CHARACTERIZATION OF EFFECTIVE ANTIOXIDANT COMOPNENTS OF TROPICAL FRUIT AND VEGETABLE SPECIES

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS……..…I

TABLE OF CONTENTS…….....III

SUMMARY……..…….....IV

LIST OF TABLES…….....XII

LIST OF FIGURES…….....XIII

ABBREVIATIONS…..…….....XVII

LIST OF PUBLICATIONS…….....XIX

APPENDIX I…….....XXIII

TABLE OF CONTENTS

PART I INTRODUCTION AND LITERATURE REVIEW.....1

1 INTRODUCTION…….....2

1.1 Benefits of consuming fruits and vegetables ....…….....2

1.2 Free radical damages…….....3

1.3 Antioxidant protections…….....5

1.4 Antioxidants in fruits and vegetable…….....10

1.5 Methods of assessing total antioxidant capacity (TAC) …….....12

1.5.1 TAC by non-inhibition assay…….....13

1.5.2 TAC by inhibition methods…….....17

1.5.3 Total phenolic contents (TPC) ……...…….....20

1.6 Identification of antioxidants in fruits and vegetables ….…….....21

1.6.1 Analysis of antioxidants in fruits and vegetables using HPLC/DAD……..…….....23

1.6.2 Analysis of antioxidants in fruits and vegetables using HPLC/MS ……...….....24

1.7 Aims and objectivity of this study…….....25

1.7.1 Study on antioxidant capacity of fruits and vegetables in the Singapore market ……...25

1.7.2 Identification of major antioxidants of selected fruits and vegetables …….....27

References…….....29

PART II EXPERIMENTAL…….....38

2 MATERIALS AND METHODS……..39

2.1 Investigation on TAC and TPC of fruits and vegetables …….....39

2.1.1 Materials……...39

2.1.2 Sample preparation……...40

2.1.3 Methods for TAC and TPC assays……....41

2.1.3.1 $ABTS^{\cdot+}$ scavenging assay…….....41

2.1.3.2 DPPH· scavenging assay…….....42

2.1.3.3 Ferric reducing/antioxidant power (FRAP) assay ……43

2.1.3.4 Determination of total phenolic contents…….....43

2.2 Measurement of the apparent stoichiometry of pure antioxidants with $ABTS^{\cdot+}$ and DPPH·…….44

2.3 HPLC/DAD analysis of antioxidants …….....45

2.3.1 Rapid analysis of L-ascorbic acid of fruits and vegetables by HPLC/DAD……..45

2.3.2 Simultaneous analysis of organic acid and phenolic compounds ….…….....46

2.4 Analysis of antioxidants in selected fruits and vegetables.…….....48

2.4.1 Analysis of antioxidants in star fruit……48

2.4.1.1 Solvent extraction of antioxidants…….48

2.4.1.2 Solid phase extraction of antioxidants……...49

2.4.1.3 Analysis of antioxidant peak in star fruit using HPLC/DAD ……....50

2.4.1.4 ESI-MS and HPLC-DAD-ESI-MS analyses of antioxidants…….....50

2.4.1.5 Anti-rancidity properties of residue extract on soya bean oil…….....52

2.4.2 Analysis of antioxidants of Lady's finger……...52

2.4.2.1 Solvent extraction of antioxidants…….....52

2.4.2.2 Solid phase extraction of antioxidants……..53

2.4.2.3 HPLC characterization of major antioxidant peaks……..53

2.4.2.4 HPLC-DAD-ESI-MS^n analysis of antioxidants of Lady's finger……..54

2.4.2.5 Isolation of pure compounds by semi-preparative HPLC…….55

2.4.2.6 Spectroscopy study of isolated compounds……..55

2.4.3 Analysis of antioxidants of salak……56

2.4.3.1 Solvent extraction of antioxidants……56

2.4.3.2 Identification of antioxidants in salak by HPLC/MS and HPLC/MS/MS…….56

2.4.4 Analysis of antioxidants in ciku king…….57

2.4.4.1 Solvent extraction of antioxidants……57

2.4.4.2 Identification of antioxidant in ciku king by HPLC/MS and HPLC/MS/MS……...58

2.4.4.3 Changes of TAC & TPC of ciku king fruit during storage……..……..58

2.4.5 Analysis of antioxidants of ulam raja……....58

2.4.5.1 Solvent extraction of antioxidants……58

2.4.5.2 Identification of antioxidants in ulam raja by HPLC/MS and HPLC/MS/MS.....59

References……60

PART III RESULTS AND DISCUSSIONS……62

3 Antioxidant Properties of Fruits and Vegetables……..63

3.1 Introduction…….....63

3.2 Antioxidant capacity and antioxidant efficiency……63

3.3 Antioxidant components of fruits and vegetables……..68

3.3.1 L-Ascorbic acid contribution to TAC of selected fruits and vegetables……..68

3.3.2 Total phenolic contents of fruits and vegetables…….....73

3.3.3 Effects of antioxidant components on TAC of fruits and vegetables……...74

3.4 Assessing antioxidant capacity of fruits, vegetables and pure antioxidants…….....76

3.4.1 $ABTS^{\cdot+}$ decolorization assay……..77

3.4.2 DPPH· scavenging assay……81

3.4.3 Ferric reducing/antioxidant power (FRAP) assay……...77

3.4.4 Comparison of methods for TAC assays ……82

3.5 Chapter summary……..90

References……92

4 Separation of Organic Acids and Phenolic Compounds by High Performance Liquid Chromatography……95

4.1 Introduction ……95

4.2 Method development……96

4.3 Method validation ……..…...100

4.4 Analysis of organic acids and phenolic compounds in apple juice Analysis ……..100

4.5 Negative effects on chromatographic profiles by sample solvents…….....102

4.6 Chapter summary…….....104

References…….105

5 Antioxidants in Star fruit (*Averrhoa Carambola* L.)……..……..……107

5.1 Introduction …….....107

5.2 Solvent extraction of antioxidants……..……107

5.3 Distribution of antioxidants in star fruit…….110

5.4 Inhibition of lipid peroxidation…..……..111

5.5 Correlations between TAC and total phenolic contents…….113

5.6 HPLC-DAD assay of antioxidant components……..113

5.7 Identification of antioxidanst by HPLC and mass spectrometry…….....116

5.8 Comparison of antioxidant capacity and phenolic profile of residue and Pycnogenol pills……...…….127

5.9 Chapter summary…...…….....130

References...…….…….132

6 Antioxidants in Lady's Finger (*Hibiscus Esculentus Linn*)……..……..……..135

6.1 Introduction …….……..135

6.2 Characterisation of major antioxidant peaks in lady's finger…….……..136

6.3 Identification of antioxidants using HPLC/MS^n ……..……..……....140

6.4 Structure confirmation using spectrometric methods……..……..…….145

6.5 Chapter summary…….……..149

References…….…….150

7 Antioxidants in Salak (*Salacca Edulis Reinw*)…….…….....152

7.1 Introduction …….……..152

7.2 Free radical active components in salak extract ……..155

7.3 Identification of antioxidants using HPLC/MS^n ……..……..…….159

7.4 Reactivity of antioxidants with free radicals……..……..163

7.5 Chapter summary……...164

References…….....165

8 Antioxidants in Ciku King (*Manilkara Zapota*)…..…….167

8.1 Introduction ……...167

8.2 Changes of TAC & TPC during storage…….168

8.3 Identification of antioxidants in ciku king using HPLC/MS^n……. …….171

8.4 Chapter summary……....186

References……...…187

9 Antioxidant in Ulam Raja(*Cosmos caudatus*)…….…….....188

9.1 Introduction ……..……...….188

9.2 Free radical active components in ulam raja……..…...…..189

9.3 Identification of antioxidants in ulam raja using HPLC/MS^n …….....197

9.4 Chapter summary……..…...….203

References……..…..…….204

PART IV CONCLUSIONS AND FUTURE WORKS……...…….....205

10 Conclusions ……..….....206

10.1 Future work……...210

SUMMARY

This research project has investigated the potential for tropical fruits and vegetables as sources of natural antioxidants in the diet. Several *in vitro* methods i.e. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) free radical ($ABTS^{\cdot+}$), 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) and ferric reducing antioxidant power (FRAP) were employed and compared for determination of total antioxidant capacity (TAC) of selected fruits and vegetables obtained from the Singapore market. Investigation of total antioxidant capacity of a variety of fruits and vegetables confirmed that most fruits and vegetables are good sources of natural antioxidants. The L-ascorbic acid contribution to TAC of fruits and vegetables varied greatly among species, from 0.06% in ciku to 70.2% in rambutan. Other than L-ascorbic acid, a variety of phenolic compounds were found to be major antioxidants in most fruits and vegetables, especially in those products with high or extremely high antioxidant capacity.

A range of common Southeast Asian fruits and vegetables including ciku (*Manilkara zapota*), ciku king (*Manilkara zapota*), ulam raja (*Cosmos caudatus)*, salak (*Salacca spp*), star fruit (*Averrhoa carambola* L.) and lady's finger (*Hibiscus esculentus*), were found to be excellent sources of natural antioxidants. The chemical structures of antioxidant compounds of selected products were systematically investigated using high performance liquid chromatography (HPLC) coupled with diode array detection, high performance liquid chromatography coupled with mass spectrometry (HPLC/MS) and nuclear magnetic resonance (NMR) spectroscopy.

A new HPLC method was developed for the separation and identification of antioxidants varying from very polar compounds such as L-ascorbic acid to moderately polar compounds e.g. flavonoid aglycones in extracts. The method is simple and straight forward to carry out as no additional sample pretreatment is required.

To characterise major antioxidants in star fruit, a new approach was developed. HPLC coupled with a diode array detector (DAD) was used to characterise antioxidant peaks in the juice or solvent extract through spiking with free radicals. By analysing the antioxidant capacity and chromatograms of fractions from solid phase extraction, it was possible to characterize the main antioxidant products. The antioxidants in star fruit included L-ascorbic acid, (-)epicatechin and proanthocyanidins which exist as dimers through hexamers. The presence of (-)epicatechin and proanthocyanidins are reported in star fruit for the first time and are preliminarily considered as the major phenolic compounds in star fruit.

Using an improved approach from the above, the major antioxidants of aqueous ethanol extract from Lady's Finger (*Hibiscus esculentus Linn*) were systematically investigated. The improved approach uses $ABTS^{+}$• prepared from oxidation of ABTS with MnO_2 to characterize antioxidant peaks, and thus reduce interferences of peaks from $ABTS^{+}$• oxidized by potassium persulphate. The improved approach was successfully used for identification of major antioxidants in Lady's finger. The major antioxidants in Lady's Finger were identified to be (-)-epigallocatechin and quercetin derivatives.

Another new approach was also developed for the rapid screening and identification of antioxidants in biological samples. This new approach was based on a significant decrease in the intensity of ion peaks obtained from high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) following reaction with $ABTS^{+}$•. HPLC/MS/MS was further applied to elucidate the structure of antioxidant peaks characterized in the spiking test. The new approach was successfully applied for the identification of antioxidants in extracts of salak, ciku and ulam raja. The antioxidants in salak were identified as chlorogenic acid, (-)-epicatechin, singly-linked proanthocyanidins that mainly existed as dimers through hexamers of catechin or epicatechin. The possible chemical structure of 24 antioxidants in extract of ciku king fruit were also elucidated using HPLC coupled with tandem mass spectrometry. Polyphenolics with basic blocks of gallocatechin or catechin or both were found to widely exist in the extract of ciku king fruit and preliminarily considered as its major antioxidants. Similarly, the chemical structures of 28 antioxidants in extract of ulam raja were elucidated using HPLC coupled with tandem mass spectrometry. The major antioxidants in ulam raja were attributed to be a number of proanthocyanidins, quercetin glycosides, chlorogenic acid and its isomers.

LIST OF TABLES

Table 1.1 Classes of phenolics

Table 2.1 Concentration of antioxidant standards

Table 3.1 AEAC of selected fruits and vegetables using $ABTS^{\cdot+}$ assay and their L-ascorbic acid content

Table 3.2 TAC and total phenolics of selected fruits and vegetables

Table 3.3 No. of mol of free radicals reduced by every mol of antioxidants

Table 4.1 Linearity range and limit of detection of carboxylic acid and phenolic compounds

Table 5.1 Antioxidant contribution in processed star fruit solution

Table 5.2 AEAC of star fruit at different extraction conditions

Table 5.3 Positive and negative ions of major antioxidant peaks

Table 5.4 Comparisons of elution times of proanthocyanidins in pycnogenol and star fruit

Table 6.1. Antioxidant activity performance of fractions.

Table 6.2 ESI-MS^n spectra of compounds **1~5**

Table 6.3 ^{13}C NMR spectral data of compounds **1–4** in DMSO-d_6 (δ: ppm)

Table 7.1 Ion peak intensities of extract and its reaction solution with $ABTS^{\cdot+}$

Table 7. 2 Positive and negative ions and their corresponding CID ions of antioxidants

Table 8.1 Positive and negative ions and corresponding CID ions of some antioxidants

Table 9.1 Positive and negative ions and their corresponding CID ions of antioxidants

LIST OF FIGURES

Fig. 1.1 Pathways for the oxidation and regeneration of vitamin E

Fig. 1.2 Chemical structures of ABTS and its free radical

Fig. 1.3 TAC by $ABTS^{\cdot+}$ or DPPH• assay

Fig. 1.4 The structure of DPPH radical

Fig. 1.5 $ABTS^{\cdot+}$ inhibition assay.

Fig. 3.1 AEAC values at different reaction time

Fig.3. 2. Comparison of reactions between pyrogallol and L-ascorbic acid with $ABTS^{\bullet+}$

Fig. 3.3 Correlation of AEAC and total phenolic content among fruits and vegetables

Fig. 3.4 Reaction of $ABTS^{\bullet+}$ and chlorogenic acid

Fig. 3.5 Kinetic curve of change of absorbance upon addition of fruit extracts

Fig. 3.6 Decrease in absorbance at 414 nm upon addition of L-ascorbic acid

Fig. 3.7 Plot of loss in absorbance against corresponding L-ascorbic acid (AA) concentrations (R^2=0.9956)

Fig. 3.8 The effects of concentration of AA on DPPH· decolorization (R2=0.9883)

Fig. 3.9 Standard calibration curve: [Fe(II)] vs A_{593} (R^2=0.9893)

Fig. 3.10 Correlation of change of absorbance in TAC assays and change of absorbance in TPC assay

Fig. 3.11 Correlation of FRAP value and AEAC by DPPH· model (R^2=0.8544)

Fig. 3.12 Correlation of FRAP value and AEAC by $ABTS^{\cdot+}$ method (R^2=0.9301)

Fig. 3.13 AEAC values of fruits and vegetables by $ABTS^{\cdot+}$ and DPPH· methods

Fig. 3.14 Stoichiometry coefficient of antioxidants with by $ABTS^{\cdot+}$ and DPPH· methods

Fig. 4.1 HPLC of the mixture of standards.

Fig. 4.2 Chromatogram of Berry apple juice showing carboxylic acid and phenolic compounds profiles: 2 (A), 0-30 min; 2 (B), 30-80 min.

Fig. 4.3 Negative effects on chromatographic profile by methanol: 5 (A): Standards dissolved in methanol/water (25/75, v/v) (B): Standards dissolved in water.

Fig. 5.1 Extraction efficiency vs solvent percentage Extraction temperature: 90 °C; extraction time: 45minutes

Fig. 5.2 $\Delta A_{414\ nm}$ vs extraction temperature**.** Extraction time: 45 min; solvent, 50% acetone solution

Fig. 5.3 $\Delta A_{414\ nm}$ vs extraction time. Extraction temperature, 90 °C; solvents: 50% acetone solution

Fig. 5.4 Effects of BHT and residue extract on peroxidation of Soya bean oil

Fig. 5.5 Correlations between loss of absorbance change and total phenolic contents

Fig. 5.6 Chromatograms of free radical spiking test. (**a**): solid line, Chromatogram of juice with water; dashed line, Chromatogram of $ABTS^{\bullet+}$ with water; (**b**): Chromatogram of reaction solution of juice and $ABTS^{\bullet+}$.

Fig. 5.7 Chemical structure of main proanthocyanidins in pycnogenol

Fig. 5.8 CID (MS/MS Scan) spectrum at m/z 291 of (-)epicatechin, collision energy: 35%.

Fig. 5.9 CID (MS/MS Scan) spectra of Pycnogenol proanthocyanidins, collision energy: 50%. **(a)** Corresponding to the parent ion(s) at m/z 579; **(b)** corresponding to the parent ion(s) at m/z 867.

Fig. 5.10 Fragmentation patterns of B-type proanthocyanidin

Fig. 5.11 HPLC-DAD-ESI-MS analysis of residue extract. (**a**): Chromatogram at 280 nm; (**b**): TIC at positive mode. Ion traces of (**c**) to (**g**) corresponding to monomers (m/z 291), dimers (m/z 579), trimers (m/z 867), tetramers (m/z 1155) and pentamers (m/z 1443), respectively.

Fig. 5.12 CID (MS/MS Scan) spectra of antioxidant peaks in residue extract, collision energy: 50%. CID spectrum corresponding to the parent ion(s) at m/z 579; (**b**) CID spectrum corresponding to the parent ion(s) at m/z 867; (**c**) CID spectrum corresponding to the parent ion(s) at m/z 1155; (**d**) CID spectrum corresponding to the parent ion(s) at m/z 1443.

Fig. 5.13 Ion traces of proanthocyanidins in pycnogenol and star fruit. (**a**) Ion traces of monomers through heptamers in pycnogenol. (**b**) Ion traces of monomers through heptamers in star fruit.

Fig. 6.1 Chromatograms of $ABTS^{+}\bullet$ solution oxidized by potassium persulfate and manganese dioxide. Solid line, $ABTS^{+}\bullet$ oxidized by manganese dioxide; dashed line, $ABTS^{+}\bullet$ oxidized by potassium persulfate.

Fig. 6. 2 Chromatograms of extract and reaction solution of extract and $ABTS^{+}$•. Solid line, 1:5 of extract/ $ABTS^{+}$• (v/v); dashed line, 1:5 of extract/0.1% formic acid (v/v). Detection wavelength: 280 nm.

Fig. 6. 3 Chromatograms of extract and FRCs. (a), 1:3 of extract/0.1% formic acid; (b), FRC 1; (c), 1:1 of FRC 2/0.1% formic acid; (d), FRC 3. Detection wavelength: 280 nm.

Fig. 6.4 ESI-MS^2 mass spectra of compounds at positive mode. (a) CID spectrum corresponds to the positive parent ion(s) at m/z 597 (MS/MS Scan), collision energy: 50%; (b) CID spectrum corresponds to the positive parent ion(s) at m/z 551 (MS/MS Scan), collision energy: 50%. CID spectra at m/z 627 and 465 are similar to those at m/z 597 and 551 respectively.

Fig. 6.5 ESI-MS^n mass spectra of compounds **4** at negative mode. (a) CID spectrum corresponds to the negative parent ion(s) at m/z 549 (MS/MS Scan), collision energy: 80%. (b) CID spectrum corresponds to the negative parent ion(s) at m/z 505 from m/z 549 (MS^3), collision energy: 80%.

Fig. 6.6 Chemical structures of compounds **1-4**

Fig. 7.1 HPLC Chromatograms of salak extract. (a) 280 nm; (b) TIC at positive mode; (c) TIC at negative mode.

Fig. 7.2 ESI-MS profiles of extract and reaction solution at stage 2 (10-20 min) under negative modes. (a) Extract:water=1:2; (b) Extract: $ABTS^{\cdot+}$ =1:2

Fig. 7.3 Extracted ion chromatograms for possible antioxidant ion peaks

Fig. 7.4 Chemical structures of proanthocyanidins in salak extract (n=1-6)

Fig. 7.5 Kinetics of $ABTS^{\cdot+}$ with (-)epicatechin and chlorogenic acid. Reaction conditions: room temperature (~28ºC); 30 μL addition of 3.1 mM of (-)epicatechin and 30 μL addition of 3.3 mM of chlorogenic acid into 3 mL of 1.0 mM $ABTS^{\cdot+}$ solution.

Fig. 8.1 Variation of total antioxidant capacity of ciku king fruits with storage time

Fig. 8.2 Variation of total phenolics content of ciku king fruits with storage time

Fig. 8.3 Correlations between TAC and TPC

Fig. 8.4 ESI/MS profiles of antioxidant elution period (6-19 min). (a) Sample with water; (b) Sample with $ABTS^{+}$•

Fig. 8.5 Extracted chromatograms of antioxidant ion peaks in ciku king extract.

Fig. 8.6 ESI-MS/MS mass spectra of gallocatechin dimers through pentamers at positive mode. (a) CID spectrum corresponds to the positive parent ion(s) at m/z 611 (MS/MS Scan); (b) CID spectrum corresponds to the positive parent ion(s) at m/z 915 (MS/MS Scan); (c) CID spectrum corresponds to the positive parent ion(s) at m/z 1219 (MS/MS Scan); (d) CID spectrum corresponds to the positive parent ion(s) at m/z 1523 (MS/MS Scan). Collision energy, 70%.

Fig. 8.7 Fragmentation patterns of gallocatechin dimer

Fig. 8.8 Chemical structures of prodelphinidins with gallocatechin as basic units in ciku king extract (n=1-6)

Fig. 8.9 Chemical structures of a dimer constituted of a gallocatechin and a gallocatechin gallate (Compound **C10**)

Fig. 9.1 HPLC chromatograms of ulam raja extract with UV and mass spectrometry detectors respectively.

Fig. 9.2 ESI/MS profiles of elution period 1 (0-10 min). (a) Sample with water; (b) Sample with $ABTS^{+}$•

Fig. 9.3 ESI/MS profiles of elution period 1 (10-20 min). (a) Sample with water; (b) Sample with $ABTS^{+}$•

Fig. 9.4 ESI/MS profiles of elution period 1 (20-30 min). (a) Sample with water; (b) Sample with $ABTS^{+}$•

Fig. 9.5 ESI/MS profiles of elution period 1 (30-40 min). (a) Sample with water; (b) Sample with $ABTS^{+}$•

Fig. 9.6 Chemical structures of compounds **U1-U18** (n=1-6)

Fig. 9.7 CID spectra compounds **19-21** from parent ions at m/z 355. (**a**), Compound **19.** (**b**), Compound **20**. (**c**), Compound **21**

Fig. 9.8 Chemical structures of compounds **19-21**

Fig. 9.9 Chemical structures of some quercetin derivatives

ABBREVIATIONS

AA	L-Ascorbic acid
AAPH	2,2'-Azobis(2-aminopropane) dihydrochloride
ABTS	2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid
AEAC	L-Ascorbic acid equivalent antioxidant capacity
AMD	Age-related Macular Degeneration
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CID	Collision induced dissociation
DAD	Diode array detector
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ESI	Electronspray ionisation
FRAP	Ferric reducing /antioxidant power
GAE	Gallic acid equIvalent
GC	Gas chromatography
HPLC	High performance liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
RDA	Recommended dietary allowance
RDA-F	Retro-Diels-Alder fission
RP-HPLC	Reverse phase high performance liquid chromatography
ROS	Reactive oxygen species

RNS	Reactive nitrogen species
SPE	Solid phase extraction
TLC	Thin layer chromatography
TPTZ	Tripydyltriazine
TAC	Total antioxidant capacity
TPC	Total phenolic content
TBHQ	*tert*-Butylhydroquinone
TEAC	Trolox equivalent antioxidant capacity
TBARS	Thiobarturic acid reactive substances
TRAP	Total radical trapping potential
TPC	Ttotal phenolic content
TIC	Total ion chromatogram

LIST OF PUBLICATIONS BASED ON THE STUDY

Journal papers (published)

1. G. Shui and L. P. Leong. "*Analysis of Polyphenolic Antioxidants in Star Fruit Using High Performance Liquid Chromatography and Mass spectrometry* ". **J. Chromatogr. A**. 1022 (2004) 67-75.

2. G. Shui and L. P. Leong. "*Rapid characterization and identification of antioxidants of salak (Salacca edulis reinw) using High Performance Liquid Chromatograph Coupled with Mass Spectrometry*". **Free Radical Biology and Medicine**. 35 (2003) sup.1, S47.

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Journal papers (submitted or in progress)

1. G. Shui, L. P. Leong and S.P. Wong. "*Ulam Raja, a mine of natural antioxidants*". **In progress** (2004).

2. G. Shui and L. P. Leong "*Residue from Star Fruit Juice Processing Waste as a value-added food ingredient and its potential as antioxidant nutraceuticals*". **In progress**(2003) **.**

3. G. Shui and L. P. Leong. "*Rapid characterization and identification of free radical-active components in biological samples using High Performance Liquid Chromatograph Coupled with Mass Spectrometry"*. **In progress** (2004).

4. G. Shui, L. P. Leong, Juli Effendy. "*A systematic comparison of two reactive nitrogen species (RNS) scavenging methods for assay of total antioxidant capacity: from apparent stoichiometry to reactivity*". **In progress** (2004).

5. G. Shui; S.P. Wong; L.P. Leong. "*Identification of antioxidants in Chiku king (Achras sapota Linn.) using HPLC/ESI/MS and Changes of its antioxidant capacity and total phenolics during natural ripening*". **In progress** (2004).

6. G. Shui and L. P. Leong. "*Antioxidant Capacity, Antioxidant Efficiency and Major Antioxidants of Some Fruits and Vegetables*". **In progress** (2004).

7. G. Shui and L. P. Leong "*Analysis of Major Antioxidants of Aqueous Ethanol Extract from Hibiscus esculentus Linn Using High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry and Confirmation of Structures Using Nuclear Magnetic Resonance*". **In progress** (2004). .

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1. G. Shui; S.P. Wong; L.P. Leong. (2004). "*Identification of antioxidants and changes of antioxidant levels during storages of Achras sapota Linn.*". **Singapore International Chemical Conference 3**: ***Frontiers in Physical and Analytical Chemistry***, p131. Singapore

2. G. Shui; L.P. Leong. (2004) "*A new approach for Identification of Major Antioxidants in Biological Samples*". **Singapore International Chemical Conference 3**: ***Frontiers in Physical and Analytical Chemistry***, P85. Singapore

3. G. Shui; L.P. Leong. (2004) "*Rapid Screening and identification of antioxidants in salak (Salacca edulis reinw) using High Performance Liquid Chromatograph Coupled with Mass*". **The 10th SFRBM Annual Meeting**, Seattle, USA.

4. G. Shui; L.P. Leong. (2004) "*A systematic comparison of two reactive nitrogen species (RNS) scavenging methods for assay of total antioxidant capacity: from real stoichiometry to kinetic reaction type*" **. The 2nd Asia-Pacific Conference and Exhibition on Anti-Aging Medicine**, p22. Singapore.

5. G. Shui and L. P. Leong (2003) "*A New Approach for Characterization of Major Antioxidative Components in Fruits and Vegetables and Its Application on Identification of Polyphenolics in Lady's Finger (Hibiscus esculentus Linn..)".* The **12th World Food Congress,** Chicago.

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PART I

INTRODUCTION & LITERATURE REVIEW

Chapter 1

Introduction

1.1 Benefits of consuming fruits and vegetables

It has long been recognised that fruits and vegetables are essential for a healthy and well balanced diet required for a healthy living. Their beneficial effects have been attributed to the fact that most fruits and vegetables are excellent sources of fibre, starch, vitamins and minerals. They are usually low in calories and fat and have no cholesterol, making them healthy additions to our diets. Along with grains, they contain complex carbohydrates which are the body's preferred sources of energy. Unlike calories from fat, which the body likes to store, calories from complex carbohydrates are used to meet immediate energy needs. In addition, fruits and vegetables give a feeling of satiety and aid in digestion. Many fruits and vegetables contain various vitamins such as folate, a B vitamin that reduces the risk of a birth defect of the brain or spinal cord.

Other than the general health effects mentioned above, fruits and vegetables have also been linked to many other specific health benefits including lowered risks for certain cancers, stroke, heart disease, and high blood pressure. High consumption of fruits and vegetables can help reduce the risk of developing some cancers, coronary heart diseases, inflammation, arthritis, immune system decline, brain dysfunction and cataracts [1-7]. These protective effects are considered, in large part, to be related to the various phytonutrients, especially antioxidants contained in such products.

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction triggered by free radicals, seem to be very important in the prevention of these diseases [8-13].

1.2 Free radical damage

A free radical (F•) is any species capable of independent existence (hence the term 'free') that contains one or more unpaired electrons [14]. Most free radicals are unstable and thus highly reactive since they need to pair their unpaired electron(s). When a free radical reacts with a more stable molecule (B:), the radical often pulls an atom from it and becomes a stable molecule itself (Eq. 1). The original molecule then becomes a free radical (B•) and will react with another molecule (C:) as such this molecule itself becomes a free radical (C•) and thus a self-propagating chain reaction is initiated (Eq. 1.2). If a radical pairs its unpaired electron by reacting with a second radical, then the chain reaction is terminated, and both radicals "neutralize" each other (Eq. 1.3).

$$F\bullet + B: \rightarrow F: + B\bullet \qquad \text{(Eq. 1.1)}$$

$$B\bullet + C: \rightarrow B: + C\bullet \qquad \text{(Eq. 1.2)}$$

$$F\bullet + B\bullet \rightarrow F:B \qquad \text{(Eq. 1.3)}$$

Radicals are produced by normal aerobic metabolism and are necessary to life. Our immune system needs free radicals to fight invading bacteria and viruses. In biological system, the main free radicals or precursors of free radicals include reactive oxygen species (ROS) such as singlet oxygen ($^1O_2^*$), superoxide ($O_2\bullet^-$), peroxide (O_2^{-2}) or hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•), and reactive nitrogen

species (RNS). They are usually formed by the reduction of oxygen molecule, ionising radiation, by reactive metals, or by enzymes and other endogenous and environmental initiators. Environmental influences that can contribute to the formation of free radicals include UV radiation, smoking, pollution and diet [14]. A superoxide and hydroxyl radicals are the most common radicals existing in the human body [14]. Superoxide radical is created in the body when one electron is added to an oxygen molecule. This free radical can be made by accident in the body involving the different reactions with oxygen, or it can be made deliberately by phagocytes which are cells in the blood that are capable of destroying bacteria. The hydroxyl radical might be the most reactive oxygen radical known in chemistry. It can be formed when water is exposed to X-rays, or when a reduced metal catalyses the breaking of the oxygen bond of hydrogen peroxide. The hydroxyl radical attacks all biological molecules as soon as it comes into contact with them. Peroxynitrite (ONOO-) is reactive nitrogen species formed at sites of inflammation by the rapid reaction of superoxide with nitrogen monoxide. It is a highly oxidising species capable of damaging lipids, protein, carbohydrate and DNA.

