grant Instruments Ltd., Cambridge, UK*).

The assay was carried out by homogenising 0.75 ml of substrate emulsion stock solution and 2.05 ml of 0.1 M air-saturated phosphate buffer pH 6.5. The reaction was conducted under continuous stirring in the closed incubation chamber. Afterward, 0.20 ml of tomato extract was injected into the reaction mixture (3 ml) by a syringe through a small hole in the stopper. During the entire procedure the tomato extracts were kept in ice bath.

In a typical assay, oxygen concentration in the system was calculated by comparing the deflections observed on the gradient of the linear part of the oxygen consumption curve using Data Logger *(PICO ADC16)* between the oxygen uptake by the sample and total oxygen dissolved in the initial reaction mixture. The oxygen consumption was monitored as a function of reaction time, assuming a dissolved oxygen concentration of 240 μM per litre at 25°C and 1 atm pressure in air-saturated distilled water (Clark, 1995).

Lipoxygenase activity was calculated with 4 replications of each stage from the initial rate, and was followed from 1 to 5 minutes, depending on the lipoxygenase activity in the samples. The results were expressed in nM of oxygen consumed per ml (of reaction mixture) per minute.

Specific activity of enzyme was calculated from the activity of lipoxygenase and the total amount of all proteins presented in the sample. The [illegible]

3.5.5 Protein determination

Protein content of the tomato extracts was measured according to the method of Bradford (1976). This method relies on the change in absorption spectrum (maximum at 595 nm) of the dye (Coomassie Brilliant Blue G-250) when bound to proteins. The protonated form of the dye is a pale orange-red colour, whereas the unprotonated form is blue. On binding to protein in acid solution, the protonation of the dye is suppressed by the positive charges of amino acid solution (principally arginine) and a blue colour result. It has been found that about 1.5-3.0 dye molecules per charge bind (Stevens, 1992; Price, 1996). A calibration curve was constructed using known amounts of bovine serum albumin (BSA) as a standard protein.

3.5.5.1 Dye reagent

0.01%	(w/v)	Coomassie Brilliant Blue G-250 (C.I. 42655), 90% dye content
4.7%	(v/v)	95% Ethanol
8.5%	(v/v)	85% Orthophosphoric acid

The reagent solution was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in a mixture of 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid and then distilled water was added to bring the final volume to 1 litre. The reagent solution was filtered through Whatman No. 1 paper to remove any insoluble materials (Price, 1996). This dye reagent was stable for several weeks when kept in the dark at room temperature.

3.5.5.2 Protein standard solution (10-100 $\mu g.100\ \mu l^{-1}$)

Bovine serum albumin (BSA) used as standard protein was obtained from Sigma. Protein standard solutions were prepared in 0.1 M phosphate buffer pH 6.5 and their concentrations (10-100 $\mu g.100\mu l^{-1}$) accurately determined spectrophotometrically for 1 $mg.ml^{-1}$ BSA has A_{280} of 0.66 (Read and Northcote,

3.5.5.3 Protein assay

The assay was performed by adding 100 µl of tomato extract sample to 5 ml of the dye reagent, vortexing and leaving the reaction for 2 minutes but before 1 hour at room temperature. The absorbance at 595 nm was measured by spectrophotometer *("ULTROSPEC 4050" LKB Biochrom, Cambridge, UK)* detecting 3 ml of the reaction in a 4 ml plastic cuvette *(10x10x45 mm, "Sarstedt" Germany)* against a reagent blank without protein (prepared from 100 µl of 0.1 M phosphate buffer in 5 ml of the same dye reagent). A calibration curve from standard protein was generated using linear regression analysis and over the established concentration range gave good fits (r^2>0.9500). The protein quantification (µg) was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein content in unknown samples and was expressed in $\mu g.g^{-1}$ FW of sample. The entire experiment was repeated four times.

3.6 Extraction, purification and analysis of total RNA

3.6.1 Extraction and purification of total RNA

RNA extraction buffer

TCI-EP (1,5-naphthalenedisulfonic acid, Na-salt)	5	g	(1%, w/v)
4-Amino-salicylic acid	30	g	(6%, w/v)
Phenol mixture	25	ml	(5%, w/v)
1.0 M Tris (pH 8.5)	25	ml	(50 mM)
adjust to 500 ml with sterile distilled water (SDW)			

Phenol mixture

Phenol crystal	500	g	(69 %, w/v)
m-Cresol	70	ml	(9.6%, w/v)
8-Hydroxyquinolene	5	g	(0.7%, w/v)
SDW, Milli Q water	725	ml	(20.7%, w/v)

Chloroform	400	ml	(25%, v/v)
Isoamyl alcohol	16	ml	(1%, v/v)

- Dissolved 400 g phenol and 0.8 g hydroquinoline in 800 ml of Tris HCl and stirred for 20-30 minutes.
- Added 400 ml chloroform and 16 ml isoamyl alcohol and stirred for 15-20 minutes.
- Left for 2 hours and then removed buffer from the top layer.
- Kept in a light-protected bottle at room temperature.

Total RNA of tomato fruit was extracted by modified methods of Smith *et al.* (1986) and Hamilton *et al.* (1998). Pericarp tissues of tomato fruit samples were diced into 0.5x0.5x0.5 cm^3 cubes and frozen immediately in liquid nitrogen before storage at –80°C until required. Two to three fruit pericarps from each stage of fruit development were combined together and used as a representative for RNA extraction.

About 10 grams of frozen pericarp tissues were ground to fine powder with a coffee grinder *("BRAUN-4041", Mexico)* and a mortar and pestle under liquid nitrogen. The sample powder was transferred into a 50 ml autoclaved-capped tube and RNA extraction buffer was added to the sample powder at ratio of 1 ml extraction buffer per g fruit tissues. An equal volume of phenol/chloroform (1:1) was added and the mixture was shaken properly. The phases were separated by centrifugation *("UNIVERSAL 16A" Hettich Zentrifugen, Germany)* at 3,000 rpm for 15 minutes at room temperature. After transferring the upper phase to a new 50 ml tube, an equal volume of chloroform was added and then shaken properly. The phases were again separated by centrifugation at 3,000 rpm for 15 minutes at room temperature as before then only the upper aqueous phase was transferred into a fresh 50 ml autoclaved-capped tube. The phenol/chloroform extraction might be repeated once more, if necessary. Total nucleic acids were precipitated by addition of 0.1 volume of 3.0 M sodium acetate (pH 5.6), and an equal volume of pre-cooled isopropanol into the

ml of sterile distilled water (SDW). The resulting viscous solution containing RNA, DNA and carbohydrates was transferred to 2 sterile Eppendorf tubes and RNA were selectively precipitated by adding an equal volume of 8.0 M LiCl and incubation at −20°C for at least 1 hour. The RNA pallets were collected by micro-centrifugation (*"EBA 12" Hettich Zentrifugen, Germany)* at 13,000 rpm at 4°C for 30 minutes and rewashed by adding 750 µl of 4.0 M LiCl. The pellet was re-collected by micro-centrifugation at 13,000 rpm at 4°C for 5 minutes and then resuspended in 750 µl SDW. Any undissolved materials were removed by micro-centrifugation again. The supernatant was transferred to a fresh Eppendorf tube and RNA was further purified by the addition of 5.0 M potassium acetate for final concentration 250 mM and 2.5 volumes of ice-cold ethanol with incubation at −20°C for at least 1 hour and was collected by centrifugation as described above. The pellets were washed with 200 µl of 75% (v/v) ethanol, dried under vacuum for 15 minutes and resuspended in 30-50 µl SDW. Any remaining insoluble materials were removed by centrifugation and the final supernatant was transferred into a fresh Eppendorf tube. RNA samples were stored at −70°C until required.

The total RNA samples were quantified for their concentration by scanning at wavelength 200-320 nm using spectrophotometer *(UV-VIS Scanning Spectrophotometer "PU 8720", Philips)* and the total RNA concentrations of each sample was adjusted with SDW to approximately 2-6 $\mu g.\mu l^{-1}$.