Although the immune system needs free radicals to fight invading bacteria and viruses, excess amounts of free radicals are harmful because of their reactivity. Radicals can damage lipids, proteins, and DNA [3, 14, 15], and by doing so, they alter biochemical compounds, corrode cell membrane and kill cells directly and completely. Increasing evidence suggests that they play a major role in the development of many diseases, like cancer, cataracts, heart diseases and aging in general [16]. Radical damage can be significant because of its ability to proceed as a chain reaction, as described earlier in this section. Increased levels of oxidative

damage to DNA, proteins and lipids have been detected, using a wide range of biomarkers, in post-mortem central nervous system (CNS) tissue sampled from patients who died with Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease [17-26]. Although all cells have some capability of repairing oxidative damage to proteins and DNA, they are not able to cope with excess damage. In view of the growing body of data on the role of oxidative stress in aging, scientists have initially focused much anti-aging research on attempts to reduce oxidative stress. One of the most widely studied ways to decrease oxidative stress is antioxidant intervention. Antioxidants are capable of neutralizing some of the free radicals that are taken in from the environment or are generated internally in mitochondria. Antioxidants are widely found in nature (especially in plant products) and constitute an extremely diversified group of molecules. In addition to reacting with another free radical to terminate the chain reaction, the free radical can also be terminated by antioxidants, scavengers and enzymes. Antioxidants are produced within the body and can also be acquired from a diet containing fruits, vegetables, seeds, nuts, meats, and oil. This study will discuss possible antioxidants contained in selected fruits and vegetables.

1.3 Antioxidant protections

According to the definition by Britton, an effective antioxidant is a molecule able to remove these radicals from the system either by reacting with them to yield harmless products or by disrupting or inhibiting free radical chain reactions [27]. Halliwell and Gutteridge define an antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delay or

prevent oxidation of that substrate" [14]. Both definitions emphasise the importance of substrate and the sources of free radicals. Antioxidant defence may include:

(1) Radical terminators or inhibitors

Antioxidants may inhibit or terminate oxidation by scavenging free radicals at various steps of oxidation. An antioxidant will become an antioxidant-derived radical after electron or hydrogen transfer to free radicals. The antioxidant-derived radical would become stable, or decay to a stable state, or be regenerated by other antioxidants. The antioxidant activity of α-tocopherols (AH_2) in the lipid oxidation process is based mainly on the α-tocopherol/α-tocopheryl quinone redox system (Fig. 1.1). α-Tocopherol (AH_2) is a radical scavenger where during lipid autooxidation process, it quenches lipid radicals L• and peroxyl radicals LOO•, thus competes with the chain propagation stage (Eq. 1.2). The quenching process may be expressed as below: [28]

$$AH_2 + L\bullet \rightarrow LH + AH\bullet \qquad \text{(Eq. 1.4)}$$

$$AH_2 + LOO\bullet \rightarrow LOOH + AH\bullet \qquad \text{(Eq. 1.5)}$$

After releasing one H atom, the formed α-tocopherol radical (AH•) releases another H atom to produce methyl tocopherol quinone, which is unstable and gives rise to tocopheryl quinone (A) as its stable product (Fig. 1.1). Two tocopherol radicals may form a molecule of α-tocopheryl quinone and a regenerated tocopherol (Eq. 1.6). [28]

$$AH\bullet + AH\bullet \rightarrow A + AH_2 \qquad \text{(Eq. 1.6)}$$

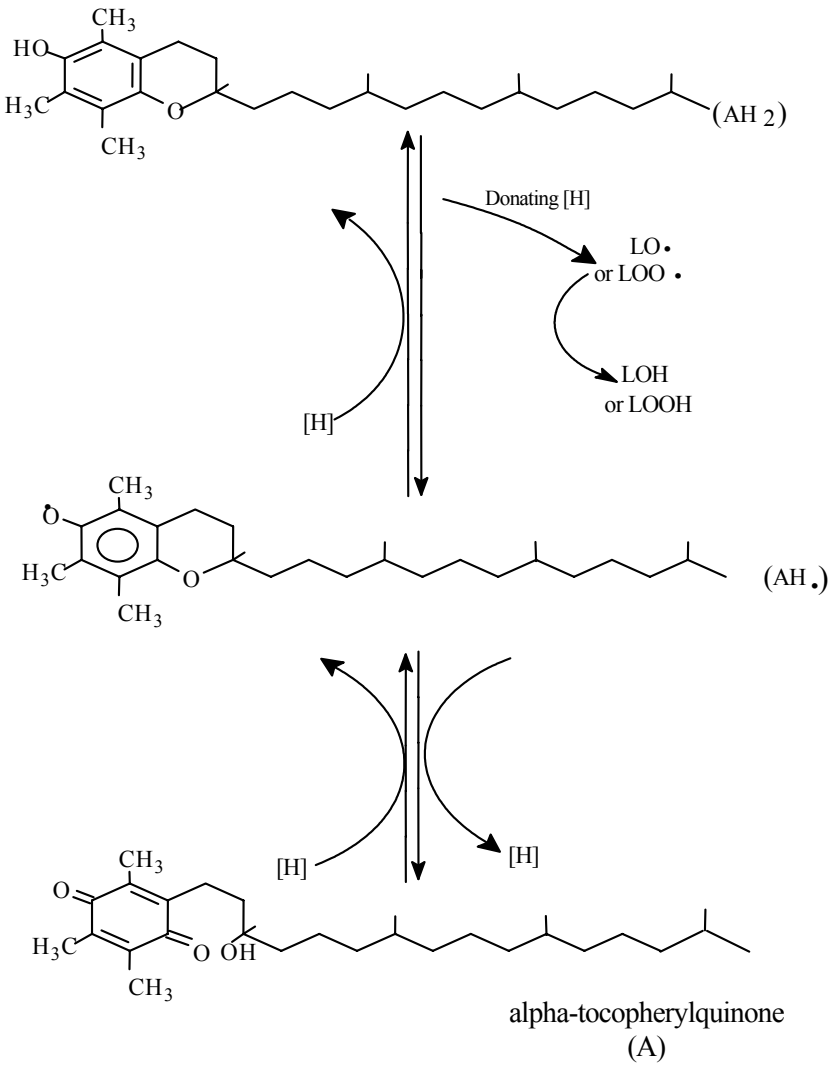

Fig. 1.1 Pathways for the oxidation and regeneration of vitamin E

α-Tocopheryl semiquinone radical (AH•) and α-tocopheryl quinone (A) may also be recovered by antioxidants such as ascorbate, urate and ubiquinol [29].

(2) Enzymatic antioxidant activities

Some enzymes can catalyse the reaction of a certain substance with oxygen and thus remove oxygen or catalyse highly reactive free radicals to more stable species. For example, superoxide dismutase (SOD) enzyme catalyses superoxide radicals to produce hydrogen peroxide and ground-state oxygen. SOD antioxidant mechanisms

might be attributed to the complexation of metal ions and SOD. For example, the catalytic ability of Cu-Zn-SOD could be explained by the following reaction [14]:

$$\text{Enzyme-Cu}^{2+} + O_2\bullet^- \rightarrow \text{Enzyme-Cu}^{+} + O_2 \qquad \text{(Eq. 1.7)}$$

$$\text{Enzyme-Cu}^{+} + O_2\bullet^- + 2H^+ \rightarrow \text{Enzyme-Cu}^{2+} + H_2O_2 \qquad \text{(Eq. 1.8)}$$

$$\text{Net reaction: } O_2\bullet^- + O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Eq. 1.9)}$$

Hydrogen peroxide is usually removed in aerobes by two types of catalases and peroxidase enzymes. Catalase directly catalyses the decomposition of H_2O_2 to ground-state oxygen (Eq. 1.10), and peroxidase enzymes remove H_2O_2 by using it to oxidize substrate (SH_2) (Eq. 1.11) [14].

$$2H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2 \qquad \text{(Eq. 1.10)}$$

$$H_2O_2 + SH_2 \xrightarrow{\text{Peroxidase}} 2H_2O + S \qquad \text{(Eq. 1.11)}$$

Glutathione peroxidase (GPX) family removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione, GSH (Eq. 1.12).

$$H_2O_2 + 2\,GSH \xrightarrow[GPX]{GPX} GSSG + 2\,H_2O \qquad \text{(Eq. 1.12)}$$

GPX enzymes are specific for GSH as a hydrogen donor. They can also act on peroxides other than H_2O_2 [14]. (Eq. 1.13)

$$LOOH + 2\,GSH \xrightarrow{GPX} GSSG + H_2O + LOH \qquad \text{(Eq. 1.13)}$$

(3) Sequestering agents

Metals such as iron and copper are very important in the human body for the synthesis of a huge range of enzymes and other proteins. However, these metal ions are potentially harmful to health since they can catalyse the autoxidation reaction, convert H_2O_2 to HO• and decompose lipid peroxides to reactive peroxyl and alkoxy radicals. Compounds such as citric acid, amino acids and phosphates exhibit little or no antioxidant activity, but they can chelate metal ions and thus greatly enhance the activity of other antioxidants [30].

(4) Oxygen scavengers

These are compounds that can react with oxygen and thus remove oxygen in a closed system. Ascorbyl palmatate, sulphites, erythorbic acids and ascorbic acid are commonly used oxygen scavengers.

(5) Singlet oxygen quenchers

Carotenoids such as β-carotene are excellent singlet oxygen quenchers. The electron rich conjugated double bond structure is primarily responsible for the excellent ability of carotenoids to physically quench singlet oxygen, the chemical reactivity of carotenoids with free radicals, and their instability towards oxidation [27, 31]. They can convert singlet oxygen to more stable ground-state oxygen through physical process quenchers (Eq. 1.14 and 1.15).

$$^1\beta\text{-carotene} + {}^1O_2{}^* \rightarrow {}^3\beta\text{-carotene}^* + {}^3O_2 \qquad \text{(Eq. 1.14)}$$

$$^3\beta\text{-carotene}^* \rightarrow {}^1\beta\text{-carotene} + \text{heat} \qquad \text{(Eq. 1.15)}$$

For protection against $^1O_2^*$ by carotenoids, it is essential that chemical quenching is only a very minor side reaction [32]. Thus, the antioxidant contribution of this chemical reaction is negligible.

1.4 Antioxidants in fruits and vegetables

Fruits and vegetables contain several classes of compounds that can potentially contribute to antioxidant activity. Most of the extracts from fruits and vegetables exhibite some antioxidant properties. One of the most widely studied antioxidants in fruits and vegetables is L-ascorbic acid (vitamin C). L-Ascorbic acid has numerous biological functions, which include the synthesis of collagen, some hormones and certain neurotransmitters [33]. It is believed that the role of L-ascorbic acid in disease prevention is due to its ability to scavenge free radicals in the biological systems. Cancer, which is due to uncontrolled cell proliferation, may be initiated by oxidative and free radical damage to DNA and cells. Since L-ascorbic acid may act as an effective antioxidant, it is able to slow down or prevent such a damage [33].

The majority of the antioxidant capacity of a fruit or vegetable may be contributed by compounds other than vitamin C. For example, carotenoids, another big family of compounds with antioxidant activities, are the most common and most important natural pigments in fruits and vegetables. They are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers. Of the approximately 600 carotenoids identified only about 50 of these have provitamin A activity, and six are considered important for human health, namely, α-carotene, β-carotene, lutein, zeaxanthin, lycopene and cryptoxanthin. The most widely studied carotenoid is β-

carotene. Others, however, like lycopene, lutein, and zeaxantin, may offer health protection equal to or better than β-carotene. This study will not cover aspects of carotenoids as antioxidants since their contents in tropical fruits and vegetables have been widely studied [34].

One of the most important antioxidants in fruits and vegetables might be phenolic compounds. Phenolic compounds are believed to significantly contribute to the antioxidant activity of fruits and vegetables. Primarily due to their redox properties, which allow them to act as reducing agents, hydrogen donors and single oxygen quenchers, phenolic compounds demonstrate strong antioxidant activity. Other than antioxidant activity, phenolic compounds have other biological effects, including antibacterial, antiviral and antithrombotic activity. More and more researchers are interested in this group of compounds due to these reasons. More data on phenolic compounds in fruits and vegetables are now available and added to nutrition tables [35]. However, related information on tropical fruits and vegetables are still sporadic and limited. Therefore, it is necessary to identify and quantify phenolic distributions in selected tropical fruits and vegetables, and it is also meaningful to provide the public with a more complete nutrition profile of their diets. This study will concentrate on phenolic compounds in selected fruits and vegetables.

The following table gives the major classes of phenolics in fruits and vegetables:

Table 1.1 Classes of phenolics

Number of carbon atoms	Basic skeleton	Class
7	C_6-C_1	Hydroxybenzoic acids
9	C_6-C_3	Hydroxycinnamic acids
		Coumarins
10	C_6-C_4	Naphthoquinones
13	C_6-C_1-C_6	Xanthones
14	C_6-C_2-C_6	Stibenes
15	C_6-C_3-C_6	Flavonoids & isoflavonoids
N		Tannins & Lignins

The classes, on which this study focuses, are hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, isoflavonoids and tannins, -such compounds are reported to predominate in a variety of fruits and vegetables.

1.5 Methods of assessing total antioxidant capacity (TAC)

Although the knowledge of the potential antioxidant compounds present in fruits and vegetables will give useful nutrition information, it does not necessarily indicate its total antioxidant capacity (TAC) or reflect on its overall reaction. This is because synergistic effects can exist in the presence of more than one antioxidant, which means that the total antioxidant effect may be greater than the sum of the individual antioxidant activities [36-38]. In addition, there are many different antioxidant components with different physical and chemical properties in fruits and vegetables, and it is relatively difficult to measure each antioxidant component separately. Thus, measuring its total antioxidant activity will provide only a general idea on the effectiveness of a crude extract obtained from fruits and vegetables. Several analytical

methods have been proposed for determining total antioxidant activity of biological extracts in order to evaluate the total antioxidant capacity of biological samples [39-48]. The methods developed to measure the total antioxidant capacity of biological samples might be classified as non-inhibition methods or inhibition methods.

In TAC assay by non-inhibition methods, the sample directly acts on reactive species, and no substrate is involved. Thus, the methods directly reflect TAC of a sample that reacts with a certain reactive species or interacts directly with an oxidant. While by inhibition assay, reactive species that are usually free radicals and an oxidizable substrate are often involved. Thus the method reflects the ability of a sample to delay the oxidation of the substrate.

1.5.1 TAC by non-inhibition assay

The non-inhibition methods include ferric reducing/antioxidant power (FRAP) assay, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) free radical ($ABTS^{\bullet+}$) scavenging assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, cyclic voltammetry method[39, 44, 45]. These methods will be discussed in detail below.

$ABTS^{\cdot+}$ assay. The $ABTS^{\bullet+}$ is initially formed by reacting 2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) with oxidants (Fig. 1.2). Hydrogen peroxide in an enzymic system and potassium persulphate are two of the most commonly used oxidants.

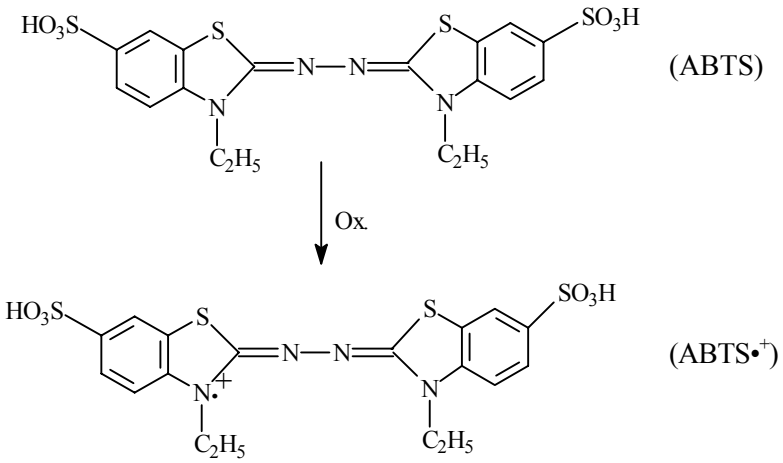

Fig. 1.2 chemical structures of ABTS and its free radical

ABTS is oxidized to ABTS•$^{+}$, which gives a blue green colour with maximum absorption at 414, 660, 734 and 820 nm [41]. When ABTS•$^{+}$ is formed which is stable in solution, antioxidants of biological samples are added into ABTS•$^{+}$ solution, and reduction of absorbance at a specific wavelength occurs (Fig. 1.3) The extent of absorbance drop reflects on the ability of samples to scavenge free radicals. This method measures the relative antioxidant capacity of fruit or vegetable extracts by comparing their ability to scavenge ABTS•$^{+}$ with a standard amount of Trolox (water-soluble vitamin E) or vitamin C.

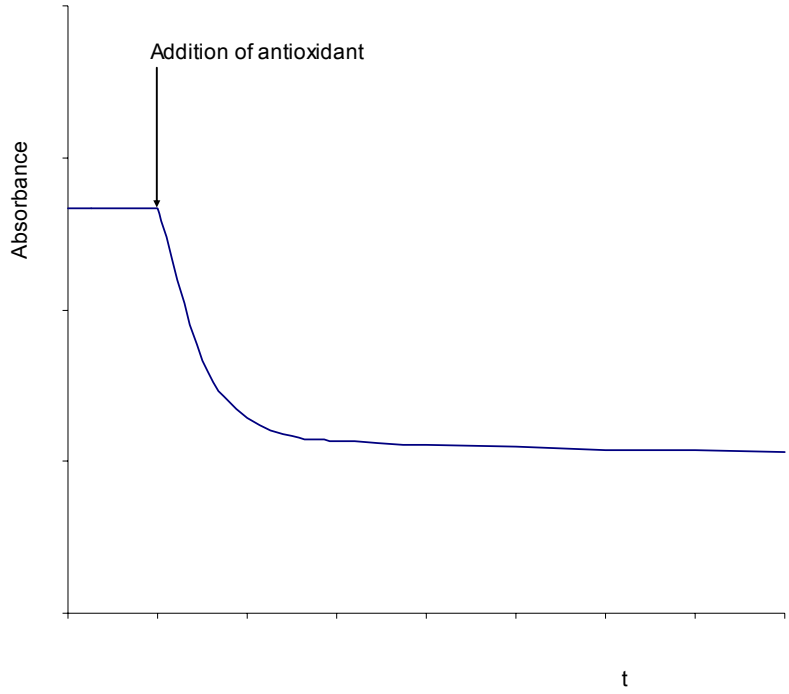

Fig. 1.3 TAC by ABTS•$^{+}$ or DPPH• assay

DPPH• scavenging assay. DPPH• is a stable free radical (Fig. 1.4) and has been widely used to measure the antioxidant capacity of many individual compounds, plant extracts and beverages [49-52]. DPPH• has a maximum absorbance at 517 nm. Similar to ABTS•$^{+}$ assay, a reduction of absorbance is observed when the unpaired electron is stabilised.

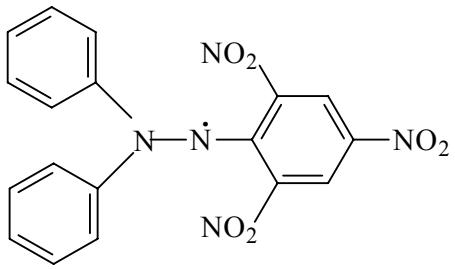

Fig. 1.4 The structure of DPPH•

For example, DPPH• reacts with phenolic substances (AH) to form DPPH-H, which do not absorb at 517 nm (Eq. 1.16).

$$DPPH\bullet + AH \rightarrow DPPH\text{-}H + A\bullet \qquad \text{(Eq. 1.16)}$$

The new radical formed (A•) can mainly follow radical-radical interaction to render stable molecules via radical disproportionation (DPPH• + A• ⟶ DPPH-A; A• + A• ⟶ A-A), although these secondary reactions are greatly hindered[53]. Therefore, the disappearance of DPPH· is an index to estimate free radical scavenging ability.

Ferric reducing/antioxidant power (FRAP) assay. Many antioxidant processes involve an oxidation-reduction reaction. The total reduction potential of a sample is related to the reducing compounds contained in the sample. Based on this, FRAP assay measures the ferric reducing ability of biological samples. At low pH (pH 3.6), ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex can be reduced to the ferrous form (Fe^{II}-TPTZ). The latter gives an intense blue colour with an absorption maximum at 593 nm. Any half reaction which has a less-positive redox potential than the Fe^{III}/Fe^{II}-TPTZ half reaction will drive the reduction of Fe^{III}-TPTZ.

However, an antioxidant that can effectively reduce pro-oxidants may not be able to efficiently reduce Fe^{III}. For example, GSH, an important antioxidant *in vivo*, will not be measured by FRAP assay [54]. Therefore, in this particular case, it may underestimate the antioxidant potential of a biological sample using the FRAP

method. On the other hand, since not all reducing agents that are able to reduce Fe^{III} are antioxidants, the TAC assay results obtained by FRAP assay might also be over-estimated. In addition, FRAP assay does not involve a pro-oxidant and an oxidizable substrate, ferric-reducing power of a biological sample may only indirectly reflect the total antioxidant capacity of the sample. Therefore, one should always be careful when results are interpreted if no other evaluation method is used together with this assay.

Although FRAP assay has some shortcomings, it is a simple and reproducible method which can be used to study the antioxidant activity of blood plasma, plant extracts, beverages and even pure dietary antioxidants. As demonstrated by Pulido and Benzie, it is extremely useful to assay phenolic antioxidants in biological samples [55, 56].

1.5.2 Inhibition methods

The widely used inhibition methods involve oxygen radical absorbance capacity (ORAC) assay [39], total radical trapping parameter (TRAP) assay [42], ferrylmyoglobin/ H_2O_2/ABTS system [41], and H_2O_2/ABTS/horseradish peroxidase inhibition assay [48]. The inhibition methods involve reactive species and an oxidizable substrate. The damage to the substrate could be inhibited or delayed in the presence of antioxidants. The inhibition time or inhibition percentage or both is measured and related to TAC of a sample. The measured TAC may have physiological importance because the pro-oxidants are pathologically important. Fig.1.5 shows the oxidation of ABTS into $ABTS^{\cdot+}$ by potassium persulphate without

any antioxidant in the medium, and the delay of $ABTS^{\cdot+}$ formation upon addition of vitamin C (unpublished data). When antioxidant vitamin C is added, oxidation of ABTS was delayed for about 5 min as the formation of $ABTS^{\cdot+}$ is inhibited by vitamin C.

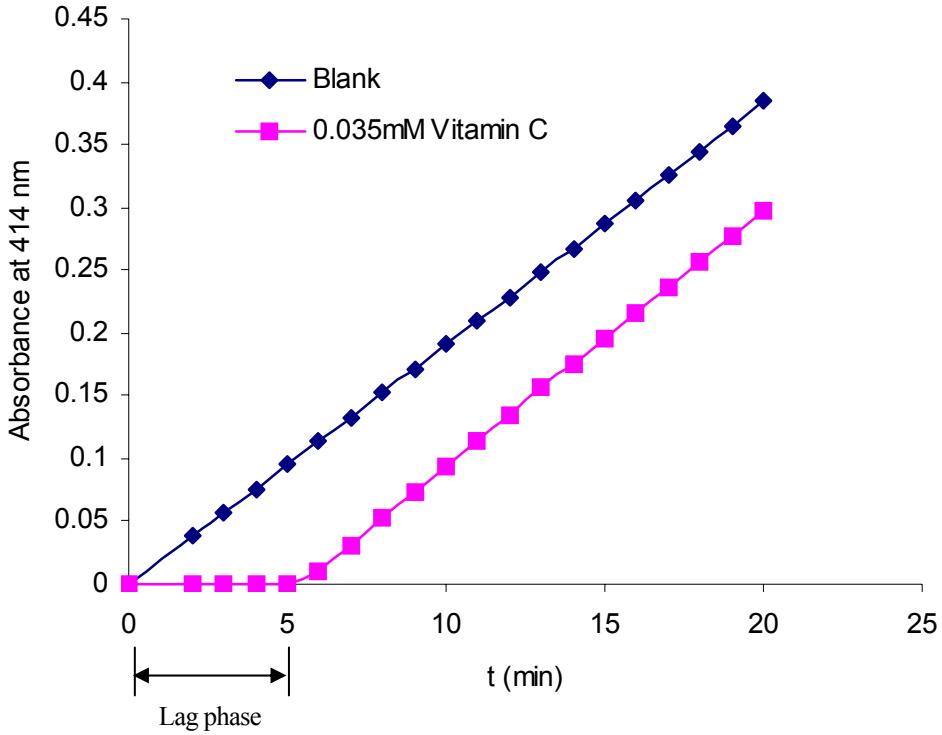

Fig. 1.5 $ABTS^{\cdot+}$ inhibition assay. Final concentration of potassium persulphate and ABTS are 0.086, 1.46 mM respectively.

TRAP method. The TRAP assay of Wayner *et al.* [42] is one of the most widely used methods for measuring total antioxidant capacity of plasma or serum. The TRAP assay uses peroxyl radicals generated from 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and peroxidizable materials contained in biological samples. After adding AAPH to the sample, the oxidation of the oxidizable materials is monitored by measuring the amount of oxygen consumed during the reaction. During the induction period, this oxidation is inhibited by antioxidants in the sample. The

length of the induction period (lag phase) is compared to that of trolox (6-hydroxyl-2, 5, 7, 8,-tetramethylchroman-2-carboxylic acid), and then quantitatively related to the antioxidant capacity of the sample. However, the major problem with the original TRAP assay lies in the oxygen electrode end point. The oxygen electrode would not maintain its stability over the period of time required [57]. At present, most widely used modified TRAP methods include luminol-based assays, dichlorofluorescin-diacetate (DCFH-DA) based assay, etc [58, 59].

ORAC method. The ORAC assay uses phycoerythrin (PE) as an oxidizable protein substrate and AAPH (2, 2'-azobis (2-aminopropane) dihydrochloride) as a peroxyl radical generator or Cu^{2+}-H_2O_2 as a hydroxyl radical generator. It is based on the measurements of fluorescence rather than absorbance; therefore, it increases sensitivity and so permits the use of a much lower molar ratio of antioxidant sample to reagents [60].

The ORAC assay works by the following principles. A sample containing antioxidant is added to a free radical-generating system. AAPH is used as a free radical generator and R-PE (R-phycoerythrin) is used as a substrate for free radical attack. Free radicals cause conformational changes in the protein structure of R-PE leading to fluorescence quenching in a dose and time-dependant manner. The inhibition of the free radical action is measured and the results calculated are related to the antioxidant capacity of the sample.

ABTS/H_2O_2/HRP Inhibition method. Arnao and co-workers [48] developed a method for measuring TAC using the enzymatic system, which composed of

horseradish peroxidase (HRP) enzyme, hydrogen peroxide, and ABTS chromophore. The method is based on the ability of antioxidants to scavenge $ABTS^{\bullet+}$ as they are formed and inhibit the formation of more free radicals. Antioxidant capacity is quantified by measuring the inhibition time in the appearance of $ABTS^{\bullet+}$ caused by the presence of the antioxidant in the system. The inhibition time will reflect efficiency of the antioxidants. The less efficient the antioxidant to scavenge free radicals as they are formed, the earlier the accumulation of the $ABTS^{\bullet+}$.

1.5.3 Total phenolic content (TPC)

Many researchers have suggested that polyphenolics contribute to total antioxidant activity because of their ability to donate hydrogen atoms and quench singlet oxygen. Phenolic compounds found in fruits and vegetables include phenolic acid (hydroxybenzoic acids and its derivatives, hydroxycinnamic acids and its derivatives), coumarins, flavonoids, tannins, etc. It is therefore useful to obtain information on total phenolic content of fruits or vegetables while using different methods to evaluate their TAC. The total phenolic content (TPC) of a fruit or a vegetable is usually determined according to Folin-Ciocalteu procedure and expressed as gallic acid equivalent (GAE). Phenolic substances are oxidised by the Folin-Ciocalteu reagent, which contains a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). The reagent becomes partly reduced resulting in the production of the complex molybden-tungsten blue, which is measured spectrophotometrically at 765 nm [61].

1.6 Identification of antioxidants in fruits and vegetables

While information on TAC of common fruits and vegetables is useful, knowledge of antioxidant compositions of fruits and vegetables is also very important as it will provide information on specific function(s) that a fruit or vegetable might have. However, there are many different antioxidant components with different physical and chemical properties in fruits and vegetables, and since plant matrices are very complex, it is relatively difficult to measure each antioxidant component separately. While the overall antioxidant capacity of fruits and vegetables could be acquired by a number of established methods, it is impossible to obtain information about what compounds are major antioxidants from the TAC value of a fruit/vegetable. In addition, the knowledge of the potential antioxidant components present in fruits and vegetables will give us useful nutritional information as well as their possible antioxidant mechanism(s). Therefore, it is very important to identify those antioxidants especially major antioxidants in those fruits and vegetables with high antioxidant capacity.

There are many challenges for identification of antioxidants in biological samples. Firstly, biological sample matrices are usually very complex and the antioxidant compounds need to be extracted as far as possible and subjected to purification and separation procedure before they are identified using the various methods such as paper chromatography, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), high performance liquid chromatography coupled with mass spectrometry (HPLC/MS), etc. Because of different chemical and physical properties among antioxidants in different fruits and

vegetables, the sample treatment for different antioxidant families will likely be different. The extraction should enable efficient extraction of antioxidants and must avoid chemical modification as much as possible. As most phenolic compounds are polar, ethanol/water solution, methanol/water solution and acetone/water are the most commonly used extraction solutions for plant phenolics. The procedures for other antioxidant families are similar to that of phenolic compounds with some variation in extraction solvents, temperature, time, and analytical methods used.

The ethanol/water or methanol/water or acetone/water extract obtained is a crude extract that contains numerous non-phenolic substances (sugar, pigments, etc.) which can interfere during separation and identification. Purification is usually important and necessary. Based on difference of polarity, solvent extraction or solid phase extraction or combination of both is usually utilized to remove unwanted matrices.

Separation of various antioxidants in an extract is an essential stage prior to identification. Chromatographic technology is the most widely used separation technology both for analytical purpose and for preparation technology. Although paper and thin-layer techniques have been used to separate various phenolics like anthocyanins, hydroxycinnamic derivatives, and flavonols, they could not provide accurate information because they have the drawbacks of not being sufficiently selective for the unambiguous identification of targeted compounds. Column chromatography is a particularlly useful technology for the isolation of large amount of the compounds in extract. For example, gas chromatography (GC) [62, 63], capillary electrophoresis [64] and HPLC [65-69] have been used for the determination of some phenolic acids. Of all column chromatographic techniques used, HPLC is

becoming the most powerful and most-widely used tool since it enables simultaneous separation, identification and quantification of this class of chemical compounds.

1.6.1 Analysis of antioxidants in fruits and vegetables using HPLC/DAD

Liquid chromatographic technique which utilizes nonpolar adsorbent surface and a polar eluent has been named reversed-phase HPLC (RP-HPLC). In the last 20 years, RP-HPLC has quickly grown in its number of applications, and now it is the most widely used HPLC mode. This success can be explained by its ability to separate a large variety of organic compounds with polarity of very polar to non-polar by changing the polarity of mobile phases. The most often used columns have been packed with C_{18} column materials. The mobile phases that have been employed with RP-HPLC columns are acetonitrile and/or methanol in combination with water containing an acid [70]. For the analysis of flavonoids in foods by HPLC, as reviewed by Merken and Beecher, columns are almost exclusively reversed phase, and elution systems are usually binary, with an aqueous acidified polar solvent and a less polar organic solvent [70]. The aqueous acidified polar solvents include acetic acid, phosphoric acid, perchloric acid, or formic acid. The elution may be isocratic or gradient. The traditional detectors for HPLC system include refractive index (RI), ultraviolate-visible (UV-Vis), fluorescence, electrochemical detector, etc. In most cases, direct identification of the peaks is possible through comparison with published data or with standard compounds. However, as different compounds might be eluted at the same time, identification based on retention times may give wrong information for quantification and identification of antioxidants in such complex mixtures.

Recently, diode array detector (DAD) has become one of the most widely used detectors for identification.

DAD detection is very powerful for monitoring phenolic compounds as it can provide UV-Vis spectra of target compounds, and may provide possible chemical structural information. Furthermore, the obtained spectrum of a target compound can also be used to select the wavelength with maximum absorbance for improving sensitivity and selectivity. HPLC/DAD appears be an ideal tool for the preliminary characterization of antioxidants. However, as UV-Vis spectra of phenolic compounds are often very similar, the possibility of faulty identification exists. With the introduction of mass detector coupled to HPLC, this technique has now evolved into a routine technique that enables collection of significant data on the structures of those compounds that show similar UV–Vis spectra [71].

1.6.2 Analysis of antioxidants in fruits and vegetables using HPLC/MS

The increasing interest in the characterization of phenolic compounds in diets has created new demands for the development of rapid, sensitive, and specific analytical methods for identification and quantification of this class of chemical compounds in fresh and processed food products.

HPLC coupled with mass spectrometry has been widely used to identify phenolic compounds, and electronspray ionization (ESI) mass spectrometric liquid interface is able to provide advantages in terms of sensitivity and capability to analyze large, thermally labile and highly polar compounds[72, 73]. In ESI-MS, deprotonated

molecular ions represent the base peak in the negative ion spectra. In contrast, protonated molecular ions are the base peaks in the positive ion mode. Collision induced dissociation (CID) spectrum obtained from positive and/or negative parental ions can further give structural information. Recently, HPLC/ESI/MS and HPLC/ESI/MS^n have been extensively applied for identification of phenolic compounds in biological samples [72-80].

1.7 Aims and objectivity of this study

1.7.1 Study on antioxidant capacity of fruits and vegetables in the Singapore market

One of the main objectivities of this study was to investigate the antioxidant capacity of fruits and vegetables available in the Singapore market and identify fruits and vegetables as potential dietary antioxidant sources. The exact link between fruits and vegetables and certain kinds of age-related diseases such as cancer is still unclear. Some studies have found a weak relationship between the two, while others have found no link [81]. While this may seem to provide an argument for fruits and vegetables being protective against these diseases, there have been more promising results by some researchers who find that certain fruits and vegetables, such as tomato, are able to reduce the risk of prostate cancer. In a study of over 40,000 health professionals, researchers found that men who ate the most tomato-based foods had a 35 percent lower risk of developing prostate cancer than those who ate the least amount of these foods [82]. While researchers have found that eating five servings per day of any fruits and vegetables is associated with a 30 percent lower risk of stroke in healthy men and women, cruciferous and green leafy vegetables, citrus fruits and their juices were particularly found to have the greatest apparent benefit [82].

It is most likely that consuming large quantities of fruits and vegetables with high antioxidant activity can lower the risks for age-related diseases such as certain cancers, stroke, heart disease, immune system decline, brain dysfunction and cataracts. Therefore, it is meaningful to determine total antioxidant capacity of local fruits and vegetables and subsequently provide the public information on antioxidant levels in their diets available from fruits and vegetables in the Singapore market.

As discussed above, both inhibition and non-inhibition methods are very useful tools to measure antioxidant capacities of fruits and vegetables. Inhibition assay might be a direct antioxidant assay, and the results might reflect physiologic importance of antioxidants. However, when inhibition methods are used, it is possible that different results might be obtained when different substrates are used. Non-inhibition assay can directly reflect TAC of a sample that reacts with a certain reactive species or interacts directly with an oxidant. As no substrate is required, it would be easier to perform when compared with inhibition assay. Since one of the objectives of this study was to find potential food sources with high antioxidant capacity, non-inhibition methods could fulfil the requirements of a preliminary investigation. Therefore, FRAP assay, $ABTS^{\bullet+}$ and DPPH• assay were utilized for screening antioxidant capacity of fruits and vegetables. Advantages and disadvantages of different assays are discussed. Furthermore, stoichiometry and reactivity of various antioxidants with $ABTS^{\bullet+}$ and DPPH• were studied.

1.7.2 Identification of major antioxidants of selected fruits and vegetables

While the overall total antioxidant capacity of fruits and vegetables could be acquired by a number of non-inhibition methods, it is also important to investigate which compounds are major antioxidants in selected fruits and vegetables as it will give useful nutritional information as well as their possible antioxidant mechanism(s). L-Ascorbic acid is a very important antioxidant existing widely in fruits and vegetables. Although its content in most fruits and vegetables has been widely studied, its contribution to total antioxidant capacity in most fruits and vegetables is still not clear. This study will cover quantification of L-ascorbic acid in selected fruits and vegetables using HPLC. With the knowledge of TAC and L-ascorbic acid content, contribution of L-ascorbic acid to TAC will be obtained.

Other than L-ascobic acid, other compounds such as polyphenolics are most likely to be major antioxidants in polar solvent extracts of many fruits and vegetables. Therefore, a complete profile of antioxidants should include as many components as possible. However, it is difficult to obtain information on a complete profile of antioxidants in a fruit/vegetable extract from existing methods. It is a challenge and meaningful to study complete antioxidant composition of a fruit/vegetable extract systematically. Therefore, a further objective of this study was to characterise and identify the major antioxidants or a complete profile of antioxidants in polar solvent extracts of selected fruits and vegetables with high antioxidant capacity. Making use of non-inhibition assay

combined with modern analytical techniques, different sample pretreatments and analytical approaches may have to be developed or modified for different source of antioxidants. Related information can be obtained from HPLC/DAD, HPLC/MS^n, NMR, etc.

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PART II

EXPERIMENTAL

Chapter 2

Materials and Methods

2.1 Investigation of TAC and TPC of fruits and vegetables

2.1.1 Materials

Fruits and Vegetables. Fruits and vegetables were purchased on separate occasions from local supermarkets and a wholesale center in Singapore. The fruits and vegetables comprised ciku and ciku king (*Manilkara zapota*), plum (*Prunus domestics*), mango (*Mangifera indica* L.), cempedak (*Artocarpus integer* Merr.), rockmelon (*Cucumis melo* var. *cantalupensis*), honeydew (*Cucumis melo* var. *inodorus*), coconut (*Cocos nucifera*), rambutan and rambutan king (*Nepthelium Lappaceum L.*), star fruit (*Averrhoa carambola* L.), kiwi fruit (*Actinidia deliciosa*), seedless grape (*Vitis vinifera*), mangosteen (*Garcinia mangostana*), banana (*Musa acuminate*), orange *(Citrus sinensis*), foot long papaya and solo papaya (*Carica papaya* L), guava (*Psidium guajava*), apple (*Malus pumila*), pomelo (*Citrus maxima*), watermelon (*Citrullus vulgaris*), tomato (*Lycopersicon esculentum* L), lemon (*Citrus limon*), avocado (*Persea Americana*), pineapple (*Ananas comosus Merr.*), salak (*Salacca edulis reinw*), strawberry (*Fragaria spp.*), blueberry (*Vaccinium corymbosum*), kang kong (*Ipomoea aquatica*), egg plant (*Solanum melongena*), lady's finger (*Hibiscus esculentus Linn*), long bean (*Vigna sesquipedalis*), bittergourd (*Mormodica charantia*), French bean (*Phaseolus vulgaris L*), beetroot (*Beta vulgaris*), lettuce (*Lactuca sativa*), red cabbage (*Brassica oleracea* L), red onion (*Allium ascalonicum*), red chilly (*Capsicum spp.*), spinach (*Spinacia oleracea)*, cucumber *(Cucumis sativus L.*) and ulam raja (*Cosmos caudatus).*

Nutraceutical product. One brand of commercial Pycnogenol, which is made from Nuvanta, Horphag's French Maritime Pine bark, was purchased from a branch of the Guardian pharmaceutical shops in Singapore.

Chemicals. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH·), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), L-ascorbic acid, α-tocopherol, fumaric acid, p-coumaric acid, vanillic acid, cysteine, tyrosine, procatechuic acid, p-hydroxyl benzoic acid, myricertin, quercetin, (-)-epicatechin, (-)-gallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (+)-catechin gallate, kaempferol, eugenol, pyrogallol, hydroquinone, catechol, potassium persulfate and glycine were purchased from Sigma (St Louis, Missouri, USA); (+)-catechin hydrate, ferulic acid, succinic acid and chlorogenic acid from Aldrich Chem. Co. (Madison, Wisconsin, USA); trans-cinnamic acid, salicyclic acid, syringic acid, caffeic acid, tripydyltriazine (TPTZ), iron (II) sulphate heptahydrate, trolox, gallic acid from Acros Organics (New Jersey, USA); iron (III) chloride hexahydrate, Folin-Ciocalteu reagent, ethanol, acetic acid, benzoic acid, malic, oxalic, L(+)tartaric acid, methanol and hydrochloric acid were purchased from Merck (Darmstadt, Germany); acetonitrile from EM Science (New Jersey, USA), anhydrous sodium carbonate from J.T.Baker (New Jersey, USA); citric acid from BDH (Poole, England).

2.1.2 Sample preparation

Fruits and vegetables were immediately prepared for analysis after purchase. Coconut water was weighed, diluted and filtered for analysis. Ciku and ciku king were tested before natural ripening. The edible portion of fruits and vegetables was homogenized

using a blender and weighed into a 50 mL centrifuge tube, and 25 mL of 50% aqueous ethanol (sonicated by Bransonics cleaner for 10 minutes) was added (1:10 w/v) and mixed in a vortex mixer for 60 s. The extract was allowed to stand for 20 min at room temperature in the absence of light and then centrifuged at 2000 *g* for 5 min at room temperature. The extract was filtered and used directly for total antioxidant capacity (TAC) and total phenolic content (TPC) assays.

2.1.3 Methods for TAC and TPC assays

2.1.3.1 $ABTS^{\cdot+}$ scavenging assay

The total antioxidant capacity assay was carried out using the Ultraspec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England). The procedure was based on a method developed by Roberta *et al.* [1] with some modification. $ABTS^{\bullet+}$ was generated by reacting ABTS (7.4 mM) with potassium persulphate (2.6 mM). The solution was diluted to obtain an absorbance of 1.5-2.0 units at 414 nm (molar extinction coefficient $\varepsilon = 3.6 \times 10^4 mol^{-1}\ l\ cm^{-1}$, [2]) with pH 4.5 HCl solution before use. In this study, it was found that $ABTS^{\bullet+}$ at pH 4-4.5 is stable even after 2 hours. An aliquot of 20~80 μL of extract or L-ascorbic acid solution was added to 3 mL of this solution. The changes in absorbance at 414 nm were recorded at timed intervals up to 60 min after mixing or until the absorbance reached a plateau. The antioxidant/fruit/vegetable extract solution was further diluted if final absorbance of reaction solution was lower than 0.5 absorbance units. Antioxidant capacity of the extract was obtained by comparing the change of absorbance at 414 nm in test reaction mixture containing extracts of fruit with that containing L-ascorbic acid.

Results were expressed as mg of L-ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of homogenate. L-Ascorbic acid equivalent antioxidant capacity was calculated using the following equation:

$$\mathrm{AEAC} = \frac{\Delta A}{\Delta A_{AA}} \times c_{AA} \times v \times \frac{100}{w}$$

where, ΔA is the change in absorbance after addition of fruit extract, c_{AA} is the concentration of L-ascorbic acid (AA) standard solution (mg/mL), ΔA_{AA} is the change of absorbance obtained from a calibration curve when the same volume of AA standard solution as that of fruit extract was added, v is the volume of filtrate (mL) and w is the weight of homogenate used for extraction (g).

Similar procedures were used for the determination of stoichiometric coefficients of pure antioxidants with ABTS•$^{+}$. The prepared ABTS•$^{+}$ solution was diluted to obtain an initial absorbance of 2-2.6 units at 730 nm (molar extinction coefficient $\varepsilon = 1.5 \times 10^{4} \mathrm{mol}^{-1}\ \mathrm{l\ cm}^{-1}$, [2]) with pH 4.5 HCl solution before use.

2.1.3.2 DPPH• scavenging assay

The DPPH• scavenging activity of fruits was measured using the method described by Brand-Williams [3] with some modification. A solution of 0.1~0.15 mM DPPH• (1, 1-diphenyl-2-picrylhydrazyl) in methanol was prepared. An aliquot of 20~80 μL of extract or L-ascorbic acid solution was added to 3 mL of this solution. The extract was diluted if final absorbance of reaction solution was lower than 0.6 absorbance units. The decrease in absorbance at 517 nm was measured at 0, 1, 5 and then every 5 min until the reaction reached a plateau. The decreased absorbance at 517 nm for

DPPH• remaining at the steady state was calculated and expressed as mg of AA equivalents per 100 g of homogenate (AEAC).

Similar procedures were used for the determination of stoichiometric coefficients of pure antioxidants with DPPH•. DPPH• was dissolved in methanol and kept in fridge for one hour before use. The initial DPPH• with an absorbance of 1.8-2.5 was used for analysis.

2.1.3.3 Ferric Reducing/Antioxidant Power (FRAP) assay

The antioxidant capacity of plant extracts was determined using a modification of the FRAP assay conducted by Benzie and Strain [4]. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6 [5]. Three millilitres of FRAP reagent, prepared freshly and warmed at 37°C, was mixed with 40 µL of plant extract and the reaction mixture was incubated at 37 °C. Absorbance at 593 nm was read relative to a reagent blank incubated at 37 °C for up to 1 hour. Aqueous solutions of known Fe (II) concentrations in the range of 0-2000 µmol/L ($FeSO_4 \cdot 7H_2O$) were used for calibration.

2.1.3.4 Determination of total phenolic contents

Total phenolic contents were determined using Folin-Ciocalteau reagents [6]. Gallic acid standard solution (2.0 mg/mL) was prepared by accurately weighing 0.01 g and dissolving in 50 mL of distilled water. The solution was then diluted to give concentrations of 1.5, 1.0, 0.5, 0.2, 0.1 mg/mL working standard solutions. Forty

microlitres of plant extract or gallic acid standard was mixed with 1.8 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allow it to stand at room temperature (about 28 °C) for 5 min; 1.2 mL of sodium bicarbonate (7.5%) was added to the mixture. After 60 min at room temperature, absorbance was measured at 765 nm. Forty microlitres of distilled water was used as blank following the same procedure as above. Increases of absorbance upon addition of different concentration of gallic acid were used to obtain the calibration curves. Total phenolic contents of a biological sample were obtained by comparing the increase of absorbance upon addition of its extract of total phenolic contents with that of gallic acid. Results are expressed as mg/g gallic acid equivalents (GAE).

2.2 Measurement of the apparent stoichiometry of pure antioxidants with ABTS•$^{+}$ and DPPH•

Both ABTS•$^{+}$ and DPPH• decolorization assays of pure antioxidants were carried out using the Ultraspec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech Ltd., Cambridge, England) or UV 1601 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). The procedures were similar to the above as described in Section 2.1.3.1 and 2.1.3.2. L-Ascorbic acid was dissolved in 2% acetic acid solution. Cysteine and tyrosine were dissolved in pure water. Standards of most phenolic compounds were dissolved in ethanol. All solutions were freshly prepared and immediately used for free radical scavenging assay. The concentration of individual standards is listed in Table 2.1.

Table 2.1 Concentration of antioxidant standards

Compound	Concentration (ppm)	Compound	Concentration (ppm)
L-Ascorbic acid	142	BHT	164
Cysteine	130	BHA	133
Syringic acid	161	TBHQ	154
p-Hydroxybenzoic acid	500	Pyrogallol	78
Procatechuic acid	183	Hydroquinone	175
Caffeic acid	162	Quercetin	76
Tyrosine	202	Quercetin glucoside	130
Coumaric acid	167	(-)Epicatechin	87
Vanillic acid	139	Myricertin	66
Ferulic acid	147	Kampferol	120
Gallic acid	87	Trolox	156

2.3 HPLC/DAD analysis of antioxidants

2.3.1 Rapid analysis of L-ascorbic acid of fruits and vegetables with HPLC/DAD

L-Ascorbic acid standard solution (1.36 g/mL) was prepared daily by accurately weighing 68 mg and dissolving it in 50 mL of 2% acetic acid. This was then diluted to give 0.068, 0.034, 0.017, 0.0085 mg/mL working standard solutions. HPLC system consisted of a Shimadzu HPLC (Model LC-10ATvp two pumps and DGU-14A degasser) equipped with a diode array detector (DAD) (Model SPD-M10A_{VP}) (Shimadzu, Kyoto, Japan) interfaced with IBM Pentium-III personal computer. The separation was performed on a Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10×4.6 mm i.d.) using 2% (v/v) acetic acid/acetonitrile (95:5, v/v) as the mobile phase at a flow-rate of 0.8 mL/min at 40 °C oven temperature. Ten microlitres of the extract prepared earlier

were injected into the HPLC. The measured L-ascorbic acid content was expressed as mg AA/100 g edible portion.

2.3.2 Simultaneous analysis of organic acid and phenolic compounds

HPLC system used was the same as above. The separation was performed on a Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10×4.6 mm i.d.). The optimum efficiencies of separation were obtained using 0.35 mL/min of pH 2.5 sulphuric acid solution (solvent A), and flow rate of methanol (solvent B) was increased from 0 to 0.45 mL/min from 15 min to 75 min and then kept at 0.45 min/L for a period of 15 min.

The individual standards were dissolved in 0.2% BHT methanol or pH 2.5 sulphuric acid and injected to determine individual retention times. Stock solutions of 500 μg/mL of tartaric acid, 2000 μg/mL quinic acid, 150 μg/mL oxalic acid, 1000 μg/mL malic acid, 100 μg/mL L-ascorbic acid, 1000 μg/mL malonic acid, 1800 μg/mL citric acid, 2000 μg/mL acetic acid, 1000 μg/mL citric acid, and 20 μg/mL fumaric acid were prepared by dissolving pure standards into pH 2.5 sulphuric acid. The stock solutions and the four diluted standards up to 10 times dilution from stock solutions were injected for linearity range and detection limit tests. Similarly, stock solutions of other individual standards were prepared by dissolving 2 mg pure standard in 0.2% BHT methanol except that ellagic acid was dissolved in ethanol. The stock solutions and the four diluted standards up to 20 times dilution from stock solutions were injected for linearity range and detection limit tests.

(-)-Gallocatechin, (-)-epigallocatechin, (+)-catechin, chlorogenic acid, (-)-epigallocatechin gallate, (-)-epicatechin, caffeic acid, (-)-catechin gallate, myricetin and quercetin, eugenol and kaempferol were dissolved in about 30 mL of 0.2% BHT methanol. Ellagic acid was dissolved in ethanol. This solution was mixed with 30 mL of sonicated water. As solvents will interfere with peak shape, the mixed solution was further evaporated to remove most of the organic solvent at room temperature under vacuum. Other compounds were dissolved in 50 mL of H_2SO_4 solution (pH 2.5), and mixed with the above solution. The final volume of this solution was made up to 100 mL. The solution was kept at 4°C and used to optimize the HPLC separation conditions. The solution was also applied to observe the within-day and between-day precisions of retention times and peak areas. All solutions were filtered through a 0.45-μm membrane filter (Iwaki Glass) before HPLC analysis, and the mobile phase solvents were degassed before use.

To verify its potential application for separation and determination of antioxidants in fruits and vegetables, the developed method was used for analysis of Brand A apple juice (Australia), which was purchased from a local supermarket. Furthermore, the established method was used for preliminary screening and identification of antioxidants in juice and solvent extracts of selected fruits and vegetables.

2.4 Analysis of antioxidants in selected fruits and vegetables

A few tropical fruits and vegetables e.g. ciku king, ulam raja, salak, star fruit and lady's finger were found to be good sources of natural antioxidants. To our knowledge, their antioxidant compositions have not previously been fully reported.

Of these selected fruits with high antioxidant capacity, star fruit is largely produced and widely consumed in Southeast Asia. This makes it possible to be a potential source of antioxidant nutraceuticals or food additives. Thus, while this study concentrated on its antioxidant compositions, its potential application was also discussed.

Both ciku and ciku king had extremely high antioxidant capacity, but only ciku king fruit was systematically investigated as it is much easier to obtain from local markets than ciku. Although unripe ciku king had high antioxidant capacity, it could not be eaten due to extremely astringent taste. To inform the public how much antioxidant capacity is usually consumed, this study will not only cover antioxidant composition but also variation of antioxidant capacity at different stages of ripening.

For other fruits and vegetables selected, their antioxidant composition will be directly studied as they are ready for eating.

2.4.1 Analysis of antioxidants in star fruit

2.4.1.1 Solvent extraction of antioxidants

Fresh star fruit obtained from the market was homogenized using a blender and filtered under vacuum. The liquid portion (juice) was kept in a fridge at 4°C immediately. The solid portion was collected and used for TAC analysis. The extraction was carried out at different temperatures (30, 50, 70 and 90°C respectively), different extraction times (15, 30, 45, 60 minutes respectively), and different ratios of solvents (acetone and ethanol respectively) to water. Total antioxidant capacity (TAC) of all extracts was determined as described in Section 2.1.3.1. Under optimum extraction condition i.e. 50% aqueous acetone at 90°C for 45 min, residue was extracted three times to test the necessity of multiple extractions. The extract was evaporated to remove the solvent under reduced pressure, and then made up to a fixed volume with water. It was found that the loss of TAC was less than 5 % after evaporation and reconstitution. The obtained solution was immediately used for other assays or kept at -18°C in a freezer for future uses. The residual extract and juice were filtered with 0.5 μm filter before HPLC analysis.

Total phenolic contents of corresponding extracts or juice were determined using the procedure described in Section 2.1.3.4.

2.4.1.2 Solid phase extraction of antioxidants

The mixture of juice and residue extract was subjected to solid-phase extraction using a 3 mL end-capped C_{18} column (Isolute) previously activated with methanol and conditioned with pure water. Two milliliters of juice were introduced into the column. Three milliliters of water were used to elute most of organic acid and sugar (FRC 1), and then 1.5 mL of methanol were used to elute and the fraction was collected (FRC

2), and then another 1 mL of methanol was used to elute large molecular compounds (FRC 3). The TAC of all fraction collections was measured by the ABTS•$^{+}$ assay.

2.4.1.3 Analysis of antioxidant peak in star fruit using HPLC/DAD

HPLC conditions used are those described as section 2.3.2. Twenty microlitres of juice or extracts were injected into the HPLC for L-ascorbic acid (AA) and (-) epicatechin assays. Three milliliters of juice/extract and 4.5 mL of ABTS•$^{+}$ solution were mixed to react for 1 h and then passed through 0.5 μm filter and injected for HPLC assay. Blanks of juice with water and ABTS•$^{+}$ with water were also analyzed. The gallic acid content in gallotannin forms was measured as described in [7]. Briefly, juice and extracts of residue were mixed according to their volumetric ratio. One millilitre of mixed solution, in triplicate, was pipetted into culture test tube, and then 0.1 mL of 22 N sulphuric acid was added to it. The contents were frozen and air was removed from these tubes by using a vacuum pump. These tubes were kept at 100 °C for 16 h to hydrolyze gallotannins to gallic acid. After hydrolysis, the volume was made up to 10 mL with pH value of 4.0±0.5 by adding 0.5 N sodium hydroxide and distilled water. The hydrolyzed supernatant was filtered through 0.5 μm filter and injected for HPLC assay of gallic acid. The contents of gallic acid in gallotannin forms were also tested respectively in juice and extracts using the procedure described above.

2.4.1.4 ESI-MS and HPLC-DAD-ESI-MS analyses of antioxidants.

A Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with TSP 4000 HPLC system, which includes UV6000LP PDA detector, P4000

quaternary pump and AS3000 autosampler was used. The heated capillary and spray voltage were maintained at 250 °C and 4.5 kV, respectively. Nitrogen was operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate. The full scan mass spectra from *m*/*z* 50–2000 were acquired both in positive and negative ion mode with a scan speed of 1 s per scan. Tandem mass spectrometry was performed using helium as collision gas, operated at 0.8 mtorr, and the collision energy was set from 10 to 80 and 50% was found to be suitable to obtain extensive fragment ions of proanthocyanidins in pycnogenol and star fruit.

For the HPLC-DAD-ESI-MS assay of juice/extract, the instrument was set to measure the following events: (1) UV chromatogram at 280 nm, which is the wavelength usually used to monitor phenolics; (2) UV spectra of individual peaks; (3) TICs; (4) zoom scan was applied for measuring isotopic distances at *m*/*z* 291, 579, 867, 1155 and 1443, respectively; (5) MS-MS was used to break down the most abundant $[M+H]^+$ or $[M-H]^-$. Chromatographic separations were done on a Shim-Pack VP-ODS column (250 mm×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10 mm×4.6 mm i.d.) under the following elution conditions: flow rate, 600 μL min^{-1}; room temperature (27°C); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50 to 90% B in 25 min, and from 90 to 20% B in 5 min for washing and reconditioning of the column.

Manual injection for ESI-MS or EIS-MS-MS was performed with a constant flow rate of 300 μL min^{-1} of 50/50 solvent A to B. The collision induced dissociation (CID) spectra of (-)epicatechin, (+)catechin, and singly-linked proanthocyanidins from pycnogenol were recorded.

2.4.1.5 Anti-rancidity properties of residue extract on soya bean oil

Twenty mL of extract solution from 0.2 g of residue was evaporated to around 0.5 mL and then thoroughly mixed with 100 g of soya Bean oil. Separate 5g aliquots of this oil were added into 15 test tubes respectively and then subjected to peroxidation at 110°C. At a specific time, the peroxide values (PV) of the test tubes were determined using the AOCS official method [8]. The PV value of blank was also obtained using pure soya bean oil while oil containing 140 ppm BHT was used for comparison.

2.4.2 Analysis of antioxidants of Lady's finger

2.4.2.1 Solvent extraction of antioxidants

Preliminary HPLC-DAD analysis implied that quercetin derivatives were the major antioxidants present, and no proanthocyanidins were identified. Thus, aqueous ethanol was selected as extraction solvent for Lady's finger instead of aqueous acetone, which could inhibit tannin-protein interaction and thus be particularly efficient for tannin rich samples. Fresh lady's finger was homogenized and immediately extracted in 70% denatured ethanol (w/v, 1:8) for 4 hours with continuous stirring in a dark bottle. The extract was filtered and kept in a fridge at 4°C. The residue was extracted again using one fourth of the first extraction volume. The two filtrates were combined and solvent was evaporated under vacuum at 35°C, and then redissolved in pure water. It was found that the loss of TAC was less than 5% after evaporation and reconstitution. Hexane was subsequently added to remove non-polar compounds *e.g.* carotenoids and

chlorophyll. The aqueous portion was collected and dried by vacuum evaporation at 35°C. There was almost no loss of antioxidant during hexane extraction process. The solution was stored in a fridge at 4°C for 2 days to further remove chlorophyll precipitated from solution. The obtained solution was centrifuged at 4°C and filtered with 0.45 µm membrane filter and then used for solid phase extraction, TAC, HPLC, HPLC/MS^n and semi-preparative HPLC analyses.