3.6.2 Northern analysis of total RNA

Agarose formaldehyde gel

Agarose powder	3	g	(1%, w/v)
1.0 M Sodium phosphate buffer (pH 6.8)	6	ml	(20 mM)
Formaldehyde (pH 7.0)	25	ml	(8.3% v/v)
Adjust to 300 ml with SDW			

1.0 M Sodium phosphate buffer (pH 6.8)	4	µl	(4%, v/v)
10.0 mM /ml Ethidium bromide	2	µl	(2%, v/v))
SDW	25.5	µl	(25.5%, v/v)

Running buffer

Formaldehyde (pH 7.0)	200	ml	(8% v/v)
1.0 M Sodium phosphate (pH 6.8)	25	ml	(10 mM)
SDW	2250	ml	

Northern-gel loading buffer

1.0 M Sodium phosphate buffer (pH 6.8)	10	ml	(5 mM)
Glycerol	5	ml	(50% v/v)
Bromophenol blue	0.02	g	(0.2% w/v)

3.6.2.1 Formaldehyde denaturing agarose gel electrophoresis of total RNA

Twenty µg of total RNA per sample was adjusted into a volume of 12.5 µl with SDW. An equal volume of 2x ethidium bromide buffer was added and the RNA mixture was denatured by incubation at 65°C for 15 minutes in an incubation block (*Techne Dri-Block "DB-2A", Techne Cambridge Ltd., Duxford, Cambridge, UK).* After cooling on ice for 5 minutes, samples were mixed with 5 µl Northern-gel loading buffer and were fractionated onto gel electrophoresis (1.0%, w/v) agarose formaldehyde gel containing 20 mM sodium phosphate buffer (pH 6.8) and 3.7% (v/v) formaldehyde using gel electrophoresis apparatus *("GNA-200" Pharmacia, Sweden).* Gels were run submerged at a constant 100 volts for 2-3 hours with continuous recirculation of the running buffer by a Pharmacia P1 peristaltic pump *("1200-VARIOPERPEX", LKB, Bromma, Sweden).* The gels were carefully removed from the gel tray after electrophoresis and the RNA was visualised under UV illumination and photograph *(UVP Imagestore-5000, Germany).*

3.6.2.2 RNA Northern blotting

Following electrophoresis, Northern transfer of total RNA to a GeneScreen

blotting stack was shown in Figure 3.5. Following overnight transfer at room temperature, the membrane was briefly rinsed in 25 mM sodium phosphate buffer (pH 6.8) to remove any residual agarose and then placed RNA-side up on dried paper. The RNA was covalently bound to the membrane using a UV Crosslinker (*UV-Stratalinker® "2400", Stratagene, Lajolla, USA*) set to an auto-crosslink at 1200 mJ for 60 seconds while damp. Both the membrane and gel were viewed under UV light to check that RNA was transferred to the membrane. The membrane was then baked at 80°C for 1.5 hours in order to drive off any remaining formaldehyde. The dried membrane was sealed in a plastic bag and stored at –20°C until required.