2.4.2.2 Solid phase extraction of antioxidants.

The extract was subjected to solid phase extraction using a reverse-phase C18 column (Isolute) that has been activated with methanol and conditioned with pure water. One millilitre of juice was introduced onto the column. Half a millilitre of water was used to elute most of the more polar components (FRC 1), and then 1 mL of methanol was used to elute and the fraction was collected (FRC 2), and then another 1 mL of methanol was used to elute possibly existing more non-polar compounds (FRC 3). The TAC of all the fractions collected and extract were measured by $ABTS^{\cdot+}$ scavenging assay. The fractions were also injected for HPLC assay as described in section 2.4.2.4.

2.4.2.3 HPLC characterization of major antioxidant peaks

Chromatographic separations were done on a Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10×4.6 mm i.d.) under the following elution conditions: flow rate = 600 µL min^{-1}; 30ºC; solvent A, 0.1% formic acid in water; solvent B, methanol; starting from

30 to 70% B in 30 min and keeping constant for 4 min, and then from 70% to 30% B in 6 min and keeping constant for 5 min for reconditioning the column.

$ABTS^{\bullet+}$ solution was prepared as previously reported by Aliaga and Lissi [9]. One mL of extract and 5 mL of $ABTS^{\bullet+}$ solution were mixed and allowed to react for 1 hour and then passed through 0.45 μm filter and used for HPLC assay. Blanks of juice with water and $ABTS^{\cdot+}$ with water were also analysed.

In addition, 20 μL of extract from fresh lady's finger was injected into the HPLC to obtain L-ascorbic acid (AA) contribution using the method described in section 2.3.2.

2.4.2.4 HPLC-DAD-ESI-MS^n analysis

The instrumentation and operation conditions for mass spectrometric detection were similar to those described in section 2.4.1.4 except that the heated capillary was set at 200°C instead of 250°C to minimize fragmentation of parent ions, and the collision energy was set from 20 to 100% and optimized collision energy was chosen for individual compounds.

For HPLC-DAD-ESI-MS assay of extract, the instrument was set to measure the following events: (1) UV spectra of individual peaks; (2) TICs; (3) zoom scan was applied for measuring isotopic distances at m/z 597, 627, 465, and 551 respectively; (4) MS-MS and MS^n were applied to break down the most abundant $[M+H]^+$ or $[M-H]^-$ and corresponding daughter ions from MS^{n-1}.

Chromatographic separations were done on a Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10×4.6 mm i.d.) under the following elution conditions: flow rate, 600 µL min^{-1}; room temperature (27ºC); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50% to 90% B in 25 min, and from 90% to 20% B in 5 min for washing and reconditioning of the column.

2.4.2.5 Isolation of pure compounds by semi-preparative HPLC

Two millilitres of sample solution were loaded on a tandem column system, which consists of a C18 Nova-Pack C_{18} (7.8×300 mm, Waters) and Shim-Pack VP-ODS column (250×4.6 mm i.d.) (Shimadzu, Kyoto, Japan). Isocratic flow rate 1.2 mL/min, 40% of Methanol and 60% of 0.1% formic acid were the eluents. Compounds **1-4** were isolated from the extract by means of repeated semi-preparative HPLC. The purity of the compounds was examined by analytical HPLC-DAD as described in section 2.4.2.3.

2.4.2.6 Spectroscopic study of isolated compounds

General UV-visible shift reagents were prepared according to Markham [10]. NMR spectra were acquired using a Bruker DPX300 spectrometer and Bruker AMX500 spectrometer**.** Samples were dissolved in DMSO-d6, and reference to the residual solvent resonance at δ_H=2.50 ppm or δ_C=39.50 ppm as appropriate.

2.4.3 Analysis of antioxidants of salak

2.4.3.1 Solvent extraction of antioxidants

Fresh salak with firm flesh and sweet taste was obtained from local markets. Salak was peeled and the seeds were removed from the flesh. Flesh was cut into small pieces and then homogenized. The homogenized flesh was extracted with 60% of aqueous ethanol for 1 hour in the dark. The extract was centrifuged and filtered. The obtained solution was dried by rotary evaporation at 35ºC and redissolved in 20% aqueous methanol. The antioxidant capacity of obtained solutions was measured, and it was found that the loss of TAC was less than 5 % after evaporation and reconstitution. The obtained solution was immediately used for other assays or kept in the refrigerator at -18ºC for further experiments. The solution was filtered with 0.45 μm membrane filter before HPLC-MS.

2.4.3.2 Identification of antioxidants in salak by HPLC/MS and HPLC/MS/MS

The instrumentation and most parameters for HPLC/MS were the same as those in section 2.4.1.4 except for elution conditions, which are flow rate = 500 μL min^{-1}; room temperature (around 27ºC); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50 to 90% B in 20 min and keeping constant for 10 min, from 90 to 20% B in 5 min, keeping constant at 20% for 10 min for reconditioning of the column. Mass spectra were recorded within 45 min.

One millilitre of extract and 2 mL of $ABTS^{\bullet+}$ solution are mixed to react for 1 hour and then passed through 0.45 μm filter and injected for HPLC assay. Blank of extract with water was used as control.

For characterised antioxidant peaks, the collision energy was set from 50% to 80% to obtain fragment ions from their corresponding parent ions.

2.4.4 Analysis of antioxidants in ciku king

2.4.4.1 Solvent extraction of antioxidants

Preliminary HPLC-MS analysis implied that tannins were major antioxidants of ciku king. Similar solvent extraction procedures to those for star fruit were carried out to optimise extraction conditions, and similar optimised extraction was obtained i.e. 50% aqueous acetone for 45 min at 90°C. The flesh of ciku king fruit was extracted under optimised conditions. After cooling to room temperature with ice, the extracts were centrifuged at 8000 rpm and the supernatant was used without further treatment for total antioxidant capacity and total phenolic content assays as described in sections 2.1.3.1 and 2.1.3.4. The obtained solution was dried by rotary evaporation at 35ºC, redissolved in 20% aqueous methanol and centrifuged again. The antioxidant capacity of supernatant was measured, and it was found that less than 5% of TAC was lost after evaporation and reconstitution. The obtained solution was kept in fridge at -18ºC for further experiments.

2.4.4.2 Identification of antioxidant in ciku king by HPLC/MS and HPLC/MS/MS

The instrumentation conditions were the same as those described in section 2.4.3.2. One mL of extract and 1 mL of $ABTS^{\bullet+}$ solution were mixed to react for 1 hour and then passed through 0.45 μm filter and injected for HPLC assay. Blank of extract with water was used as the control. For characterised antioxidant peaks, the collision energy was set from 50% to 80% to obtain fragment ions from their corresponding parent ions.

2.4.4.3 Changes of TAC & TPC of ciku king fruit during storage

Five batches of fresh unripe ciku king fruits were purchased from a local supermarket at different occasions. For each batch, fruits of the same size with a similar appearance and hardness were selected for one test. For all replicates, unripe ciku king fruits were placed at room temperature (around 27ºC) to leave them to ripen naturally. For each batch, the TAC and TPC of ciku king fruits were measured at the same time of 0, 1, 2, 3, 4, 5, 6 and 7 days, respectively.

2.4.5 Analysis of antioxidants of ulam raja

2.4.5.1 Solvent extraction of antioxidants

Preliminary HPLC-MS analysis implied that proanthocyanidins and quercetin were the major antioxidants of ulam raja. To maximally extract antioxidants from it, leaves

of ulam raja were dried at 40ºC, homogenized and immediately extracted with boiling water, 50% aqueous ethanol at 80ºC and 50% aqueous acetone at 80ºC for 45 min respectively. The extracts were centrifuged at 6400g, and the supernatants were directly used for TAC assay as described in section 2.1.3.1. Fifty percent of aqueous acetone at 80ºC for 45 min was found to give maximum antioxidant extraction. The extract with the highest antioxidant capacity was dried by rotary evaporation at 35 ºC and reconstituted into 20% aqueous methanol solution. The antioxidant capacity of the reconstituted solution was measured, and it was found that the loss of TAC was less than 5 % after evaporation and reconstitution. The obtained solution was centrifuged at 6400g at 4°C and filtered with a 0.45 μm membrane filter and then used for HPLC/MS and HPLC/MS/MS analyses.

2.4.5.2 Identification of antioxidants in ulam raja by HPLC/MS and HPLC/MS/MS

The instrumentation and most parameters for HPLC/MS were almost the same as those described in section 2.4.3.2. One millilitre of extract and 4 mL of $ABTS^{\bullet+}$ solution were mixed to react for 1 hour and then passed through a 0.45 μm filter and injected for HPLC assay. Blank of extract with water was used as the control. For characterised antioxidant peaks, the collision energy was set from 30 to 100% to obtain fragment ions from their parent ions.

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PART III

RESULTS AND DISCUSSIONS

Chapter 3

Antioxidant Properties of Fruits and Vegetables

3.1 Introduction

One of the main objectivities of this study was to investigate total antioxidant capacity (TAC) of selected fruits and vegetables in Singapore market and to find antioxidant rich items for further study on their antioxidant compositions and possible health effects. While information on total antioxidant capacity of a fruit or vegetable is important, information on its antioxidant efficiency might also be important as it indicates how fast those antioxidants react with free radicals. It is reasonable to assume that antioxidant property of a fruit or vegetable depends mainly on its major antioxidants. This part will address the antioxidant property of fruits and vegetables using different non-inhibition methods and compare various methods of analysis.

3.2 Antioxidant Capacity and Antioxidant Efficiency

One of the most common methods used to study the antioxidant capacity of a fruit or vegetable is to study the drop of absorbance due to the loss of $ABTS^{\bullet+}$ scavenged by the antioxidant in question. The drop of absorbance gives the quantity of $ABTS^{\bullet+}$ scavenged after a specific time. Unless the choice of time used is supported by a series of well studied experiments, the drop of absorbance recorded will not be meaningful if the reaction is not completed for all the samples. However, since the

time taken for the reaction to come to completion can be invariably long, a convenient but arbitrary time is usually chosen to calculate and compare the total antioxidant capacity (TAC) of a sample. As different times are often applied by different researchers, the TAC obtained can only be compared if the same time is used. For the same reasons, ranking of a series of compounds based on their TAC obtained in this manner will not be very meaningful.

Considering an absorbance *vs*. time plot of two different antioxidants reacting with $ABTS^{\bullet+}$, the activity of the antioxidants can be compared based on the initial rate of reaction or the quantity of radicals they are able to scavenge. The initial rate of reaction with $ABTS^{\bullet+}$ provides information on the efficiency of the antioxidant, while the quantity of radicals scavenged provides information on the capacity of the antioxidant. Based on this, if the efficiency and capacity of an antioxidant is constantly higher than the other at any time interval, ranking of the antioxidants by taking the change of absorbance after a certain time will be accurate.

In the event where the total drop of absorbance caused by two antioxidants are the same, they have the same antioxidant capacity. However, the time taken for the absorbance to drop to a level where it does not change any more may be different. In this case, the efficiencies of the two antioxidants are different.

It is also possible that two different antioxidants may scavenge free radicals at the same rate initially. However, one may react with more radicals compared with the other, thus have a higher capacity. Therefore, when absorbance drop is taken during the initial part of the reaction, they will give the same antioxidant capacity, while

taking the change of absorbance after completion of the reaction will result on one antioxidant having a higher antioxidant capacity than the other.

In the worse case scenario, both the efficiency and the capacity of the two antioxidants in question are different. Thus, taking the change of absorbance before the crossover point will reflect the efficiency of the antioxidant while if the absorbance change is considered after the crossover point, this will reflect more accurately on the capacity of the antioxidants.

It was found that if the entire reaction was considered for fruits and vegetables extracts expressed as L-ascorbic acid equivalent antioxidant capacity (AEAC) *vs.* reaction time, a fruit extract may exhibit higher AEAC at a given time and a lower value at another time of another fruit extract. This means that the AEAC ranking can be changed when different reaction times were chosen. An example is shown in Fig. 3.1, where the AEAC value of guava is larger than that of plum before 56 min but lower after 56 min. Similar phenomena were also observed comparing the AEAC of kiwi fruit and mango before and after 20 min (Fig. 3.1).

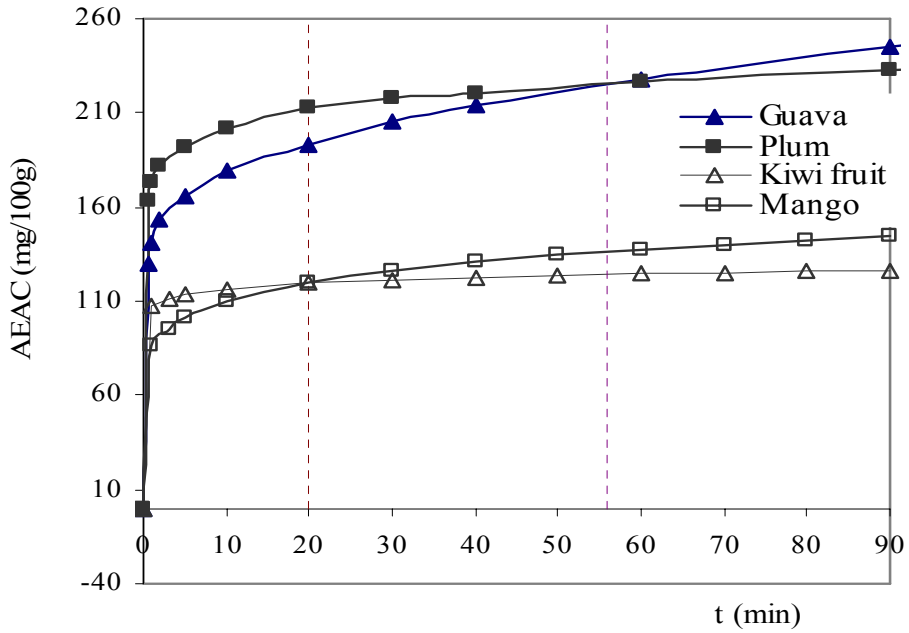

Fig. 3.1. AEAC values at different reaction time for plum, guava, kiwi fruit and mango extracts

Therefore, ranking natural antioxidant according to their TAC by considering the change of absorbance at a specific time or arbitrary time, may provide information not only on antioxidant capacity but also their efficiency. However, the two parameters cannot be differentiated if TAC is calculated considering the drop of absorbance at a specific time. In addition to this, different biological samples contain a variety of antioxidants at different concentration levels. As such, antioxidant capacity is a sum of the individual compounds. Thus, it is more meaningful to identify their major components and understand the kinetics of their reaction with free radicals individually.

In addition to the above, the reaction rate of antioxidants with free radicals may differ significantly from one another because of their chemical structures. For example, the reaction between L-ascorbic acid and $ABTS^{\bullet+}$ is essentially complete after 1 min. Similar results are obtained when $ABTS^{\bullet+}$ reacts with Trolox and

α-tocopherol, while some other compounds like luteolin, quercetin, naringenin and glutathione are still reacting with ABTS•$^{+}$ after 4 min [1]. In fact, many phenolic compounds e.g. chlorogenic acid are still reacting with ABTS•$^{+}$ and DPPH• after a few hours or even days (data not shown).

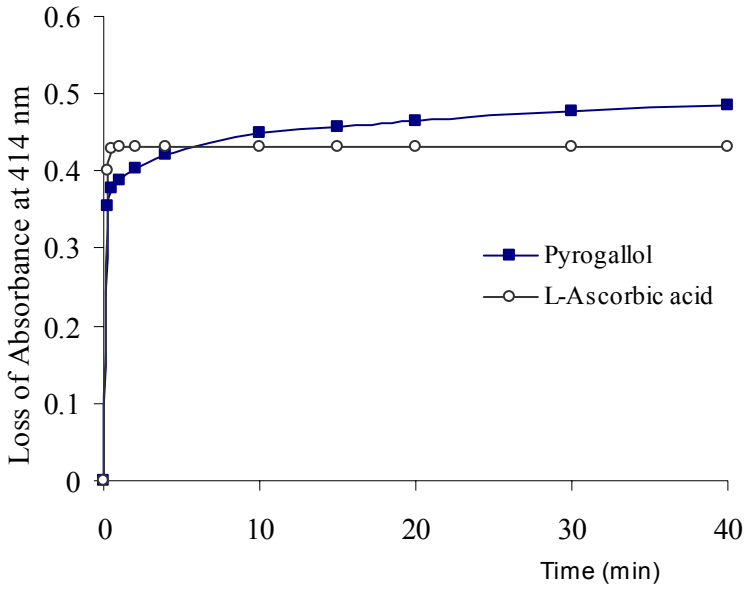

Fig.3. 2. Comparison of reactions between pyrogallol and L-ascorbic acid with ABTS•$^{+}$

As shown in Fig.3.2, L-ascorbic acid reacts with ABTS•$^{+}$ completely after 1 min, while pyrogallol is still reacting with ABTS•$^{+}$ after 40 min. Therefore, it is reasonable that different fruits exhibit different kinetic curves because of the difference in variety and concentration of antioxidants in every sample. As a result, it is better to analyse the kinetics of the reaction on the whole to compare the TAC of fruits with similar antioxidant capacity. For example, as shown in Fig. 3.1, antioxidants in guava react with ABTS•$^{+}$ faster than those in plum, which indicates that antioxidants in guava may be more efficient than those in plum. On the other hand, the knowledge of major antioxidant(s) in a fruit or vegetable will help one to understand the contribution to

the kinetic curve observed for the extract. Further discussion on this aspect is presented in Section 3.3.

3.3. Antioxidant components of fruits and vegetables

The knowledge of the antioxidant components in a fruit or vegetable will help the understanding of the kinetics of antioxidant reactions with radicals such as $ABTS^{\bullet+}$ and DPPH•. Every antioxidant component will have a different influence on the kinetics of the mixture with radicals. Presumably, the effects of these antioxidants are additive, this will mean that if the quantity of each antioxidant and their kinetic parameters are known, the total antioxidant capacity can be predicted. Although there is evidence that antioxidants act synergistically [2-4], it is reasonable that the major antioxidants will play a major role on the kinetics of the whole extract. Antioxidants reported in fruits may include L-ascorbic acid, tocopherols, phenolic acids, flavonoids and amino acids. Contents of tocopherols in plant oil, fish oil, nuts, seeds and grains are normally quite high, but low in most fruits and vegetables [5]. L-Ascorbic acid and phenolic compounds are found widely in fruits and vegetables and are probably two of the most effective antioxidants present.

3.3.1 L-Ascorbic acid contribution to TAC of selected fruits and vegetables

Table 3.1 shows total antioxidant capacity expressed as AEAC of fruits tested, and values vary 300 fold from the lowest to the highest. Based on these values, the activity of the fruit extract to scavenge free radicals is arbitrarily classified into

four categories. The fruit with an AEAC of over 600 mg AA_{eq}/100g is classified as containing extremely high antioxidant capacity. On the other hand, fruits with AEAC from 200 to 600 mg AA_{eq}/100g, 70 to 200 AA_{eq}/100g and less than 70 mg AA_{eq}/100g are classified as containing high, medium and low antioxidant capacity respectively. On the basis of the wet weight of fruit (edible portion), ciku shows the highest antioxidant capacity, followed by ciku king, ulam raja, blueberry, strawberry, plum, star fruit, guava, seedless grape, salak, mangosteen, avocado, oval orange, solo papaya, mango, kiwi fruit, cempedak, pomelo, lemon, pineapple, apple, foot long papaya, rambutan, rambutan king, banana, coconut pulp, tomato, rockmelon, honeydew, watermelon and coconut milk. The total antioxidant capacity of ciku, ciku king, ulam raja and blueberry was found to be extremely high. Several tropical fruits from Southeast Asia, such as guava, star fruit and salak, are very good sources of antioxidants. Others fruits like avocado, papaya, mangosteen, pomelo, pineapple, cempedak, lemon and rambutan also contain considerable amounts of antioxidants. Wang *et al.* had previously measured the total antioxidant activity of 12 fruits using automated oxygen radical absorbance capacity (ORAC) assay [6]. Of the eight fruits that were mutually tested, the ranking orders of total antioxidant capacity were as follows: strawberry>plum>orange>kiwi >banana>apple>tomato>honeydew melon. The ranking order was found to be the same here except apple was found to have a higher AEAC compared with banana. The difference could be related to seasonal variations or variety.

As mentioned in Chapter 1, L-ascorbic acid is very important for human health. Because of different criteria of adequacy and different interpretations of

experimental evidence, the recommended dietary allowance (RDA) for L-ascorbic acid varies between 30 and 80 mg/day. The RDA of L-ascorbic acid for adult men and women is 30, 60 and 80 mg/day in Singapore, United States and Netherlands respectively [7, 8]. It is likely that the current RDA for L-ascorbic acid to prevent deficiency disease is not sufficient to optimally protect against the chronic disease mentioned above, therefore, Carr and Frei suggested a new RDA of 120 mg L-ascorbic acid /day [9].

Table 3.1 AEAC of selected fruits and vegetables using ABTS•$^{+}$ assay and their L-ascorbic acid content*

Variety	AEAC [a] (mg/100g)	AA content by HPLC [a] (mg/100g)	Percentage contribution of AEAC by AA (%) [b]	Classification by AEAC
Ciku	3396 ± 387	2.0 ± 0.7	0.1	Extremely high
Ciku King	2800 ± 456	3.6 ± 0.9	0.1	
Ulam Raja	2400 ± 297	19.0 ± 3.2	0.7	
Blueberry	760.4 ± 56.3	13.0 ± 5.4	1.7	
Strawberry	471.5 ± 92.9	53.9 ± 11.2	11.4	High
Plum	312.4 ± 23.2	8.2 ± 2.3	2.6	
Star fruit	277.5 ± 22.3	5.9 ± 1.8	2.1	
Guava	270.2 ± 18.8	130.5 ± 18.2	48.3	
Grape seedless	264 ± 83.6	0.5 ± 0.2	0.2	
Salak	260.2 ± 32.5	2.4 ± 1.5	0.9	
Red onion	180 ± 9.5	13.0 ± 4.5	7.2	Medium
Mangosteen	149.5 ± 23.3	4.1 ± 1.2	2.7	
Avocado	143.3 ± 16.5	9.0 ± 2.1	6.3	
Orange (oval)	141.8 ± 22.6	36.1 ± 15.9	25.5	
Solo papaya	140.9 ± 26.7	67.8 ± 12.6	48.0	
Mango	139.1 ± 21.5	19.7 ± 9.1	14.2	
Kiwi fruit	136.4 ± 18.2	52.8 ± 22.5	38.7	
Cempedak	126.2 ± 19.1	6.2 ± 0.9	4.8	
Lady's Finger	118 ± 16.3	10.0 ± 4.1	8.5	
Pomelo	103.6 ± 34.7	36.0 ± 7.5	34.7	
Lemon	93.3 ± 9.8	49.6 ± 6.8	53.2	
Pineapple	85.6 ± 21.3	54.0 ± 7.9	63.0	
Apple	78.9 ± 2.7	2.1 ± 0.9	2.7	
Foot long	72.5 ± 2.6	45.2 ± 10.3	62.3	
Rambutan	71.5 ± 7.6	50.2 ± 6.5	70.2	
Rambutan king	70.6 ± 8.2	49.5 ± 8.8	70.0	
Banana	48.3 ± 1.2	2.1 ± 0.8	4.4	Low
Eggplant	55 ± 8.6	1.7 ± 0.6	3.1	
Coconut pulp	45.8 ± 6.5	0.9 ± 0.3	19.7	
Tomato	38.0 ± 1.7	11.0 ± 2.6	29.1	
Rockmelon	26.2 ± 3.5	2.7 ± 0.6	10.3	
Honeydew	19.6 ± 0.8	3.9 ± 0.4	19.9	
Watermelon	11.9 ± 0.1	3.7 ± 0.2	31.0	
Coconut milk	11.5 ± 2.2	0.7 ± 0.3	6.1	

[a] Mean of three determinations ± SD (standard deviation).

[b] Percentage contribution of AEAC by AA (%) = $([AA]_{mean}/AEAC_{mean})\times100\%$

*Most fruits and vegetables were obtained from August 2000 to February 2001.

As can be also seen from Table 3.1, the L-ascorbic acid contribution to scavenge ABTS$^{\bullet+}$ varies extensively up to 200 times the lowest value. L-Ascorbic acid accounts for high percentage contribution to ABTS$^{\bullet+}$ scavenging activity in rambutan and rambutan king (70%), pineapple (63%), guava (48.3%), lemon (53.2%) and solo papaya (48%), foot long papaya (62.3%), kiwi fruit ((38.7%), pomelo (34.7%), watermelon (31.0%), tomato (29.1%) and oval orange (25.5%). Wang *et al.* suggested the contribution of L-ascorbic acid to ORAC activity of a fruit was usually less than 15% except for kiwi fruit and honeydew melon [6]. However, in this study, it was found that several fruits tested had high contribution of L-ascorbic acid to their total antioxidant capacity. Since Wang *et al.* did not test the antioxidant capacity of fruits having high L-ascorbic acid contribution in this study, the real L-ascorbic acid contribution to ORAC activity among these fruits needs to be confirmed. The contribution of L-ascorbic acid to AEAC among other fruits was low, especially for ciku, plum, star fruit, salak, seedless grape, mangosteen, apple and cempedak. It seems that fruits with high AEAC value are more likely have a lower percentage contribution from L-ascorbic acid to AEAC except for guava.

This set of data shows that the contribution of L-ascorbic acid to the AEAC of fruits can differ extensively from one fruit to another. Where the contribution of L-ascorbic acid is low, it must be that other compounds present are the main antioxidant components. Therefore, contribution of other compounds to AEAC of these fruits must not be neglected. For example, the contribution of L-ascorbic acid to TAC of plum is less than 5%. This is consistent with the kinetic curve of plum, which shows that the radicals are gradually being scavenged instead of the fast reaction type of L-ascorbic acid with free radicals. Therefore, contribution of other compounds to

AEAC of these fruits should not be neglected. Among the most important compounds that contribute to the high AEAC value of these fruits are likely to be polyphenolic compounds.

3.3.2 Total phenolic contents of fruits and vegetables

Phenolic compounds may significantly contribute to TAC of many fruits and vegetables. Therefore, it is necessary to investigate the correlations between antioxidant capacity and total phenolic contents of fruits and vegetables.

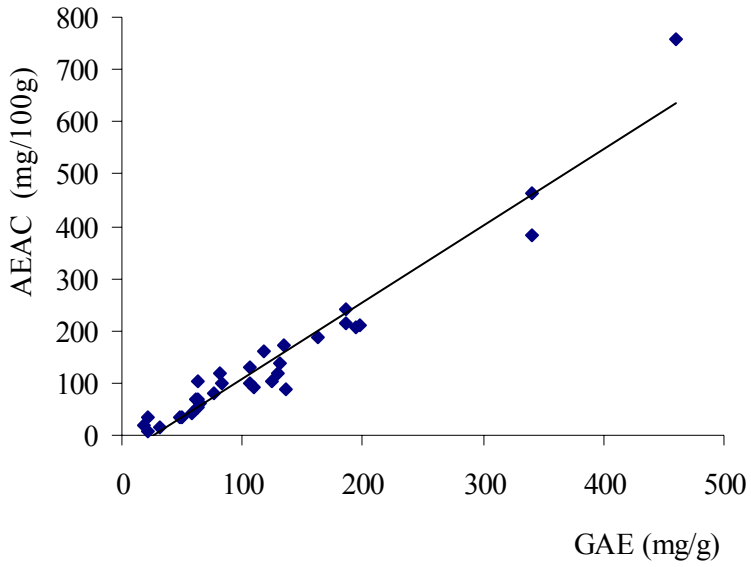

Fig. 3.3. Correlation of AEAC and total phenolic content among fruits and vegetables (R^2 =0.9381)

As can be seen from Fig. 3.3 and Table 3.2, of the fruits and vegetables tested in this study a good correlation (R^2 =0.9381) between total phenolic content and AEAC value by $ABTS^{\bullet+}$ scavenging assay was obtained. The high correlation coefficient

between TAC and total phenolic content suggested that free radical scavenging ability of most fruits and vegetables is most likely contributed by phenolic compounds. A similar result was reported by Kaur and Kapoor [10], who noticed that antioxidant activity of selected Asian vegetables, which was obtained by using a model system consisting of beta-carotene and linoleic acid, correlated significantly with their total phenolic contents. A linear relationship was also observed between antioxidant capacity measured by oxygen radical absorbance capacity (ORAC) method and total phenolic contents of certain selected medicinal herbs (R = 0.919) and culinary herbs (R = 0.986) as reported by Zheng and Wang [11].

3.3.3 Effects of antioxidant components on TAC of fruits and vegetables

Many reports suggest phenolic compounds are one of the main antioxidant components in fruits and vegetables [10, 11]. As reported by Stacewicz-Sapuntzakis and co-workers, chlorogenic acid and neo-chlorogenic acid are major antioxidants in plum [12]. Reaction at room temperature between chlorogenic acid and $ABTS^{\bullet+}$ has a similar trend to plum extract and is not completed even after 15 hours (Fig. 3.4). This is consistent with the results of kinetic curve of chlorogenic acid rich plum extract. Therefore, identification of the major antioxidants in a fruit or vegetable will help predict the shape of its kinetic curve with free radicals.