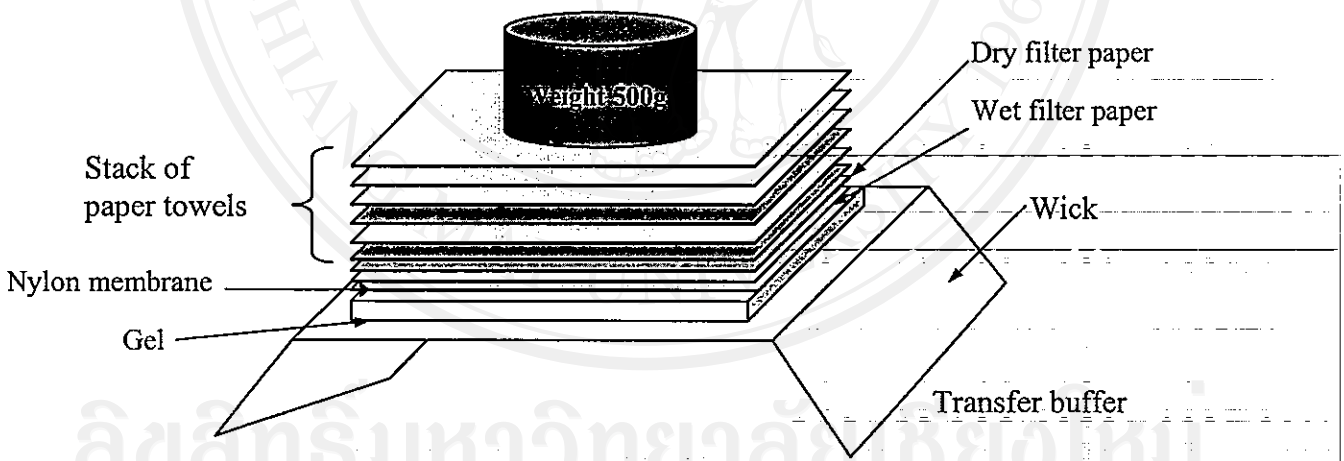


Figure 3.5 A blotting stack for Northern blotting.

3.6.3 Radioactively labeled nucleic acids as probes

3.6.3.1 Random-primed DNA probe synthesis

The method of DNA labeling depends on ability of Klenow fragment of DNA polymerase I to copy a single-stranded template DNA primed with random hexa-nucleotides at numerous sites along DNA length. DNA synthesis is initiated from these primers and radiolabeled nucleotides are then incorporated.

^{32}P-labelled specific probes generated from cDNA sequences *(TomloxA: 147-1414; TomloxB: 585-1435; TomloxC: 841-2345; TomloxD: 44-604 and TomloxE: 1-790, (NCBI, 2002))* were synthesized using the "Rediprime™ II random prime labeling system" *(Amersham Pharmacia Biotech UK Limited)* and essentially followed the manufacturer's protocol. This system allows the labeling of template DNA to the same high specific activity but at a greatly accelerated rate. This rapid labeling is achieved by using of random primers. Random priming is useful where only a small amounts of probe template is available and where high specific activities are required. However, probes are generally shorter than those made by nick translation and the template DNA remains as an unlabelled competitor in the final probe solution.

DNA fragment template was diluted to a concentration of 25 ng in 47 µl of TE buffer in a sterile Eppendorf. The DNA sample was denatured by heating at 95°C for 5 minutes in a heating block and then snapped cool by placing on ice for 5 minutes. The DNA solution was centrifuged briefly to bring the contents to the bottom of the tube. The denatured DNA solution was added to the reaction tube (containing buffered solution of dATP, dGTP, dTTP, exonuclease free Klenow enzyme and random primers in a dried, stabilized form). Five µl of Redivue [^{32}P] dCTP were added into the reaction, mixed gently by pipetting up and down about 12 times, moving the pipette trip around in the solution. The mixture was then incubated at 37°C for 20 minutes. To denature the probe, the Eppendrof lid was punctured and the tube was placed in a heating block at 95°C for 5-10 minutes and then on ice for 5

3.6.3.2 Preparation of denatured sheared salmon sperm DNA

Salmon sperm DNA *(Sigma)* (200 mg) was added to 100 ml SDW and boiled to dissolve by string in a stirrer for about 30 minutes. After the addition of 2 ml of 5.0 M NaCl, the DNA solution was extracted with an equal volume of phenol/ chloroform (1:1) with continual agitation for 20 minutes and subsequent centrifugation (10,000g for 15 minutes at room temperature). The aqueous phase was then extracted with an equal volume of chloroform followed by centrifugation as before. The aqueous layer was transferred to a clean tube and the DNA was precipitated by addition of 2.5 volumes of ethanol and incubation at –20°C for at least 1 hour. The DNA was pelleted by centrifugation (10,000g for 30 minutes at 4°C) and the pellet was washed with 80% ethanol, partially air-dried and dissolved in 15 ml SDW. To shear the DNA, the solution was pulled 35 times through a 21-gauge needle using a syringe. The sheared DNA solution was heated to 100°C for 15 minutes, diluted to a final concentration of 5 $mg.ml^{-1}$ and aliquoted into 1.5 ml Eppendorf tube and kept at –20°C until required.