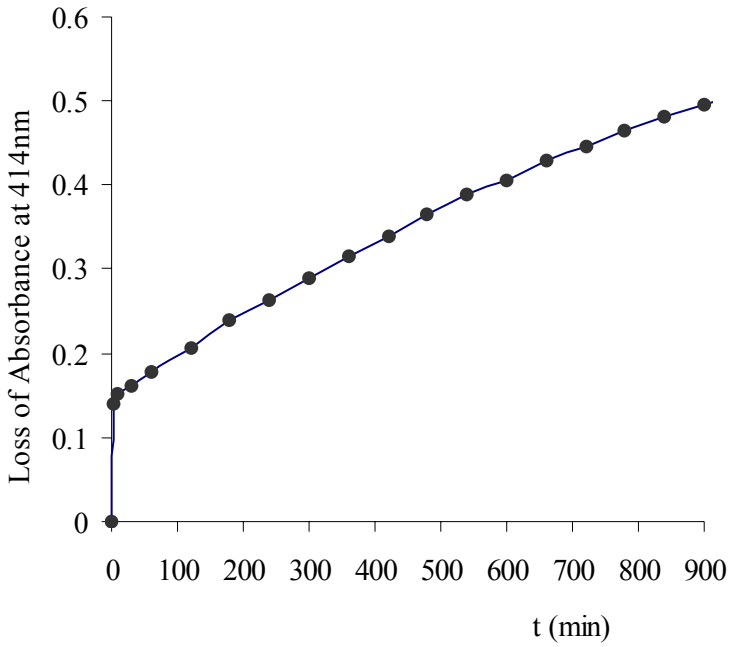

Fig. 3.4. Reaction of ABTS•$^{+}$ and chlorogenic acid

Knowledge of major antioxidants in fruits/vegetables and the kinetics of their reaction types with free radicals are very important for the understanding of their antioxidant property. It is more meaningful to report both antioxidant capacity and antioxidant efficiency of a biological sample in future research as antioxidant efficiency, which indicates how fast the antioxidants react with the free radicals and complements that of total antioxidant capacity and is necessary for characterizing a potential antioxidant source. Most recently, Cevallos-Casals and Cisneros-Zevallos have reported both antioxidant capacity and also kinetics of antioxidants in Andean purple corn and sweet potato [13].

3.4 Assessing antioxidant capacity of fruits, vegetables and pure antioxidants

In this study, it was found that, for extracts of many fruits and vegetables, scavenging of free radical continues for over 1 hour (Fig. 3.5). Since one of the main objects of this study is to find potentially antioxidant rich dietary sources and identify their antioxidant profiles, it was not necessary to wait for the reaction to be completed. For extracts of most fruits and vegetables, the rate of loss of absorbance using free radical assay at 60 min was less than 5% every 5 min after 60 min. Antioxidant capacity of a sample could be calculated from loss of absorbance at 60 min as 5% of TAC could be thought as bing not a significant contribution to the overall antioxidant capacity. Similar results were found in the FRAP assay. In addition, it was also found that no difference was observed for ranking fruits and vegetables according to their antioxidant capacity after 60 min. Therefore, one hour will be used as the assessing time for investigation of TAC of fruits and vegetables. This part of the work addresses total antioxidant capacity of fruits and vegetables using three different methods, namely $ABTS^{\bullet+}$ scavenging assay, DPPH• scavenging assay and FRAP assay, respectively. Furthermore, antioxidant activity of a variety of pure antioxidants with both free radicals was investigated, and more reactive free radical was chosen for further experiments.

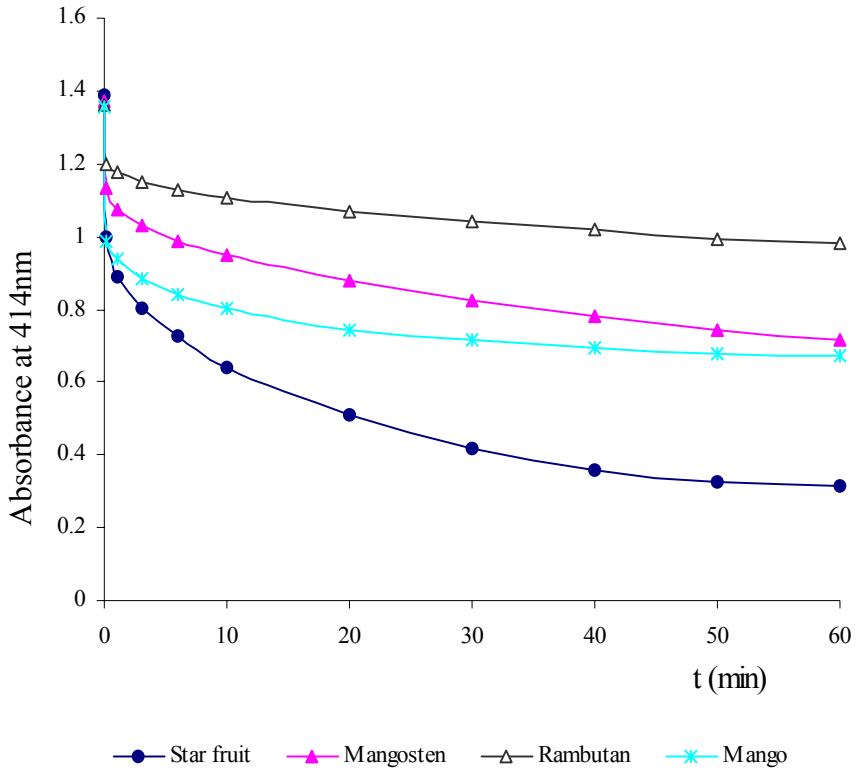

Fig. 3.5. Kinetic curve of change of absorbance upon addition of fruit extracts

3.4.1 $ABTS^{\cdot+}$ decolorization assay

$ABTS^{\cdot+}$, usually generated by potassium persulfate, is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavengers of lipid peroxyl radicals). Rice-Evans and co-workers [14, 15] and Roberta [1] have demonstrated that $ABTS^{\cdot+}$ assay can be used to measure the antioxidant activity of a broad diversity of substances. This method usually measures the relative antioxidant ability of fruits to scavenge the radical $ABTS^{\cdot+}$ in the aqueous phase as compared with a standard antioxidant such as Trolox, an analogue of vitamin E.

Fig. 3.6 shows that the reaction between L-ascorbic acid and $ABTS^{\bullet\cdot+}$ is very fast and essentially complete after 30 s. Fig. 3.7 shows that loss of absorbance at 414 nm is proportional to the amount of L-ascorbic acid (AA) added into the mixture. This allows L-ascorbic acid to have a potential to be a suitable reference for expressing antioxidant activity of other pure antioxidants and biological samples as it could provide repeatable stoichiometry with free radicals. The TAC of a fruit/vegetable can then be obtained from the graph by considering the drop of absorbance at a particular time, and expressed as AEAC.

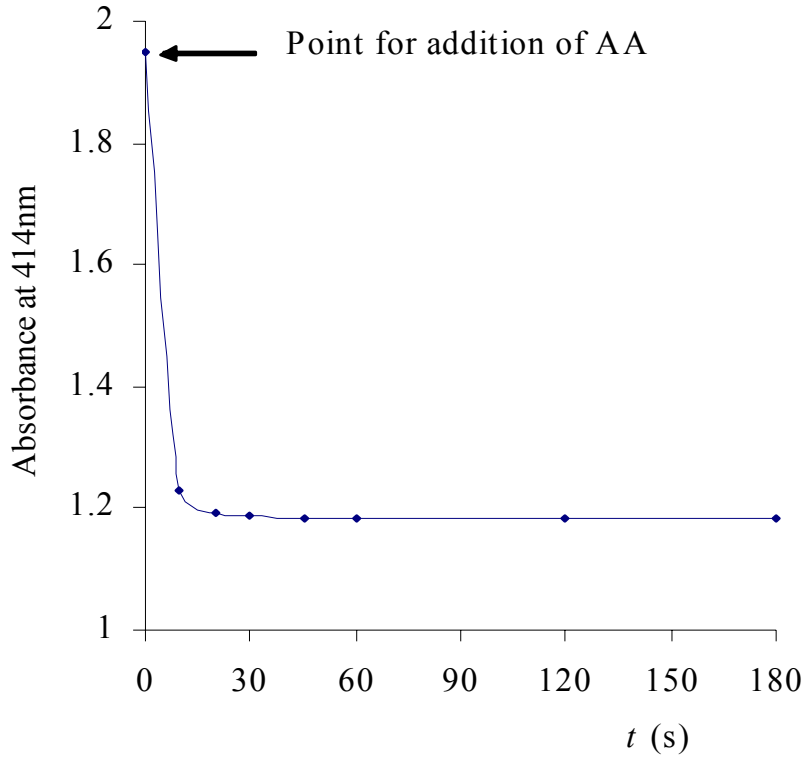

Fig. 3.6. Decrease in absorbance at 414 nm upon addition of L-ascorbic acid (final concentration in medium: 0.011 mM)

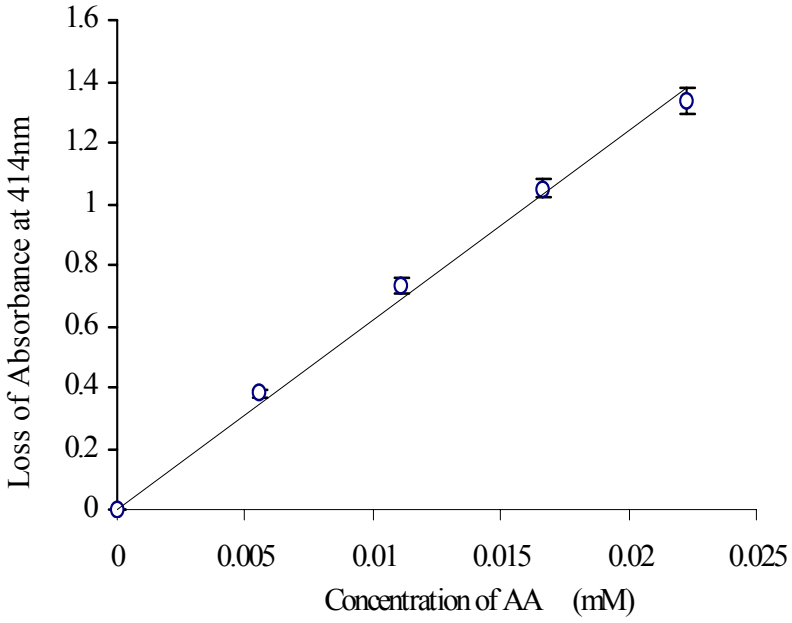

Fig. 3.7. Plot of loss in absorbance against corresponding L-ascorbic acid (AA) concentrations (R^2=0.9956; for each point, n=3).

The antioxidant defence system of the body is composed of a mixture of antioxidants. Fruit and vegetables are good sources of antioxidants that may be more effective and economical than supplements in protecting the body against oxidative damage under different conditions**.** Fruit and vegetable antioxidants, which include L-ascorbic acid, tocopherols, carotenoids and phenolics, vary greatly in their contents and profile among various fruits. As a result, the antioxidant capacity of one fruit or vegetable differs considerably from one another. By using the ABTS•$^{+}$ decolorization assay, we have measured, for the first time, the antioxidant capacity of a variety of fruits and vegetables, which were purchased from local markets during the period from January 2001 to June 2001 (Table 3.2).

Table 3.2. TAC and total phenolics of selected fruits and vegetables*

	Total phenolic contents mg/g GAE [a]	$AEAC_{ABTS}$ mg/100g [a]	$AEAC_{DPPH}$ mg/100g [a]	FRAP μmol/g [a]
Rambutan	0.62 ± 0.06	69.95 ± 2.06	29.46±1.12	13.24±1.03
Star fruit	1.86 ± 0.10	214.06 ± 11.16	215.37±8.97	29.03±1.26
Kiwi fruit	1.31 ± 0.09	137.57 ± 4.23	129.43±6.84	24.26±1.32
Seedless grape	1.35 ± 0.07	170.34 ± 9.55	170.01±7.55	22.23±1.06
Mangosteen	1.10 ± 0.08	91.74 ± 5.54	63.87±2.93	14.01±0.85
Banana	0.65 ± 0.04	61.45 ± 3.65	37.57±1.04	6.58±0.23
Orange	1.37 ± 0.09	88.25 ± 5.27	67.86±4.03	16.52±0.78
Foot long papaya	0.76 ± 0.06	81.58 ± 5.94	55.48±3.16	13.19±0.47
Guava	1.86 ± 0.11	241.89 ± 17.52	163.68±5.51	34.19±1.19
Apple	1.18 ± 0.07	159.85 ± 12.33	117.89±4.23	15.93±0.43
Pomelo	0.84 ± 0.06	98.00 ± 3.25	32.60±0.86	11.40±0.47
Watermelon	0.19 ± 0.03	20.49 ± 0.97	10.87±0.39	3.16±0.11
Tomato	0.21 ± 0.03	35.89 ± 0.89	23.79±0.47	7.68±0.19
Lemon	1.24 ± 0.09	101.72 ± 9.32	84.07±4.06	30.49±1.03
Avocado	0.62 ± 0.05	102.07 ± 10.64	25.57±1.27	12.35±0.44
Pineapple	0.63 ± 0.04	69.20 ± 4.28	43.60±2.13	11.20±0.45
Salak	3.40 ± 0.13	383.20 ± 21.13	429.36±19.74	57.68±2.85
Strawberry	3.41 ± 0.16	463.77 ± 23.29	362.32±12.67	60.59±2.87
Blueberry	4.60 ± 0.14	758.72 ± 32.73	884.25±21.06	85.13±0.35
Kang kong	0.49 ± 0.05	33.65 ± 2.26	12.96±0.32	6.02±0.21
Egg plant	0.64 ± 0.03	55.31 ± 3.49	37.72±0.56	8.84±0.20
Lady's finger	0.81 ± 0.05	118.01 ± 5.20	100.29±5.37	11.91±0.31
Long bean	1.06 ± 0.06	131.81 ± 8.37	135.87±5.69	21.05±0.48
Bittergourd	0.49 ± 0.04	35.91 ± 3.06	19.38±0.95	8.66±0.56
Cucumber	0.22 ± 0.02	7.45 ± 0.23	2.55±0.25	0.56±0.08
French bean	0.32 ± 0.02	16.44 ± 0.31	6.43±0.18	2.91±0.11
Beetroot	1.94 ± 0.09	206.44 ± 8.57	118.87±5.47	37.62±1.21
Spinach	0.59 ± 0.04	42.09 ± 1.39	6.84±0.18	8.05±0.09
Lettuce	1.06 ± 0.08	99.63 ± 4.53	71.48±3.83	16.59±0.62
Red cabbage	1.62 ± 0.08	189.34 ± 6.92	135.17±5.17	33.81±0.79
Red onion	1.30 ± 0.06	118.08 ± 5.52	40.77±1.13	13.57±0.68
Red chilly	1.98 ± 0.11	209.90 ± 10.49	133.32±3.69	33.01±1.14

[a] Mean of three determinations ± SD (standard deviation)

* Fruits and vegetables were purchased during the period from January to June of 2001.

3.4.2 DPPH· scavenging assay

The model of scavenging stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability [16].

Similar to TAC assay by ABTS•$^{+}$ decolorization method, the TAC of a sample is obtained by comparing its ability to scavenge DPPH• with that of L-ascorbic acid. The rate of reaction between L-ascorbic acid and DPPH• is fast and the reaction is almost completed in one minute.

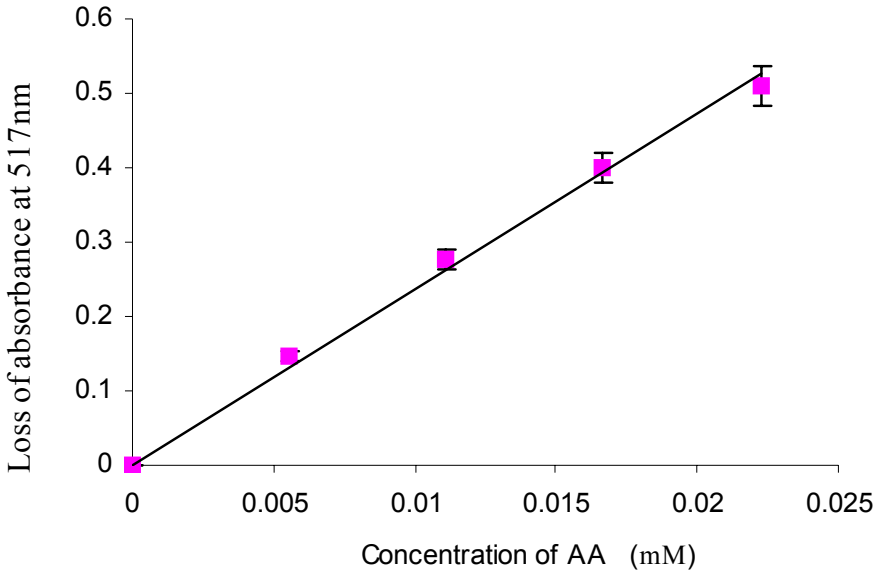

Fig. 3.8. The effects of concentration of AA on DPPH· Decolorization (R2=0.9883; for each point, n=3)

Fig. 3.8 shows that the loss of absorbance at 517 nm is proportional to the amount of L-ascorbic acid in the medium. Therefore, the TAC of a sample could be obtained

from its ability to scavenge DPPH• and expressed as AEAC. The AEAC of fruits and vegetables by DPPH• scavenging assay is shown in Table 3.2.

3.4.3 Ferric reducing/antioxidant power (FRAP) assay

In most cases for assessing antioxidant activity, a common mechanism involving a redox reaction takes place. Based on that, Benzie and Strain [17] developed a simple method for determining the reduction of a ferric-tripyridyltriazine complex to its ferrous-tripyridyltriazine complex in the presence of antioxidants. The TAC of a sample, expressed as the amount of Fe (II) reduced, offers an index of antioxidant potential. Fig. 3.9 shows the standard calibration curve of Fe (II) versus A_{593}. The reducing ability of selected fruits and vegetables, as a measure of their antioxidant capacity, is shown in Table 3.2.

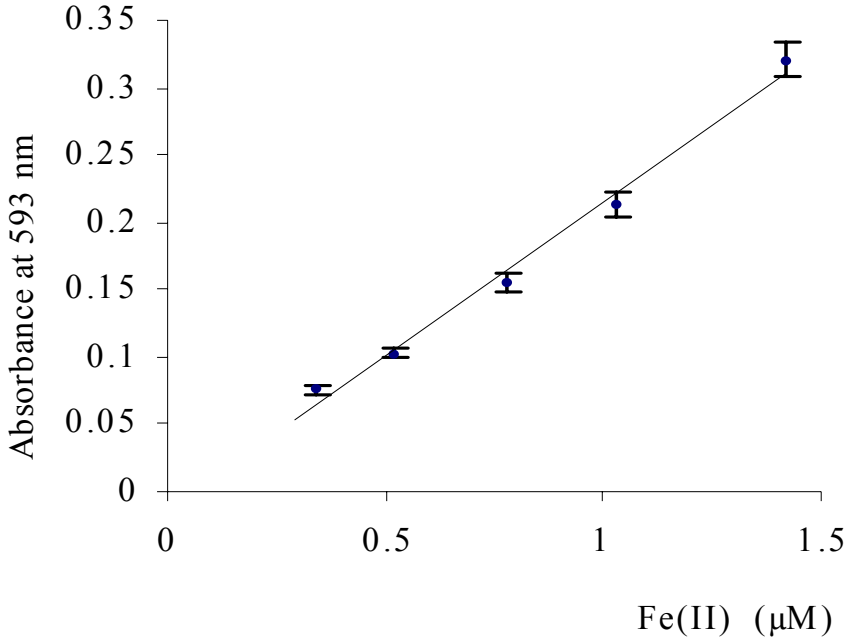

Fig. 3.9. Standard calibration curve: [Fe(II)] vs. A_{593} (R^2=0.9893; for each point, n=3)

3.4.4 Comparison of methods for TAC assays

As can be seen from Fig. 3.10, good correlation was obtained between total phenolic content and TAC using different assays. The correlation coefficients (R^2) were 0.9605, 0.9381 and 0.8581 for FRAP, $ABTS^{\bullet+}$ and DPPH• assay, respectively. The high correlation coefficients between TAC assay and total phenolic content suggest that both the ferric reducing antioxidant power and free radical scavenging ability of the extract of most fruits and vegetables might be mainly contributed by phenolic compounds. Here, the method used for TPC assay is based on an oxidoreduction between phenolic compounds and Folin-Ciocalteu reagent, which contains a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). The reagent becomes partly reduced resulting in the production of the complex molybden-tungsten blue, which is measured spectrophotometrically at 765 nm [18]. FRAP assay measures the ferric reducing ability of biological samples. Any half reaction which has a less-positive redox potential than the Fe^{III}/Fe^{II}-tripydyltriazine (TPTZ) half reaction will drive the reduction of Fe^{III}-TPTZ. Probably due to similar reaction mechanism to TPC assay, TAC from FRAP assay shows highest correlation coefficient with TPC of fruits and vegetables (Fig. 3.10).

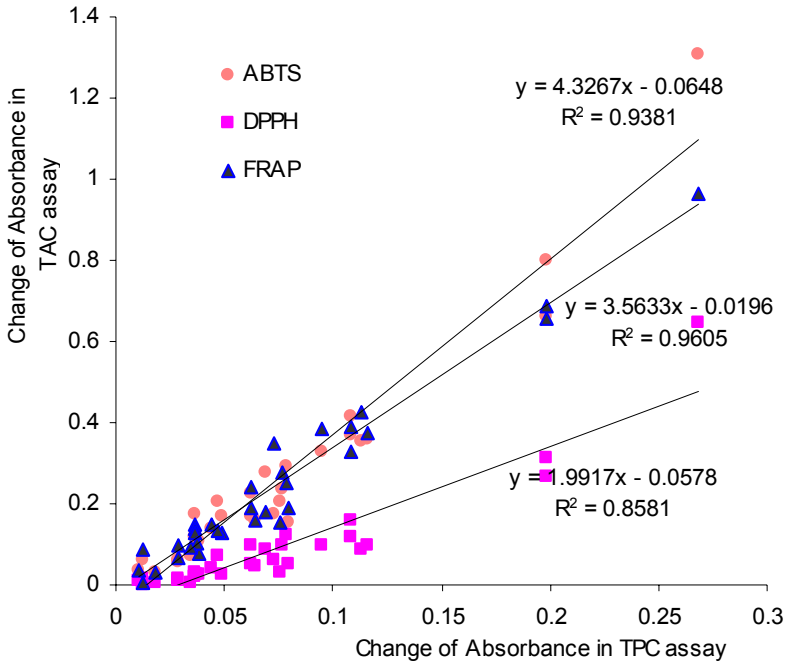

Fig. 3.10 Correlation of change of absorbance in TAC assays and change of absorbance in TPC assay

As addressed by Prior and Cao, although FRAP was claimed to be a direct test of the total antioxidant power, it had a lot of problems for TAC assay of biological samples [19]. The use of ABTS•$^{+}$ and DPPH• methods provides an easy way to evaluate the antiradical activities of antioxidants. As shown in Fig. 3.11 and 3.12, most fruits and vegetables tested with high AEAC also have high FRAP values. A very good correlation was obtained between FRAP value and AEAC by ABTS•$^{+}$ assay (R^2=0.9301), but a relatively worse correlation was observed between FRAP and DPPH• method (R^2=0.8544).

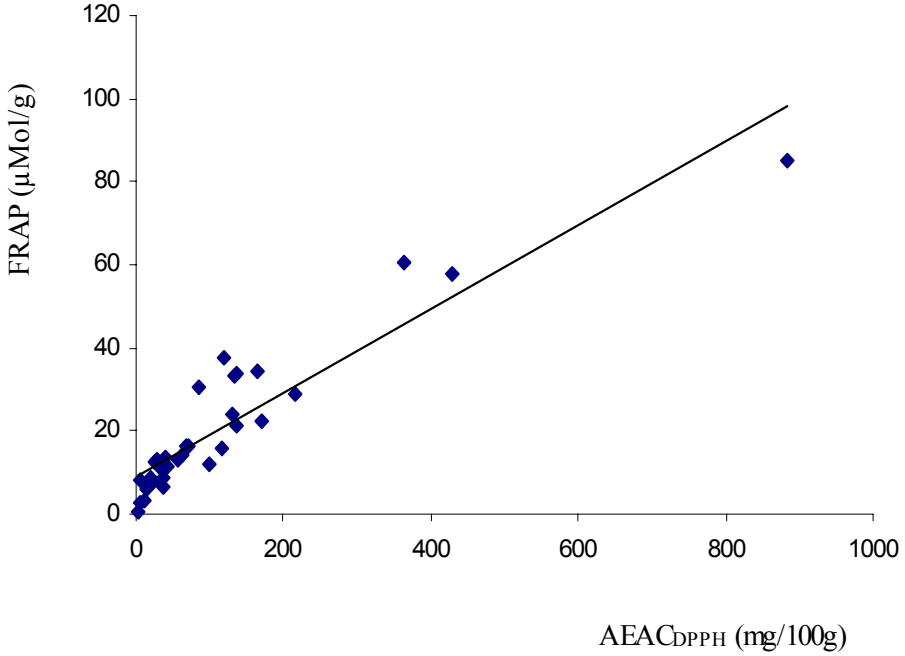

Fig. 3.11 Correlation of FRAP value and AEAC by DPPH• model (R^2=0.8544)

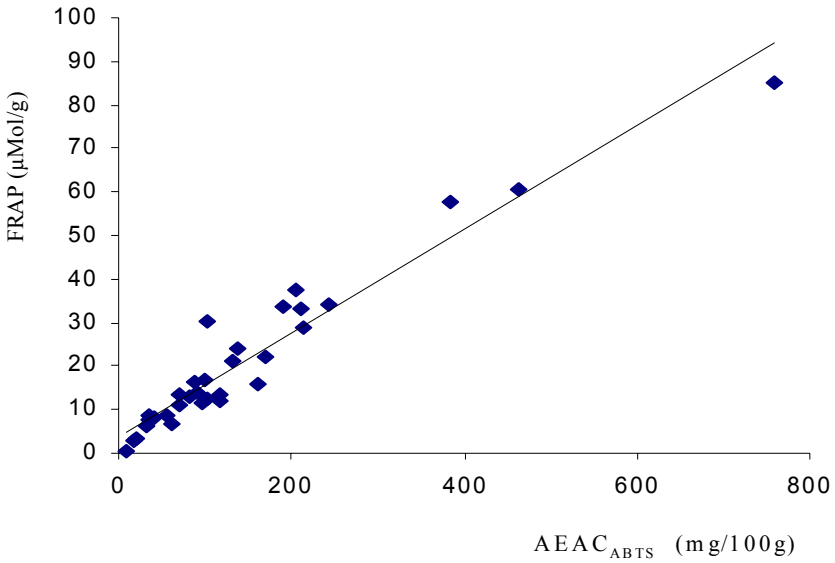

Fig. 3.12 Correlation of FRAP value and AEAC by ABTS•$^+$ method (R^2=0.9301)

Fig. 3.13 shows that AEAC values of most fruits obtained from ABTS•$^+$ assay were quite close to those from DPPH• assay. Most fruits and vegetables tested with high

AEAC in ABTS•$^{+}$ method also have high AEAC in DPPH• method. This might suggest that, in most cases, both methods are compatible when used to assess free radical scavenging activity. However, as can also be seen from Fig. 3.13, most points fall under the curve y=x. This means that $AEAC_{ABTS}$ is almost always higher than $AEAC_{DPPH}$. As L-ascorbic acid has the same stoichiometry when reacting with both ABTS•$^{+}$ and DPPH•, it is most likely that some antioxidants in most fruits and vegetables are more reactive to ABTS•$^{+}$ than to DPPH•. To further verify this, the reactivity of a variety of pure antioxidants with both ABTS•$^{+}$ and DPPH• will be studied.

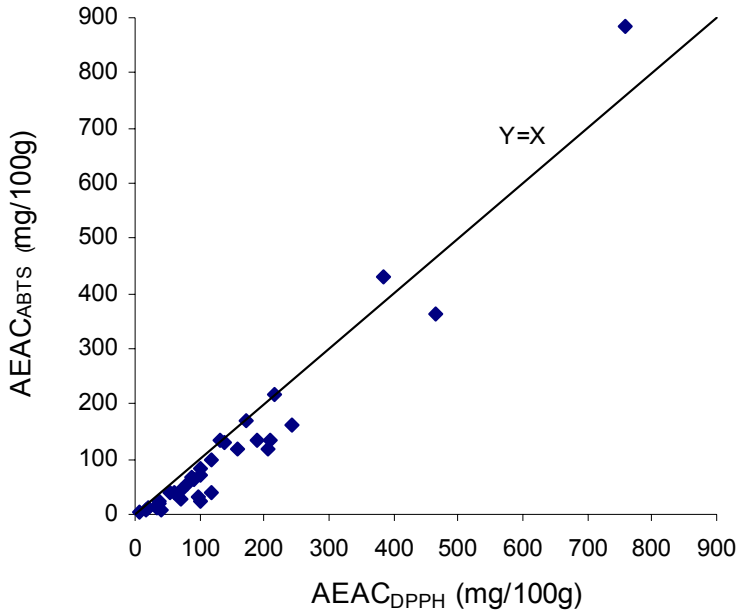

Fig. 3.13 AEAC values of fruits and vegetables by ABTS•$^{+}$ and DPPH• methods

Other than the fact that ABTS•$^{+}$ correlated better with FRAP than DPPH• and that it was more reactive to most antioxidants, at pH 4.3 it was found to be quite stable even after a number of hours and even a few days while DPPH• in methanol remained stable only within a short period.

The fact that AEAC of most fruits/vegetables from ABTS•$^{+}$ assay were almost always higher than those from DPPH• assay could be due to the fact that stoichiometry coefficients of most antioxidants with ABTS•$^{+}$ were close to or higher than those with DPPH•. Fig.3.14 shows that the stoichiometry coefficients of most antioxidants with ABTS$^{\cdot+}$ were higher than or similar to those with ABTS•$^{+}$.

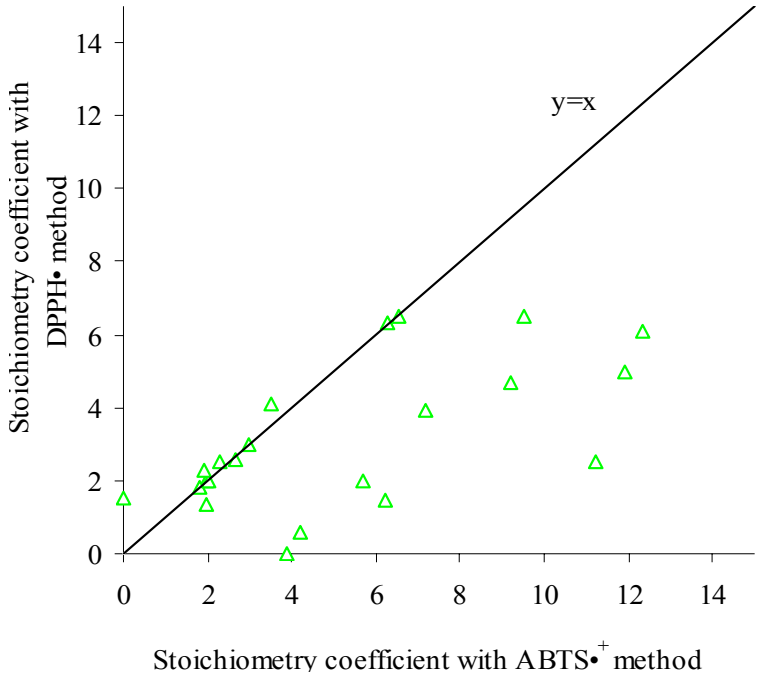

Fig. 3.14 Stoichiometry coefficient of antioxidants with by ABTS$^{\cdot+}$ and DPPH$^{\cdot}$ methods

Here, it is found that 1 mol of L-ascorbic acid (AA) reacts with approximately 2 mol of ABTS•$^{+}$ or DPPH•. The result is consistent with that 1 mol of L-ascorbic acid reducing 2 mol of ABTS radicals obtained by Cano *et al.* [20]. Similarly, 1 mol of cysteine reacts with approximately 2 mol of ABTS•$^{+}$, which is consistent with that reported by Aliaga and Lisa. [21]; 1 mol of gallic acid reacts with 6.34 mol of DPPH•, which is close to 6.25 reported by Brand-Williams and co-workers [22]; 1 mol of gallic acid reacts with 6.34 mol of ABTS•$^{+}$, which is close to TEAC of 3.01 reported

by Rice-Evans *et al*. [15]; 1 mol of BHA reacts with 2.5 mol of DPPH•, which is close to 2.63 reported by Brand-Williams and co-workers [22]. Stoichiometry coefficients of other pure antioxidants are given in Table 3.3.

Stoichiometric factors between some antioxidants and free radicals have been proposed [20-22, 23-25]. For example, L-ascorbic acid was thought to react firstly with one $ABTS^{\bullet+}$ to give one ABTS and a monodehydroascorbic acid, which reacts with a second $ABTS^{\bullet+}$ to produce another ABTS and a dehydroascorbic acid [20]; a similar reaction mechanism might also be applied for trolox or other antioxidants, which are compounds bearing two labile hydrogen atoms and having a stoichiometry factor of around two with free radicals. The following scheme explains the main features of those reactions (Eq. 3.1, 3.2, 3.3).

$$ABTS^{\bullet+} + AH_2 \rightarrow ABTS + H^+ + AH^{\bullet} \quad \text{(Eq. 3.1)}$$

$$ABTS^{\bullet+} + AH^{\bullet} \rightarrow ABTS + A \quad \text{(Eq. 3.2)}$$

$$2ABTS^{\bullet+} + AH_2 \rightarrow 2ABTS + 2H^+ + A \quad \text{(Eq. 3.3)}$$

The main features of the reaction between $ABTS^{\bullet+}$ and monophenols that gives stoichiometric factors from one to two have been explained in a simplified mechanism (Eq. 3.4, 3.5 and 3.6) [23].

$$ABTS^{\bullet+} + PhOH \rightarrow ABTS + H^+ + PhO^{\bullet} \quad \text{(Eq. 3.4)}$$

$$ABTS^{\bullet+} + PhO^{\bullet} \rightarrow \text{Non-radical products} \quad \text{(Eq. 3.5)}$$

$$PhO^{\bullet} + PhO^{\bullet} \rightarrow \text{Non-radical products} \quad \text{(Eq. 3.6)}$$

For those antioxidants e.g. polyphenolics having high stoichiometric factors with free radicals, the whole mechanism is still not clear. Chemical studies of tea catechin, flavanols and their derivatives have revealed the dimerization of polyphenolic radicals [24, 25]. High stoichiometric factors between polyphenolics and free radical could be due to the facts that more hydroxyl groups contained in polyphenolics and that non-radical products (Eq. 3.5 and 3.6) are still reactive to free radical species.

As proposed by Brand-Williams and co-workers, reaction between antioxidants and DPPH might have similar mechanisms to those of ABTS assay [22].

Table 3.3 No. of mol of free radicals reduced by every mol of antioxidants

Compound	ABTS•$^{+}$ (mol)	DPPH• (mol)	Compound	ABTS•$^{+}$ (mol)	DPPH• (mol)
L-Ascorbic acid	1.92±0.04	1.96±0.08	BHT	3.01±0.07	2.99±0.05
Cysteine	1.96±0.04	1.32±0.02	BHA	2.51±0.03	2.49±0.07
Syringic acid	2.66±0.03	2.55±0.05	TBHQ	1.81±0.01	1.83±0.04
p-Hydroxybenzoic acid	0	1.54±0.03	Pyrogallol	6.93±0.14	4.01±0.06
Procatechuic acid	9.20±0.08	4.70±0.05	Hydroquinone	2.00±0.06	2.02±0.05
Caffeic acid	12.35±0.06	6.10±0.04	Quercetin	9.52±0.08	6.50±0.08
Tyrosine	3.77±0.05	0	Quercetin glucoside	3.52±0.05	4.10±0.09
Coumaric acid	4.53±0.03	1.48±0.04	(-)Epicatechin	6.56±0.09	6.48±0.04
Vanillic acid	4.41±0.05	0.61±0.00	Myricertin	11.90±0.18	5.02±0.07
Ferulic acid	11.20±0.23	2.51±0.03	Kampferol	5.70±0.04	2.00±0.02
Gallic acid	6.34±0.15	6.28±0.23	Trolox	1.98±0.07	2.13±0.09

As can be seen from Table 3.3, some antioxidants investigated, e.g. L-ascorbic acid, Trolox, hydroquinone, kampferol, (-)epicatechin, gallic acid and syringic acid gave approximate stoichiometric coefficient when analysed using ABTS•$^{+}$ and DPPH• methods respectively. However, a number of antioxidant had higher stoichiometry coefficients with ABTS•$^{+}$ than those with DPPH•. It had been reported previously that

TAC obtained by these two methods might be significantly different [26, 27]. As described in Chapter 1, both $ABTS^{\bullet+}$ and DPPH• are reactive nitrogen species with different chemical structures, but the latter is more nonpolar and is almost not soluble in water but soluble in methanol, which could cause the difference in stoichiometry between two free radicals with some antioxidants. Therefore, a linear correlation between two models may not be obvious among some other biological samples that contain a variety of main antioxidants that have significant different stoichiometry with $ABTS^{\bullet+}$ and DPPH•.

3.5 Chapter Summary

The AEAC of fruits and vegetables showed significantly different antioxidant capacity. The fruits and vegetables can be classified to extremely high, high, medium and low antioxidant capacity. Several tropical fruits and vegetables, such as ciku, ciku king, ulam raja, salak, star fruit, etc, could be ideal sources of natural antioxidants. Their antioxidant compositions should be systematically studied. The contribution of L-ascorbic acid to AEAC varies considerably from one fruit to another, but is generally low in those fruits and vegetables with high antioxidant capacity except that in guava.

Antioxidant properties including antioxidant capacity and antioxidant efficiency of a fruit/vegetable might mainly depend on its major antioxidants. As it takes the factors of reaction rate and reaction time into consideration, the kinetic curve of antioxidant

capacity describing the antioxidant status of a fruit or vegetable was more complete and provided more information than TAC at a specific time.

Of the two reactive nitrogen scavenging methods investigated, $ABTS^{\bullet+}$ assay was proved to be a better tool to characterise antioxidants in biological samples than DPPH• assay due to its stability and activity.

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Chapter 4

Separation of Organic Acids and Phenolic Compounds by High Performance Liquid Chromatography

4.1 Introduction

Organic acids are widely distributed in fruits and vegetables. The content of organic acids in fruit juices not only influences their flavour but also their stability, nutrition, acceptability and keeping quality. Of a variety of organic acids contained in fruits and vegetables, L-ascorbic acid is one of the most important acids with great nutritional value for human health probably due to its strong antioxidant activity. Phenolic compounds exist widely in fruits and vegetables and are reported to have multiple biological effects, including antioxidant activity [1], antitumor [2], antimutagenic [3], and antibacterial and angioprotective properties [4]. This group of compounds are distributed in fruits and vegetables in the form of hydroxybenzoic acids, hydoxycinnamic acids, flavonoids and isoflavonoids. The determination of some phenolic acids using chromatographic methods includes gas chromatography (GC) [5, 6], capillary electrophoresis (CE) [7] and high performance liquid chromatography (HPLC) [8-12]. For the analysis of flavonoids in foods by HPLC, as reviewed by Merken and Beecher [13], columns are almost exclusively reversed phase, and elution systems are usually binary, with an aqueous acidified polar solvent and a less polar organic solvent. The aqueous acidified polar solvents include acetic acid, phosphoric acid, perchloric acid, or formic acid. The elution may be isocratic or gradient. Further chromatographic separation and quantification of phenolic acids and flavonoids in natural fruits and beverages has been reported by Zuo *et al*. [14, 15].

In most of the previously developed methods, significant restrictions were placed on the compounds to be analysed, either for polar organic acid or one or two subclasses of phenolic compounds. In addition, chemical derivizations and solid phase extraction (SPE) are usually involved in the assay of these target compounds as large amounts of organic acid contained in fruits and vegetables give rise to low resolution among the organic acids and phenolic compounds. In addition, large differences in the levels of phenolic compounds in a juice or extract usually complicate the simultaneous analysis of different classes of phenolic compounds.

The purpose of this part of the study was to establish a method to simultaneously separate a variety of phenolic compounds and very polar organic acids such as L-ascorbic acid in extracts of biological samples using HPLC with diode array detection, which could be used to identify compounds not only by their retention times but also their individual spectra. The developed method will be applied for rapid screening and identification of antioxidants including very polar organic acid e.g. L-ascorbic acid and phenolic compounds in a fruit/vegetable extract and commercially available fruit juice e.g. apple juice.

4.2 Method development

As different fruits and vegetables might contain different organic acids at different levels, it is necessary to establish an elution program that allows a good separation among L-ascorbic acid, common carboxylic acids and phenolic compounds. After multiple preliminary assays, an elution program using methanol-H_2SO_4-water as

solvent was chosen. This program allowed very polar organic acids to be eluted in 30 minutes followed by the phenolic compounds. Fig. 4.1 illustrates the separation of a standard mixture of 29 acids and phenolic compounds. The established program provides a good separation for 29 compounds within 80 min, which indicates that it has the potential application for rapid screening antioxidants including L-ascorbic acid and phenolic compounds.

It is essential to keep the flow rate of solvent A (pH 2.5 sulphuric acid solution) at 0.35 mL/min during the whole run and increase the flow rate of solvent B (methanol) to 0.45 mL/min from 15 min to 75 min to achieve good resolution of the target compounds since both solvent polarity and pH are very important for separation of target compounds. Table 4.1 lists the retention times of individual carboxylic acids and phenolic compounds.

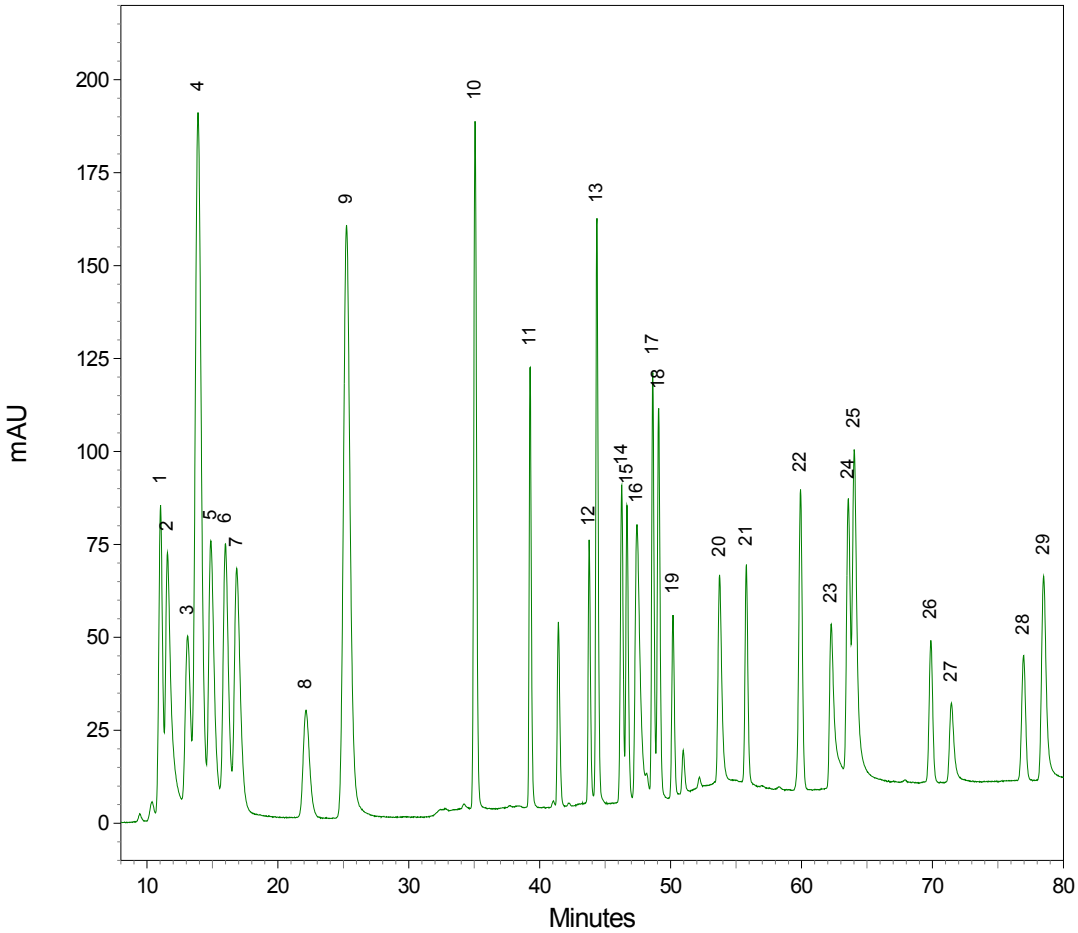

Fig. 4.1. HPLC of the mixture of standards. Detection at 215 nm. 1, Tartaric acid; 2, Oxalic acid; 3, Malic acid; 4, L-Ascorbic acid; 5, Malonic acid; 6, Lactic acid; 7, Acetic acid; 8, Citric acid; 9, Fumaric acid; 10, Gallic acid; 11, (-)-Catechin gallate; 12, (-)-Epigallocatechin; 13, (+) Catechin; 14, p-Hydroxybenzoic acid; 15, Chlorogenic acid; 16, (-)-EGCG; 17, (-)-Epicatechin; 18, Caffeic acid; 19, Syringic acid; 20, (-)-catechin gallate; 21, Ferulic acid; 22, Benzoic acid; 23, Ellagic acid; 24, Salicyclic acid; 25, Myricetin; 26, Trans-cinnamic acid; 27, Quercetin; 28, Eugenol; 29, Kaempferol.

Table 4.1 shows that some compounds such as (-)-epicatechin and *p*-coumaric acid may be eluted with similar retention times, and thus it is difficult to identify and quantify them by simply using retention time and spiking test. In this experiment, HPLC coupled with a diode array detector (DAD) was used to obtain the spectrum of 31 compounds. As can be seen from Appendix 1, different compounds might have different UV-Vis spectra, which could be used to further confirm the unknown peaks in samples.

Table 4.1. Retention time, linearity range and limit of detection of carboxylic acid and phenolic compounds*

Common name	R_t (min)	Concentration range (mg/l)	a	b	r^2	LOD (mg/l)
Tartaric acid	11.24	50~500	4109	48457	0.9987	2.20
(-)-Quinic acid	11.37	200~2000	1073	514	0.9992	12.50
Oxalic acid	11.92	15~150	20333	10319	0.9999	0.94
DL-Malic acid	13.58	100~1000	1964	24849	0.9902	5.17
L-Ascorbic acid	14.58	10~100	53183	10213	0.9957	0.25
Malonic acid	15.30	100~1000	2478	-	0.9988	6.00
Lactic acid	16.26	180~1800	1417	3499	0.9999	9.64
Acetic acid	17.06	200~2000	1224	-	0.9993	10.91
Citric acid	22.88	100~1000	2424	13039	0.9993	5.93
Fumaric acid	25.61	2~20	26367	64360	0.9987	0.12
Gallic acid	35.28	1.0~20	34023	57743	0.9903	0.02
(-)-Gallocatechin	39.32	1.0~20	16918	35273	0.9999	0.04
(-)-Epigallocatechin	43.73	1.0~20	11727	-	1.0000	0.06
(+)-Catechin	44.47	1.0~20	16318	14636	0.9998	0.04
p-Hydroxbenzoic	46.25	1.0~20	10518	83096	0.9972	0.09
Chlorogenic acid	46.61	1.0~20	79339	88640	0.9922	0.12
(-)-EGCG	47.70	1.0~20	22071	73747	1.0000	0.09
(-)-Epicatechin	48.56	1.0~20	17188	95802	0.9977	0.04
p-Coumaric acid	48.79	1.0~20	18939	43283	0.9989	0.04
Caffeic acid	49.15	1.0~20	14786	-	0.9919	0.05
Syringic acid	50.20	1.0~20	21776	92128	0.9991	0.03
(-)-catechin gallate	53.76	1.0~20	19861	-	0.9912	0.05
Ferulic acid	55.51	1.0~20	10298	-	0.9972	0.08
Benzoic acid	59.61	1.0~20	62388	47227	0.9997	0.18
Ellagic acid	62.04	1.0~20	44703	8268	0.9992	0.29
Salicyclic acid	63.50	1.0~20	10838	18898	0.9965	0.13
Myricetin	64.05	1.0~20	13901	18664	0.9960	0.06
Trans-cinnamic acid	69.57	1.0~20	13027	-7697	0.9974	0.06
Quercetin	71.39	1.0~20	11428	91952	0.9999	0.03
Eugenol	76.67	1.0~20	66253	55598	0.9999	0.16
Kaempferol	78.26	1.0~20	90207	8556	1.0000	0.15

* $A=a \times c + b$. A, peak area; a, slope; b, intercept; c, concentration

4.3 Method validation

The within-day repeatability (n=3) and between-day precision (n=10) of retention times were within 0.3 and 1.6% relative standard deviation (RSD), respectively. The repeatability (n=3) and between day precision (n=3) of peak area except L-ascorbic acid, which was not very stable, were all within 5.0% RSD.

The accuracy of the method was confirmed by analyzing the mixture prepared by adding suitable amounts of standard mixture to juices with known contents of these target compounds. The recoveries of target compounds were between 85 and 106%. The limit of detection (LOD, S/N=3) of individual compounds at 215 nm is given in Table 1. Compounds, such as L-ascorbic acid, benzoic acid and p-hydroxybenzoic acid, will have lower LOD at their maximum absorbance wavelength than those at 215 nm.

4.4 Analysis of organic acids and phenolic compounds in apple juice

Apple juice contains a variety of organic acids and phenolic compounds such as malic acid, ascorbic acid, chlorogenic acid and flavonoids. Fig.4.2 shows the chromatographic profile of Brand A apple juice.

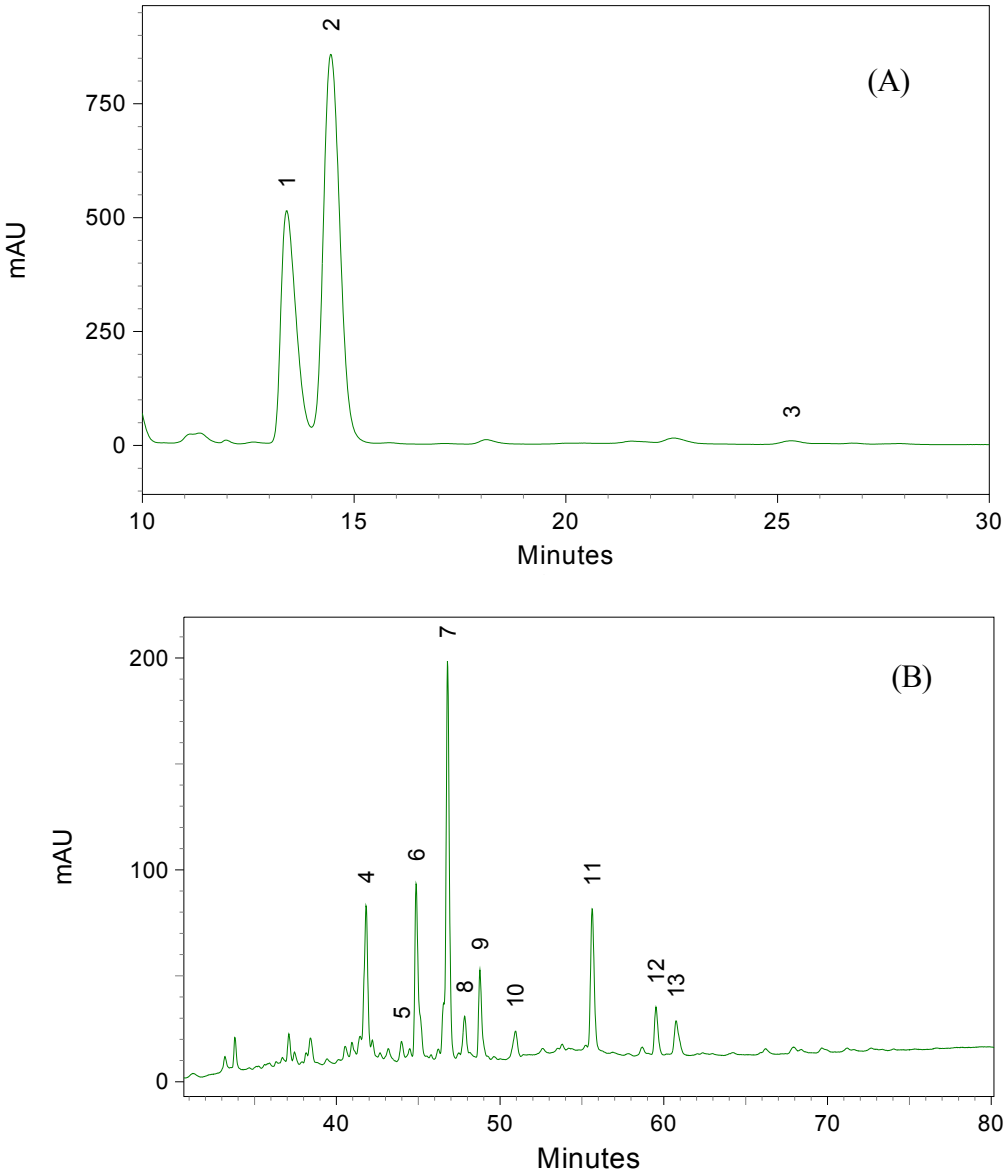

Fig. 4.2. Chromatogram of Brand A apple juice showing carboxylic acid and phenolic compounds profiles: (A), 0-30 min; (B), 30-80 min. 1, Malic acid; 2, L-Ascorbic acid; 3, Fumaric acid; 4, UC 1; 5, (-)-Epigallocatechin; 6, UC 2; 7, Chlorogenic acid; 8, (-)-EGCG; 9, (-)-Epicatechin; 10, UC 3; 11, UC 4; 12, UC 5; 13, UC, 6.

As can be seen from Fig. 4.2(A), the good separation of L-ascorbic acid and malic acids is achieved under the optimised chromatographic conditions. Fig. 4.2(B) shows the chromatographic profile of phenolic compounds in Berri juice, a commercial available apple juice. Chlorogenic acid, (-)-epicatechin, (-)-EGCG and (-)-epigallocatechin were identified in Berri apple juice. In addition, several other peaks, named unidentified compounds (UC), have similar spectra to phenolic compounds.

UC1 and UC 4 have similar spectra to that of benzoic acid. UC 2 has a similar spectrum to that of (+)-catechin while UC 3, UC 5 and UC 6 have similar spectra to that of cinnamic acid or syringic acid. In addition, UC2, 3, 5 and 6 all have a maximum absorbance wavelength of 280 nm, indicating their possibilities as phenolic compounds.

4.5 Negative effects on chromatographic profiles by sample solvents

It was found that when an organic solvent such as methanol was used in sample treatment, the resolution of quinic acid, oxalic acid, malic acid, L-ascorbic acid and malonic acid peaks was decreased (Fig.4.3). It was found that if methanol is less than 10%, the resolution is still acceptable although the Rt values are slightly reduced. This might be due to close distribution coefficients and the competitive adsorption behaviour of methanol and components between mobile phase and stationary phase. However, no obvious solvent effects were observed on the resolution of phenolic compounds. As most phenolic compounds are more non-polar than those carboxylic acids, they have much stronger adsorption affinity with C18 stationary phase than that of carboxylic acids, and thus influence on the resolution of eluted phenolic compounds could be much less than that on carboxylic acids.

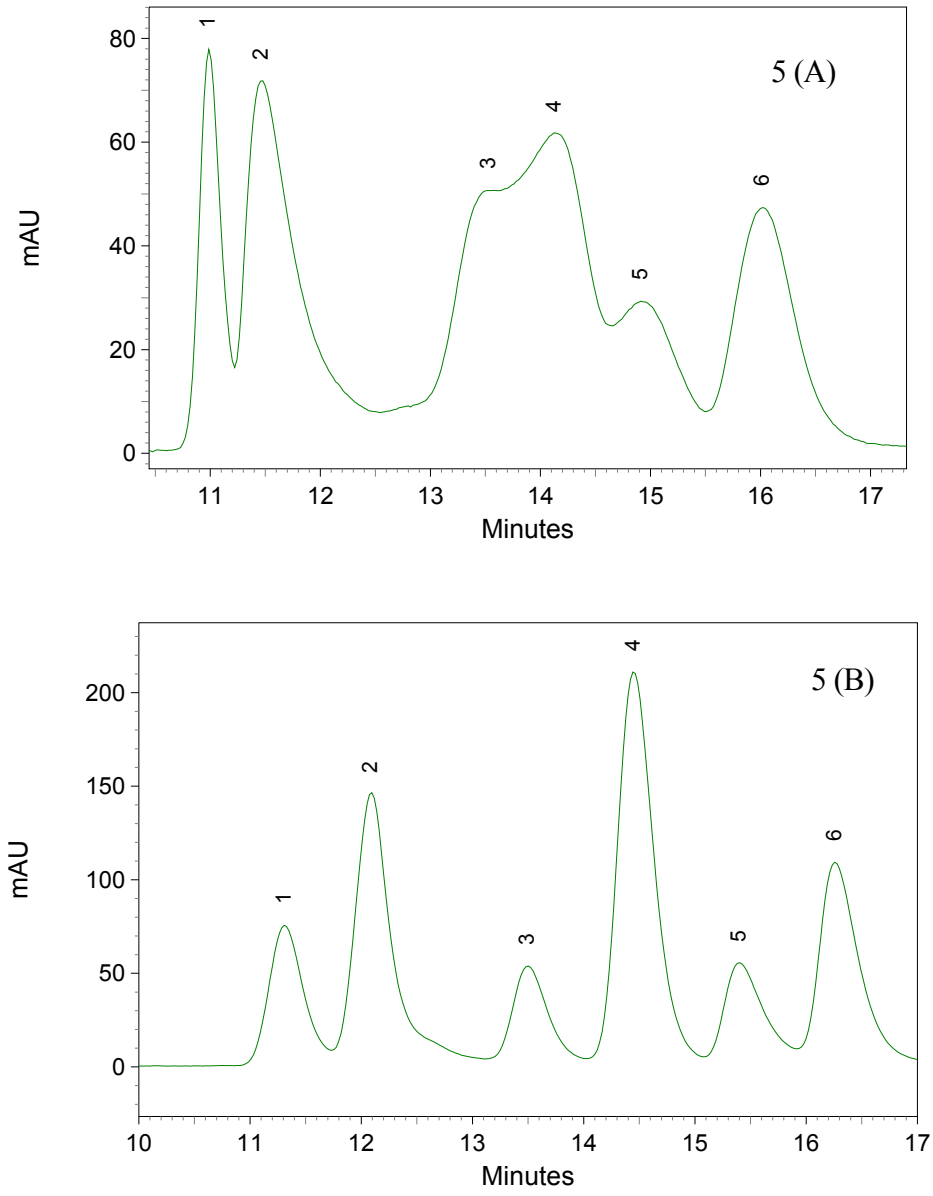

Fig. 4.3. Negative effects on chromatographic profile by methanol: 5 (A): Standards dissolved in methanol/water (25/75, v/v) (B): Standards dissolved in water. 1, Tartaric acid; 2, oxalic acid; 3, malic acid; 4, L-ascorbic acid, 5, malonic acid; 6, lactic acid.

Ethanol and acetone, two other frequently-used extraction solvents, may reduce retention time of organic acids or influence their chromatogram profiles before 30 min, but not phenolic compounds. Therefore, it is necessary to remove most of these organic solvents before HPLC assay if the peaks of interest fall within these time frames.