3.6.4 Nucleic acid hybridization and detection

Pre-hybridized buffer

10% SDS	5	ml	(1%, v/v)
20x SSC	12.5	ml	(5 x SSC)
1.0 M Sodium phosphate buffer (pH 6.8)	5	ml	(0.1 M)
Deionised formamide	25	ml	(50%, v/v)
Sodium pyrophosphate	0.005	g	(0.01%, w/v)
Dextran sulphate (MW 500,000)	5	g	(10%, w/v)

- Dissolved sodium pyrophosphate with 1 ml SDW and heated at 65°C for 1 minute.
- Mixed all together by continuously stirring on hot plate (40-50°C) for 30 minutes.
- Added 100 $ug.ml^{-1}$ of sheared and denatured salmon sperm DNA (by heating at 95°C for 8 minutes and cooling immediately).
- Kept in a refrigerator until required.

Washing membrane solution

10% SDS	5	ml	(0.1%, v/v)
SDW	500	ml	

3.6.4.1 Pre-hybridization, hybridization and washing of membrane

The nylon membranes, where RNA were transferred and fixed, were rinsed in SDW and equilibrated in 5xSSPE. The membrane was rolled and placed into a clean, preheated hybridization bottle *(Techne, "FHB11" Techne Cambridge Ltd., Duxford, Cambridge, UK)*. The bottle was filled with 15 ml of pre-hybridization buffer containing 100 $\mu g.ml^{-1}$ sheared and denatured salmon sperm DNA, which was pre-heated to 42°C. The bottle was sealed and placed in a pre-heated rotary hybridization oven *(Techne Hybridiser, "HB-1D" Techne Cambridge Ltd., Duxford, Cambridge, UK)* at 42°C for pre-hybridization at least 2 hours.

Denatured radioactively labelled RNA probes were added to the pre-hybridization buffer in the hybridization bottle, which was then sealed and returned to the rotary oven. Hybridization was carried out by continuing incubation at 42°C for overnight.

After hybridization, the radioactive liquid in the bottle was poured off and non-specifically bound probe was removed by sequentially washing the membrane with 300 ml of washing solution I (2x SSPE, 0.1% (w/v) SDS) for 2 minutes at room temperature and wash again with 300 ml washing solution I for 30 minutes at room temperature. The membrane was rewashed by 2x300 ml of washing solution II (0.2x SSPE, 0.1% (w/v) SDS) at 65°C for 30 minutes and finally with 300 ml of washing solution I for 2 minutes. The membrane was then removed from the hybridization bottle and rinsed in 500 ml SDW. The membranes were sealed inside polyethylene bags and hybridizing signals were detected by autoradiography.

3.6.4.2 Detection of hybridization signal-autoradiography

After hybridization, the signals generated by membrane bound ^{32}P-labelled probes were detected by autoradiography. The membrane was sealed wet in plastic

determined using a hand held monitor *(mini-monitor series 900, Mini Instruments Ltd., UK)* were exposed at −70°C for overnight, while membranes with counts much greater than 100 cps were exposed at room temperature for a few minutes to a few hours, as necessary.

The film was developed after exposure under safe red light (GBX-2), by agitation for 5 minutes in a 1:4 dilution of film developer *(Kodak X-ray developer "LX24"),* rinsed briefly in water and then fixed by agitating in a 1:9 dilution of film fixer *(Kodak X-ray fixer "AL4")* for several minutes or until the film became clear, rinsed briefly in continuous running water. The film was air-dried and the position of wells and orientation marks on the membrane were aligned with the dried autoradiogram.

For the development of the film, if the level of exposure was considered too high or low, the membrane was re-exposed for a shorter or longer time, respectively. This process was repeated until a satisfactory set of autoradiograpm was produced.

3.7 Statistical data analysis

Unless otherwise stated, data were analyzed by Analysis of Variance (ANOVA) using SPSS version 10.0.1 (1999) *(SPSS Inc., Chicago, IL, USA)* and the differences between treatments were analyzed by a Least Significant Difference (LSD) comparison. Level of significant differences was indicated with the following superscripts: *p = 0.05, **p = 0.01 and ***p = 0.001.