4.6 Chapter summary

A simple method for simultaneous separation and determination of very polar organic acids including L-ascorbic acid and phenolic compounds in juices and drinks by HPLC with diode array detection was developed. Ten non-phenolic acids and 21 phenolic compounds were eluted in 80 min. The established method was successfully used to measure a variety of organic acids and phenolic compounds in fruit juice. As no special sample pre-treatment was required, this method could be used for preliminary screening antioxidants and obtaining chromatographic profile in extracts of selected fruits and vegetables.

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Chapter 5

Antioxidants in Star fruit (*Averrhoa Carambola* L.)

5.1 Introduction

As described in Chapter 3, star fruit is a good source of natural antioxidants. It is known that star fruit is rich in vitamin C, however, vitamin C only accounts for a small proportion of total antioxidant capacity. Setiawan and co-workers also reported the content of carotenoids in star fruit [1], which exhibit their antioxidant activity mainly by physical quenching effects. In this part of the study, research was carried out on more polar antioxidants in star fruit. One objective of this study was to investigate whether phenolic compounds are the main antioxidants in star fruit. Furthermore, antioxidant components of star fruit were studied in order to understand their antioxidant mechanism and amend its nutrition data. To some extent, HPLC could be the most useful tool for identification and quantification of phenolic compounds [2-6]. In the present work, HPLC assay was used with solid phase extraction (SPE) to characterize those major antioxidants in star fruit, and further identify those antioxidant peaks by HPLC-ESI-MS-MS.

5.2 Solvent extraction of antioxidants

As a fruit or vegetable contains a variety of compounds, which might not be completely dissolved in its juice, it is necessary to use aqueous organic solvents to

extract them from its residue. Selection of extraction conditions for obtaining the maximum extraction of antioxidants would provide a more complete antioxidant profile of a biological sample.

The effects of solvents and the percentage of water in it on total antioxidant capacity are shown in Fig.5.1. Acetone/water extraction system was able to provide a higher extraction efficiency than ethanol/water system. The 50% aqueous acetone gave the highest extraction efficiency of antioxidant activity. Extraction efficiency on homogenized star fruit at room temperature gave similar trends on another four batches of star fruits. Therefore, 50% of aqueous acetone was chosen as the extraction solvent.

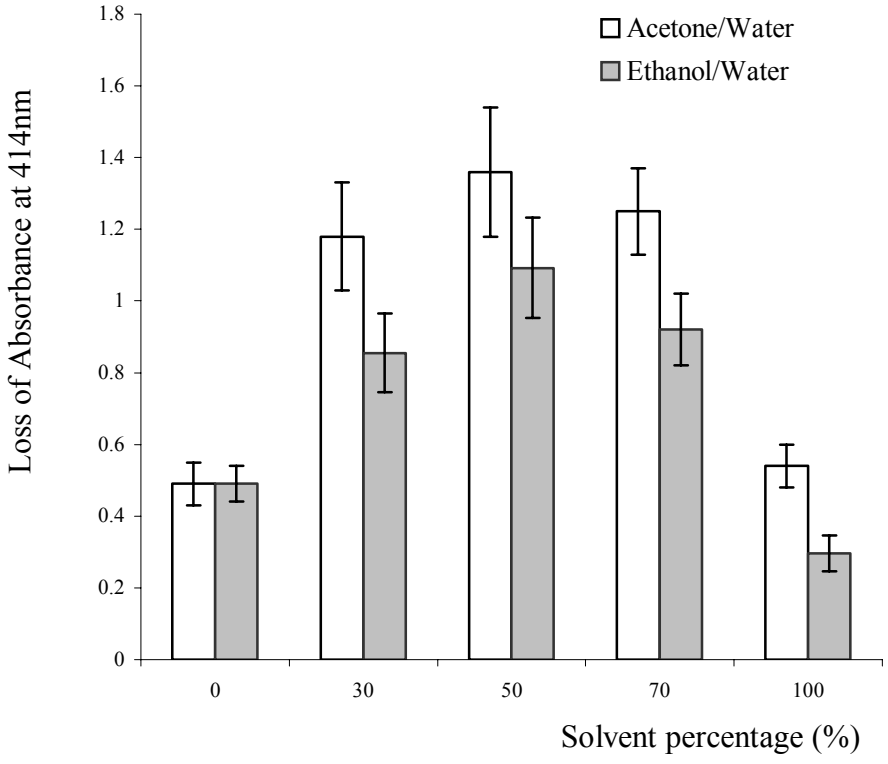


Fig. 5.1. Extraction efficiency vs. solvent percentage. Extraction temperature: 90°C; extraction time: 45 minutes

The effect of temperature on extraction efficiency based on total antioxidant capacity is shown in Fig.5.2. Of the five batches of star fruits used for extraction temperature test, the extraction efficiency increased with increased extraction temperature. Considering stability of antioxidant at high temperature, 90°C was chosen as the extraction temperature.

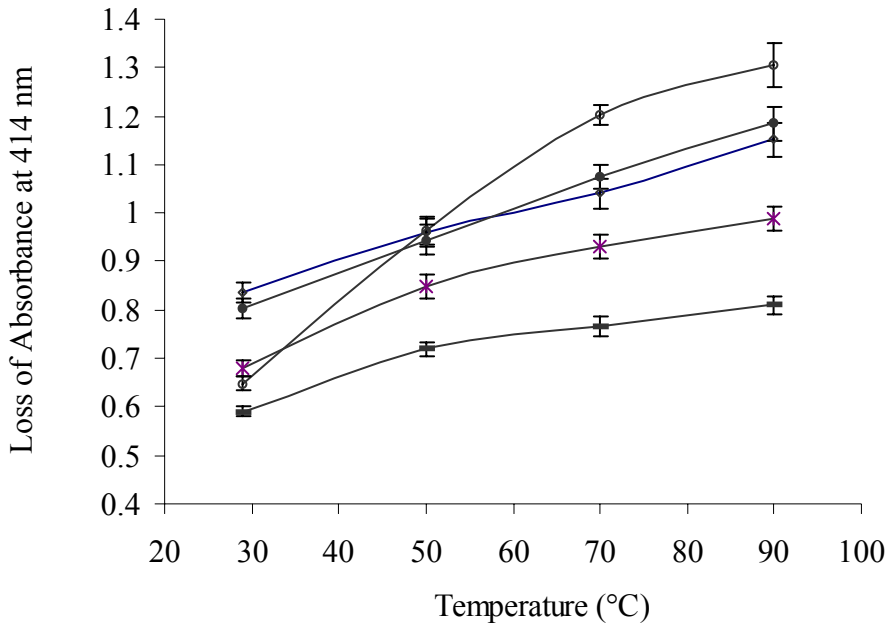


Fig. 5.2. $\Delta A_{414\,nm}$ vs. extraction temperature**.** Extraction time: 45 min; solvent, 50% aqueous acetone solution

Fig.5.3 shows the effect of extraction time on total antioxidant capacity. Extraction efficiency initially increased with time and reached the highest value from 30 to 60 min. Of the five batches of star fruits tested, four gave the highest extraction efficiency at 45 min; only one batch gave highest value at 30 minutes. Therefore, 45 min was chosen as the extraction time.

Based on the above results, the optimum extraction module was 50% acetone as extraction solvent, 90°C as extraction temperature and 45 minutes as extraction time.

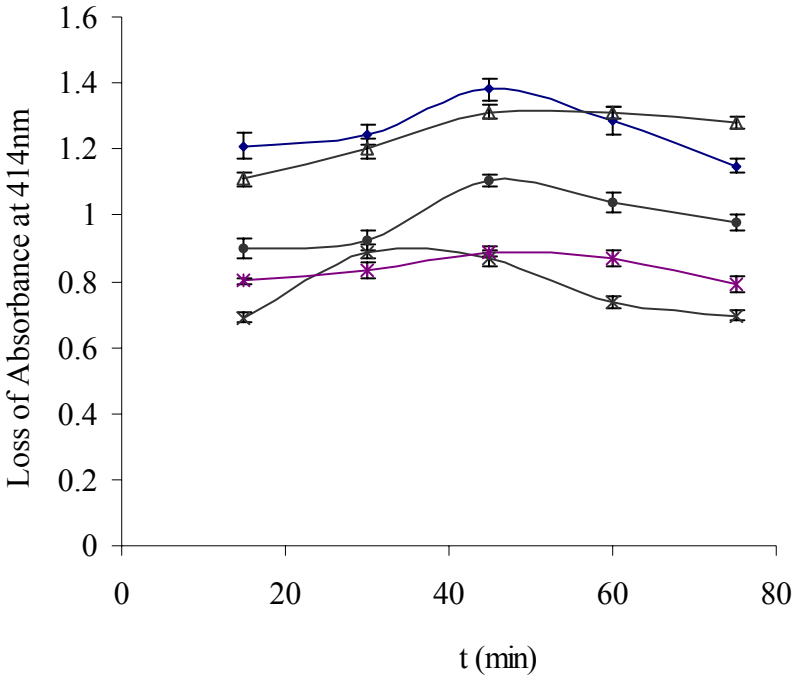


Fig. 5.3. $\Delta A_{414\,nm}$ vs. extraction time. Extraction temperature, 90°C; solvents: 50% aqueous acetone solution

5.3 Distribution of antioxidants in star fruit

In the tropics, star fruit is usually eaten whole when ripe. Occasionally, the juice is extracted as a refreshing drink while the pulp is thrown away. Antioxidants can be found in the juice as well as in the residual pulp. The residue was extracted three times to assess the efficiency of the extraction and to draw conclusion on the distribution of antioxidant in the juice and residual pulp.

As shown in Table 5.1, the first extract of residue accounted for 72.87% of total antioxidant activity in star fruit, and the second extract and the third extract accounted for 7.39% and 2.47%, respectively. Juice accounted for only 17.3% of TAC while it may account for over 90% of the total weight. Therefore, the majority of antioxidants existed in residue rather

than juice. This indicated that pulp residue may have commercial value as a source of natural antioxidants. Its possible application will be discussed later.

Table 5.1 Antioxidant contribution in processed star fruit solution

	Juice	Residue			TAA
		Extract (1^{st})	Extract (2^{nd})	Extract (3^{rd})	
ΔA*	0.855	1.380	0.14	0.078	
Volume (mL)	153	400	400	240	
ΔA×Vol	130.8	552	56	18.7	757.5**
Percentage (%)	17.3	72.87	7.39	2.47	100
			82.7		

* Absorbance change at 414 nm upon addition of 10μl of solution
** TAA is the sum of ΔA×Vol of juice, first extract and second extract and the third extract.

5.4 Inhibition of lipid peroxidation

Results shown in Chapter 3 show that star fruit extract is not only an effective scavenger of ABTS•$^{+}$ but also DPPH•. The ability of residual extract of star fruit may suggest that it is also able to scavenge radicals produced during lipid peroxidation. Peroxides, hydroperoxides, and other oxidative products of lipids may be detrimental to health. In foods, the breakdown products give off-flavors and eventually lead to rancidity. Antioxidants prevent the formation of these products and their further breakdown. Thus, it is able to provide health benefits and increase shelf life of foods.

Butylated hydroxytoluene (BHT) is a synthetic antioxidant widely used in the food industry to slow the development of off-flavors, off-odors and colour changes caused by oxidation, mostly in foods with high fats and oils content. For the purpose of safety, the U.S. Food and

Drug Administration (FDA) limits the use of BHT to 0.02% or 200 parts per million (ppm) of the oil or fat content of a food product.

In section 5.3, it was found that a large proportion of antioxidants were distributed in the residue rather than the juice. Antioxidants extracted from residue were tested for their ability to prevent rancidity of oil. Here it was found that the residue extract significantly slowed the process of rancidity of oil to a larger extent, compared with BHT (Fig.5.4). This result showed that antioxidants obtained from star fruit had the potential to be used for prevention of oil rancidity.

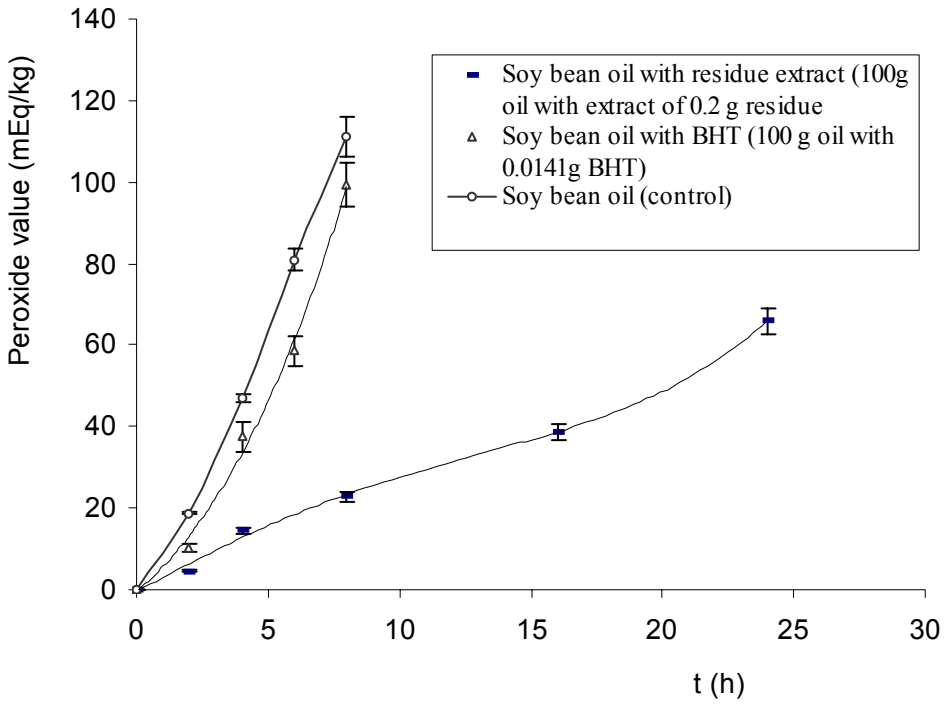


Fig. 5.4. Effects of BHT and residue extract on peroxidation of Soya bean oil

One application of synthetic antioxidant such as BHT is to prevent rancidity of oil used for frying in industrial processes. Synthetic antioxidants are often used much more compared to

natural antioxidants due to their stability at high temperature. Since polyphenolic compounds are also stable at high temperature, they may also be used for this purpose.

5.5 Correlations between TAC and total phenolic contents

The total antioxidant activity of juice and residual extract correlated well with their total phenolic content (Fig.5.5). This indicated that phenolic compounds were likely to be the major antioxidants in star fruit.

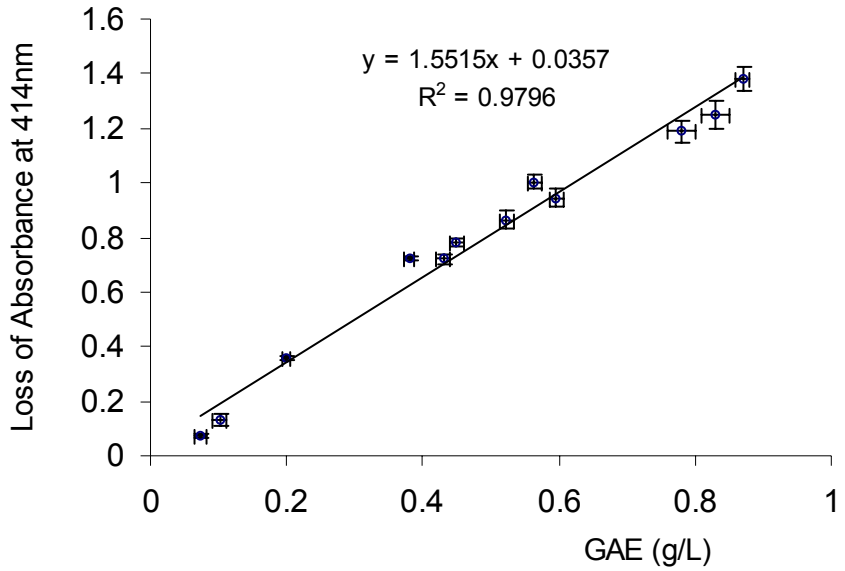


Fig. 5.5. Correlations between loss of absorbance change and total phenolic contents. Data obtained juice, single residue extracts at 30, 50, 70 and 90°C, single residue extracts at different percentage of acetone at 90°C, and individual multiple extracts at 90°C respectively,

5.6 HPLC-DAD assay of antioxidant components

To investigate the major antioxidants in star fruit, antioxidant peaks were first identified by comparing the chromatogram of juice/extract to that of reaction solution of juice and a free radical. Secondly, the percentage contributions of TAC by compounds, which could be

identified by HPLC-DAD, were calculated using their reaction stoichiometry coefficients with free radicals.

Star fruit is a good source of vitamin C (Chapter 3). However, vitamin C only contributes about 3% of TAC. This is consistent with the present results where most of the antioxidants are found to be contributed by phenolic compounds. In SPE fractions, FRC 2, which mainly contains peaks eluted after 30 min, accounted for around 80% of TAC. FRC 1, which mainly collected polar organic acids prior to 30 min including vitamin C, accounted for less than 20% of TAC. FRC 3, which may mainly collect those more non-polar components, accounted for less than 5% of TAC. Moreover, those peaks circled in Fig. 5.6(a), which include L-ascorbic acid (R_t = 14.3 min), (-)epicatechin (R_t = 48.3 min) and several other peaks with a maximum absorbance at around 280 nm, disappeared or significantly diminished after spiking with $ABTS^{\cdot+}$. Similar antioxidant peaks were observed in residue extract except for content of L-ascorbic acid, which was found to be mainly present in juice. As (+)catechin and (-)epicatechin were eluted at 44.3 and 48.3 min, respectively, in this program (Chapter 4), this indicates that those compounds, which were eluted between (+) catechin and (-) epicatechin, were the major antioxidants in star fruit. In addition, other than $ABTS^{\bullet+}$, DPPH• could also be used as a tool for rapidly characterizing antioxidants in biological samples [7]. Here, similar results for star fruit could be obtained from DPPH• assay. However, care should be taken when only DPPH• is used as some antioxidants such as certain phenolic compounds [8] have $ABTS^{\bullet+}$ scavenging activity but are not reactive to DPPH•. Results from Chapter 3 also indicated that most antioxidant were more reactive to $ABTS^{\bullet+}$ than DPPH•. Thus an antioxidant, which is reactive to $ABTS^{\bullet+}$, might not be identified using DPPH• assay.

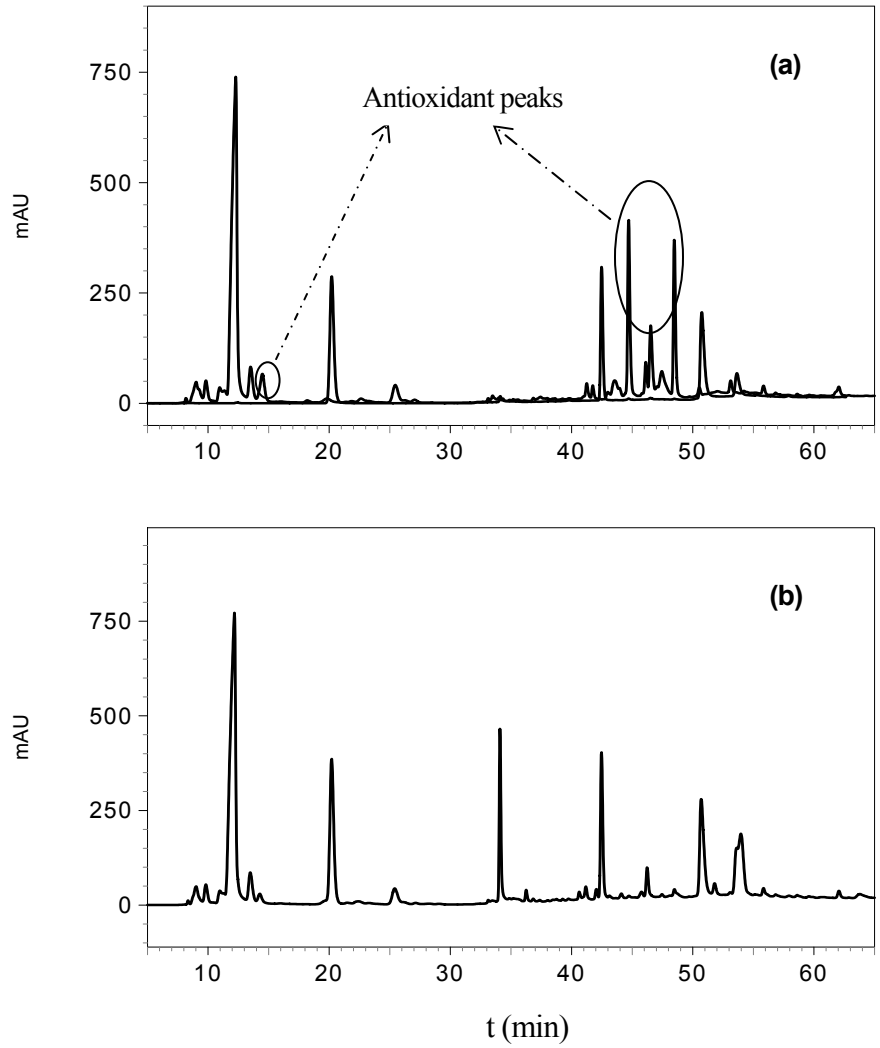


Fig.5.6. Chromatograms of free radical spiking test. HPLC conditions as described in [2], wavelength, 215 nm. (**a**): solid line, Chromatogram of juice with water; dashed line, Chromatogram of ABTS•$^{+}$ with water; (**b**): Chromatogram of reaction solution of juice and ABTS•$^{+}$.

As can be seen from Table 5.2, optimum extraction afforded 29% higher AEAC value than that at room temperature. Of the three compounds quantified by diode array detection, their total contribution was less than 10% of TAC both at room temperature and under optimum extraction conditions. This indicates that other antioxidants unidentified by HPLC-DAD mainly be responsible for TAC of star fruit extract. As no available standard matched those possible antioxidant peaks, those antioxidant peaks were not able to be identified using a DAD detector. A mass detector might provide useful information such as molecular weight and structural information.

Table 5.2. AEAC of star fruit at different extraction conditions

	AEAC mg/100 g	Vitamin C*		Epicatechin**		Gallic acid	
		Concn. ppm	% TAC	Concn. ppm	% TAC	Concn. ppm	% TAC
Juice with residue extract at optimised condition	293.8	80.5	2.74	73.1	4.2	19.5	2.1
Juice with residue extract at 30°C	227.8	80.5	3.53	70.8	5.3	*n.d.*	*n.d.*

* Two and 5.8 were used as stoichiometry of the reaction of vitamin C and (-) epicatechin with $ABTS^{\bullet+}$ respectively.
** Calculated using content of hydrolysed gallic acid content and stoichiometry of 6.3 for reaction between gallic acid and $ABTS^{\cdot+}$.
n.d.: not determined.

5.7 Identification of antioxidants by HPLC and mass spectrometry

HPLC coupled with mass spectrometry has widely been used to identify phenolic compounds, and electronspray ionisation (ESI) mass spectrometric liquid interface was thought to provide advantages in terms of sensitivity and capability to analyze large, thermally labile and highly polar compounds [9, 10]. Initial total ion chromatograms (TICs) of star fruit juice/extract, which were obtained by HPLC-ESI-MS at both positive and negative modes, indicated that the major antioxidant peaks were proanthocyanidins.

To confirm the existence of proanthocyanidins, mass spectrometry of proanthocyanidins in pycnogenol was investigated and compared with those of star fruit juice/extract. Pycnogenol is an extract from French maritime pine bark (PBE). It is a highly standardized mixture of polyphenolic compounds. Proanthocyanidins (Fig. 5.7), which are formed by catechin and epicatechin units with a degree of polymerization of up to heptamer, constitute

75% of its weight [11]. The CID mass spectrum of proanthocyanidins will be discussed below and compared with those of star fruit juice/extract.

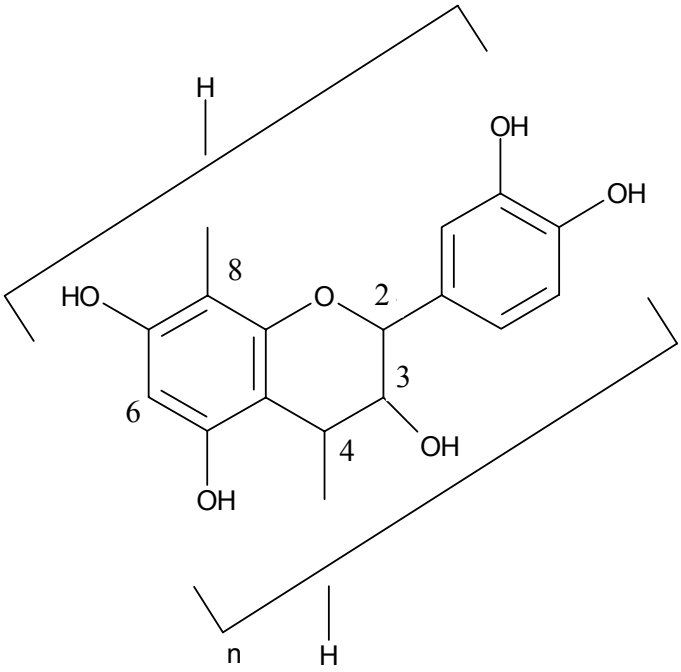


Fig. 5.7. Chemical structure of main proanthocyanidins in pycnogenol

In ESI-MS, deprotonated molecular ions represent the base peak in the negative ion spectra. In contrast, protonated molecular ions are the base peaks in the positive ion mode. Collision induced dissociation (CID) spectrum obtained from positive and/or negative parental ions can further give structural information.

Proanthocyanidins in wine have been reported to be better detected in the negative mode than in the positive mode [12]. Recently, RP-HPLC-ESI-MS-MS was successfully applied to analyse commercial vegetable tanning agents, and fragmentation pattern at negative mode was proposed [13]. Fragmentation patterns at both positive and negative modes have also been discussed by Rhor and coworkers, who have reported that fragmentation occurred more extensively in the positive mode because the abundance of molecular ions in the negative mode was more pronounced [14]. In addition, in this study, it was found that, the

signal intensity of antioxidant peaks at positive mode was significantly higher than that at negative ion mode, probably due to differences in the experimental and instrumental conditions such as HPLC mobile phase and mass spectrometric conditions. Therefore, the positive ion mode was chosen to elucidate their mass spectrometric behaviour because it provided more extensive fragment ions and appeared more sensitive for further LC–MS-MS analysis of phenolic compounds in star fruit juice/extract**.**

As (-) epicatechin and (+) catechin are constitutive units of proanthocyanidins, the fragmentation pattern of (+) catechin or (-) epicatechin may provide useful information for identification of proanthocyanidins. The CID spectrum of parent ion at m/z 291 for (-) epicatechin is displayed in Fig. 5.8 and consists of daughter ions at m/z 139, 123, 165. (+)Catechin gave the same fragment ions, since mass spectrometry cannot distinguish between stereoisomers. The fragment ions peaks obtained here are similar to those obtained by using thermospray-MS-MS [15]. As described by Rhor and co-workers, the fragment ion m/z 139 resulted from retro-Diels-Alder fission (RDA-F) of the heterocyclic ring system, and the fragment ion m/z 123 resulted from cleavage between C2-C3 and O-C2 of the pyran ring [14]. Another main fragment ion m/z 165 might might have resulted from cleavage between C4-C5 and O-C2 of the pyran ring.

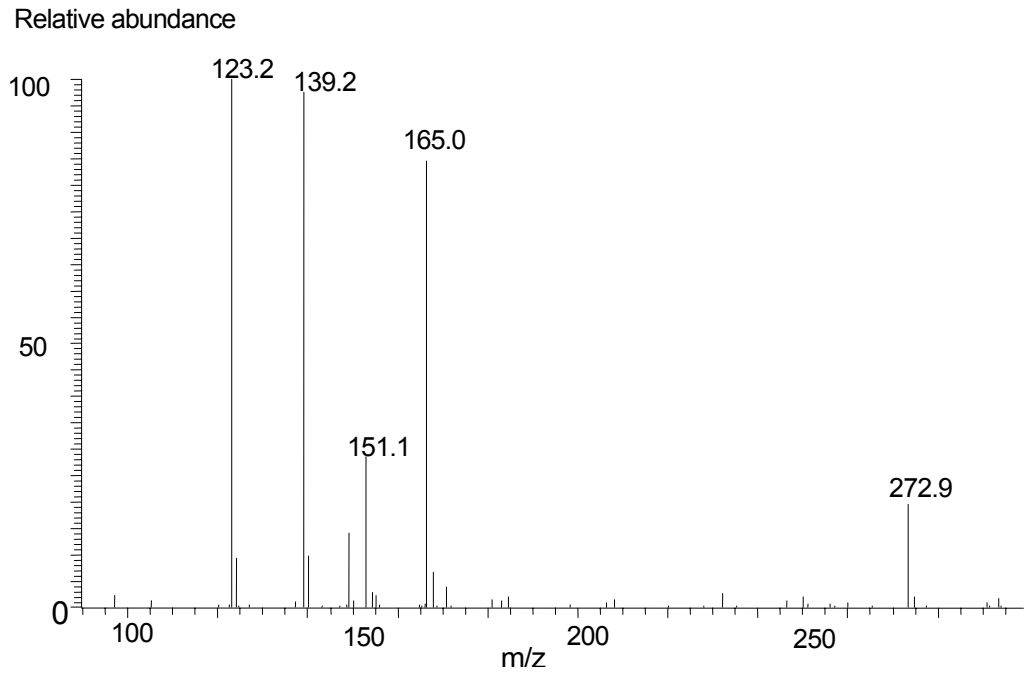


Fig. 5.8. CID (MS/MS Scan) spectrum at m/z 291 of (-)epicatechin, collision energy: 35%.

The CID spectra of proanthocyanidin dimers (m/z 579) and trimers (m/z 867) in commercial pycnogenol are given in Fig. 5.9. The main fragment ions of parent ions at m/z 579 were m/z 427 $[M+H-152]^+$ from RDA-F of the heterocyclic rings, 409 $[M+H-170]^+$ from RDA-F of the heterocyclic rings and loss of water, m/z 291 $[M+H-288]^+$ from interflavanic bond cleavage, 453$[M+H-126]^+$ from cleavages between C4-C5 and O-C2 of one of pyran rings (Fig. 5.10). Similarly, fragment ions of m/z 867 included those at m/z 579 $[M+H-288]^+$ from interflavanic bond cleavage, m/z 715 $[M+H-152]^+$ from RDA-F of the heterocyclic rings, m/z 697 $[M+H-170]^+$ from RDA-F of the heterocyclic rings and loss of water, m/z 427$[M+H-288-152]^+$ from interflavanic bond cleavage plus RDA-F of the heterocyclic rings, m/z 409 $[M+H-288-170]^+$ from interflavanic bond cleavage and RDA-F of the heterocyclic rings and loss of water, etc. In addition, another main fragment ion m/z 577$[M+H-290]^+$ might arise from interflavanic bond cleavage following the quinone-methide mechanism [16].

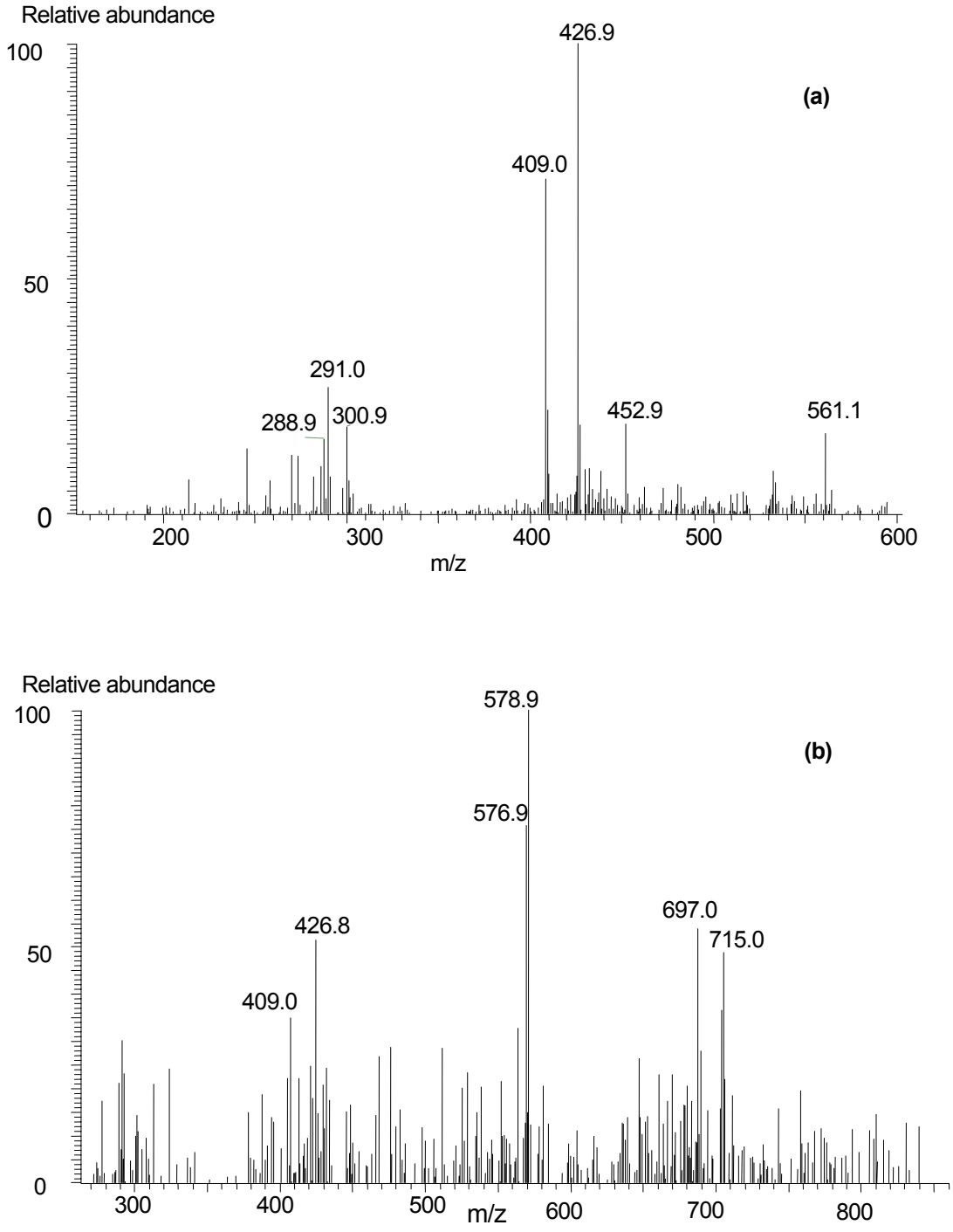


Fig. 5.9. CID (MS/MS Scan) spectra of Pycnogenol proanthocyanidins, collision energy: 50%. **(a)** Corresponding to the parent ion(s) at m/z 579; **(b)** corresponding to the parent ion(s) at m/z 867.

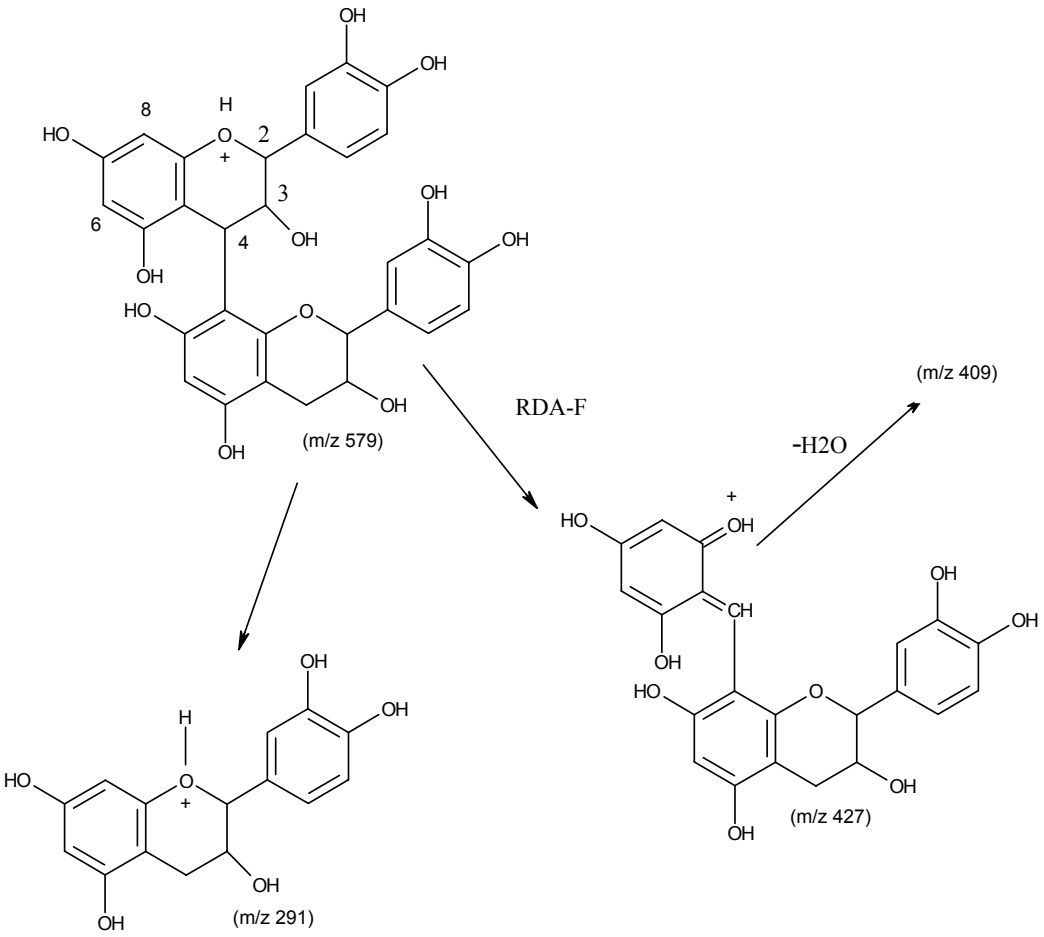


Fig. 5.10. Fragmentation patterns of B-type proanthocyanidin

It was found that the contribution of TAC by the residue of star fruit was 82.7%, and that of its juice accounted for only 17.3% while it may account for over 90% (w/w) of total weight. Therefore, major antioxidants existed in the residue rather than the juice. Fig 5.11 shows the results of HPLC-DAD-ESI-MS analysis of residue extract. As discussed above, the main antioxidants in juice/residue were found to be those of peaks near (+) catechin (R_t =13.8 min) and (-) epicatechin (R_t=18.2 min). The negative and positive ions of those major peaks are listed in Table 5.3. Each major antioxidant peak identified was named compound **1** to **5**, respectively. As the parent ions and fragment ions observed at negative ion mode were not as sensitive as those observed at positive ion mode, they were not used for structural elucidation but only for confirming the existence of compounds of interest. One ion peak at m/z 291 (R_t =3.7 min), which was eluted before vitamin C (Rt=5.7 min),

was not identified as a catechin or epicatechin isomer because no corresponding ion peak at m/z 289 was observed and it did not have a CID spectrum similar to that of catechin. In addition, this peak was also not identified as an antioxidant peak because no peak before vitamin C was identified as antioxidant peak in the spiking test.

Table 5.3. Positive and negative ions of major antioxidant peaks

Rt (min)		ESI MS m/z		Major CID fragments of $[M+H]^+$					Chemical structure
	Mode	$[M\pm H]^{+/-}$	$[M+Na]^+$	$[M\pm H-170]^{+/-}$	$[M\pm H-152]^{+/-}$	$[M\pm H-288]^{+/-}$	$[M\pm H-126]^{+/-}$	Other m/z ions	
13.98 **(1)**	+	579	601	409	427	291	453		Dimer
	-	577		407	425	289	451		
15.42 **(2)**	+	867	889	697	715	579	741	Similar to dimer	Trimer
	-	865		695	713	577	739		
15.67 **(3)**	+	1155	1177	985	1003	867		Similar to trimer and dimer	Tetramer
	-	1153			1001				
15.96 **(4)**	+	1443	1465	1273	1291	1155	1317	Similar to tetramer, trimer and dimer	Pentamer
	-	1441							
18.21 **(5)**	+	291			139		165	123, 151,273	Epicatechin
	-	289						245, 205, 179	

In HPLC-ESI-MS, compound **1** showed a $[M+H]^+$ ion at m/z 579, a $[M+Na]^+$ at 601 and a $[M-H]^-$ at 577. Distances between isotopic peaks are 1 amu, thus supporting that the molecular ions were singly charged species. Therefore, the molecular weight of compound **1** is around 578. As shown in Fig.5.12 and Table 2, CID spectrum of compound **1** at m/z 579 gave fragment ions at m/z 427$[M+H-152]^+$ from RDA-F of the heterocyclic rings, m/z 409 $[M+H-170]^+$ from RDA-F of the heterocyclic rings and loss of water, and m/z 291$[M+H-288]^+$ from interflavanic bond cleavage, etc. This fragmentation pattern is the same as proanthocyanidin dimer from pycnogenol. In addition, the UV spectrum of compound **1** is similar to that of (+)catechin/(-)epicatechin. Therefore, compound **1** was identified to be a singly-linked proanthocyanidin dimer. As shown in Fig.5.11, besides

compound **1**, other minor isomers with retention times of 12.00, 12.61, 15.5 and 26.2 min, respectively, were detected at m/z 577 and showed fragmentation patterns similar to that of compound **1**.

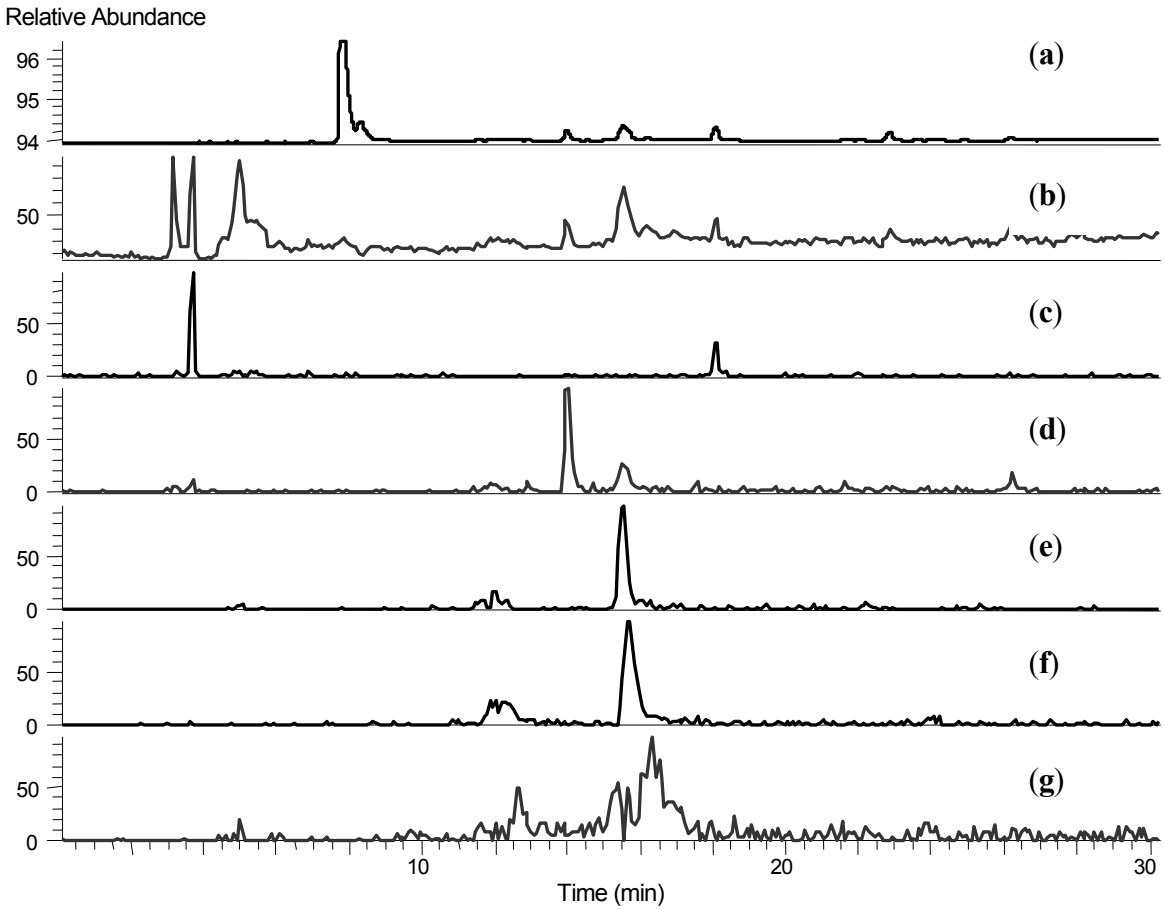
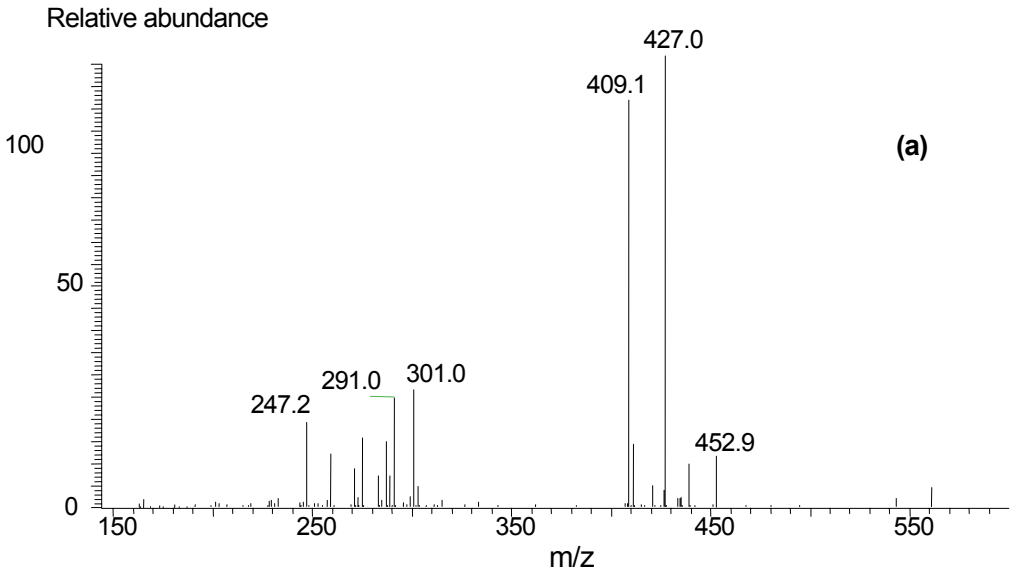


Fig. 5.11. HPLC-DAD-ESI-MS analysis of residue extract. (**a**): Chromatogram at 280 nm; (**b**): TIC at positive mode. Ion traces of (**c**) to (**g**) corresponding to monomers (m/z 291), dimers (m/z 579), trimers (m/z 867), tetramers (m/z 1155) and pentamers (m/z 1443), respectively.

Similarly, the molecular weight of compound **2** was around 866. It shows an UV spectrum similar to that of (+)catechin/(-)epicatechin and a molecular weight of [290×3 - (n-1)×2]. As shown in Fig. 5.12 and Table 2, CID spectrum of compound **2** at m/z 867 gave fragment ions at m/z 715$[M+H-152]^+$, 697$[M+H-170]^+$, 579$[M+H-288]^+$, 577$[M+H-290]^+$, *etc*. The fragment ions were similar to those of proanthocyanidin trimer, and the fragmentation pattern should also be similar to that of proanthocyanidin trimer from pycnogenol. Based

on the above results, compound **2** was tentatively identified as a proanthocyanidin trimer. The isomers of compounds **2** in both juice and extract might exist at low concentrations as intensities of their ion peaks were much lower than compound **2** (Fig. 5.11).

Compounds **3** has a molecular weight of around 1154 [290×4 - (4-1)×2] and UV spectrum similar to those of (+)catechin/(-)epicatechin. As shown in Fig.5.12 and Table 5.3, CID spectrum of compound **3** at m/z 1155 gave fragment ions at m/z 1003$[M+H-152]^+$, m/z 985[M+H-170, m/z 867$[M+H-288]^+$,579[M+H-288×2], m/z 865$[M+H-290]^+$ and 577$[M+H-288-290]^+$. The fragment ions might arise from the fragmentation pattern similar to those of dimers or trimers. Therefore, compound **3** was tentatively identified as a singly-linked proanthocyanidin tetramer. A few isomers of compound **3** with retention times of 11.87, 12.26 and 23.8 min were also observed with much lower intensities in all residue extracts and most batches of juice, while two dimers with retention times of 11.87 and 12.26 min were detected to have around 50% intensity of compound **3** in two batches of juice. However, the ion intensities of the two isomers in whole star fruit were still much lower than that of compound **3.** The results agreed with the above result, which shows that the contribution of TAC by the residue was 82.7% and major antioxidants exited in the residue rather than the juice. Therefore, compound **3** was identified as the major tetramer in star fruit.


Relative abundance
427.0
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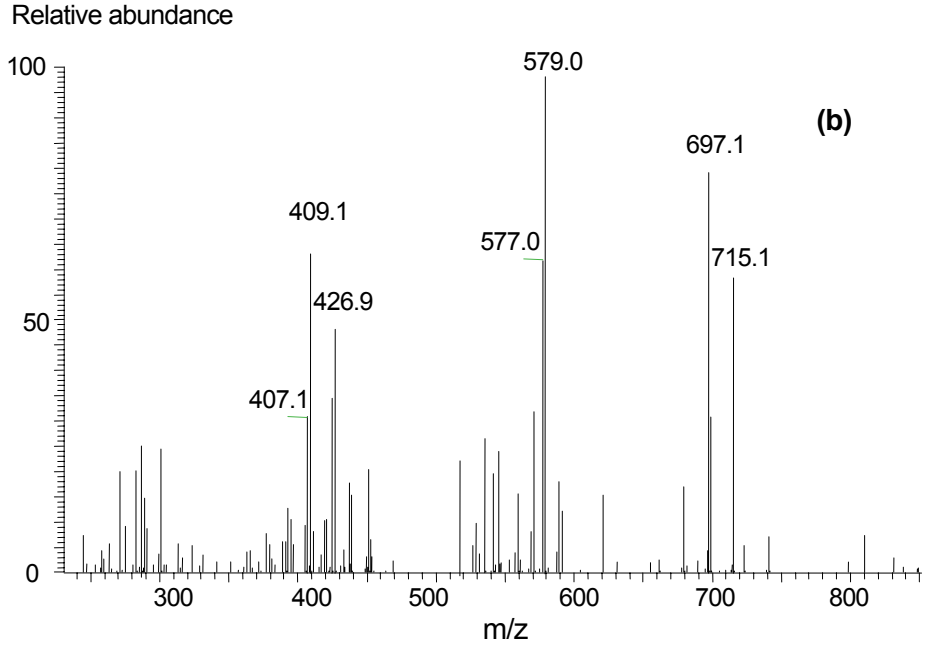
100
(a)
50
291.0
301.0
247.2
452.9
0
150
250
350
450
550
m/z


Relative abundance
100
579.0
(b)
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715.1
426.9
50
407.1
0
300
400
500
600
700
800
m/z

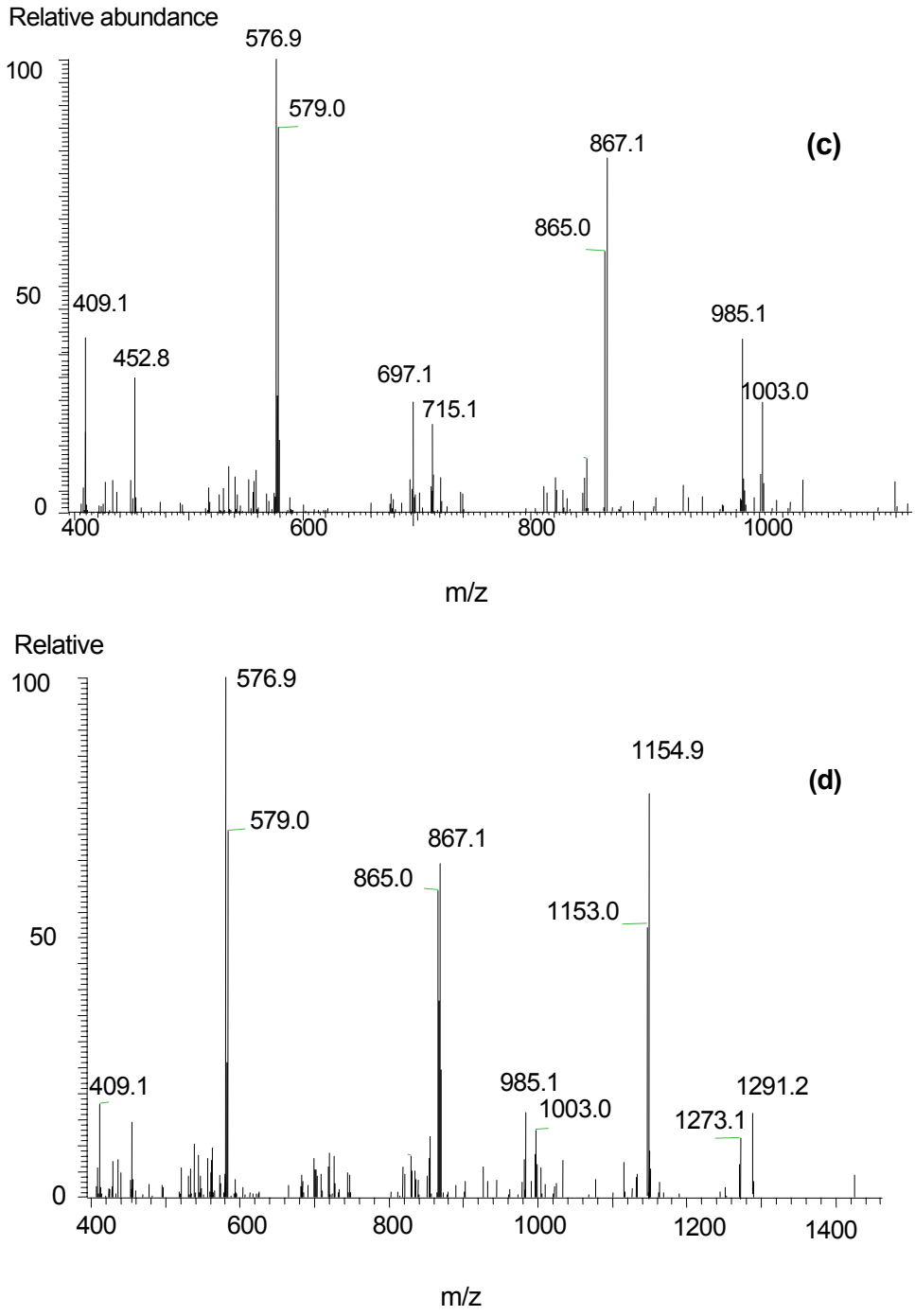

Fig. 5.12. CID (MS/MS Scan) spectra of antioxidant peaks in residue extract, collision energy: 50%. (**a)** CID spectrum corresponding to the parent ion(s) at m/z 579; (**b**) CID spectrum corresponding to the parent ion(s) at m/z 867; (**c**) CID spectrum corresponding to the parent ion(s) at m/z 1155; (**d**) CID spectrum corresponding to the parent ion(s) at m/z 1443.

For compound **4**, its molecular weight was around 1442 [290×5 - (5-1)×2] and its UV spectra was similar to those of (+)catechin/(-)epicatechin. As shown in Fig.5.12 and Table 5.3, CID spectrum of compound **4** at m/z 1443 gave fragment ions at m/z 1291[M+H-

152]$^+$, m/z 1273[M+H-170]$^+$, 1155[M+H-288]$^+$, 867[M+H-288×2]$^+$, 579[M+H-288×3], 1153[M+H-290]$^+$, 865[M+H-288-290]$^+$ and 577[M+H-288×2-290]$^+$. The fragment ions might arise from the fragmentation pattern similar to those of tetramers or trimers in pycnogenol or star fruit. Therefore, compound **4** was identified as a singly-linked proanthocyanidin pentamer. While compound 4 was the major pentamer in star fruit, a few of isomers with retention times of 12.6 and 15.2 min were also observed.

Compound **5** was identified as (-)-epicatechin by comparison of the UV and CID spectra with authentic standard.

In addition, the precipitation of bovine protein after addition of star fruit extract/juice indicated the existence of tannins. The results from solvent extraction experiment showed that acetone/water gave more effective extraction of antioxidants than alcoholic solvents. This could be due to the fact that acetone inhibits tannin-protein interaction and thus increases extraction efficiency of tannins. Based on the above results, the major antioxidants were preliminarily attributed to be condensed tannins *i.e*. proanthocyanidins.

5.8 Comparison of antioxidant capacity and phenolic profile of residue and pycnogenol pills

In this study, it was found that $AEAC_{ABTS}$ of residue from ten star fruits was close to that of a bottle of pycnogenol (40 pills) while the cost of the latter was eight times more than the former. As the residue in juice drink processing is usually discarded, its commercial potential may be significantly underestimated.

As described above, both star fruit and pycnogenol are rich sources of proanthocyanidins; the proanthocyanidins existed as dimers through pentamers in the star fruit. Results from current mass spectral profiles of dimers through pentamers were similar to the above results. However, here, $[M+H]^+$ of the heptamer ion at m/z 1731 was also observed (Fig. 5.13). This is probably due to seasonal changes of star fruit or different cultivars of star fruit used in different experiments.

Table 5.4. Comparisons of elution times of proanthocyanidins in pycnogenol and star fruit

Compounds		Retention times and relative abundance of isomers			
Monomers	Pycnogenol	14.05(100)			
	Star fruit	18.28(100)			
Dimers	Pycnogenol	10.73(100)	16.20(17)		
	Star fruit	14.10(100)	21.74(26)	26.26(50)	
Trimers	Pycnogenol	10.40(100)	11.37(40)		
	Star fruit	15.66(100)	12.00(44)	22.31(12)	
Tetramers	Pycnogenol	8.26(100)	10.13(40)		
	Star fruit	15.58(100)	11.92(50)	12.27(55)	23.86(35)
Pentamers	Pycnogenol	10.20(100)			
	Star fruit	16.01(100)	15.06(50)	11.13(35)	12.53(15)
Heptamers	Pycnogenol	11.81(100)			
	Star fruit	16.18(100)	15.28(90)		

Fig. 5.13 shows chromatographic elution profiles of proanthocyanidins in pyconogenol and star fruit residue extract. As shown in Fig. 5.13 and Table 5.4, the chromatographic profiles of proanthocyanidins in star fruit were quite different from those of pycnogenol. For example, (-)epicatechin was the major monomer, and (+)catechin was not detectable in star fruit. On the contrary, (+)catechin was the major monomer, and (-)epicatechin was not detectable in pycnogenol. In addition, (+)catechin (Rt=14.05 min) was eluted faster than (-)epicatechin (Rt=18.28min), other major proanthocyaidin isomers in pycnogenol were also eluted faster than their corresponding isomers in star fruit. This may imply that the major constituent of proanthocyanidins in star fruit is (-)epicatechin while (+)catechin is the major constituent of proanthocyanidins in pycnogenol.

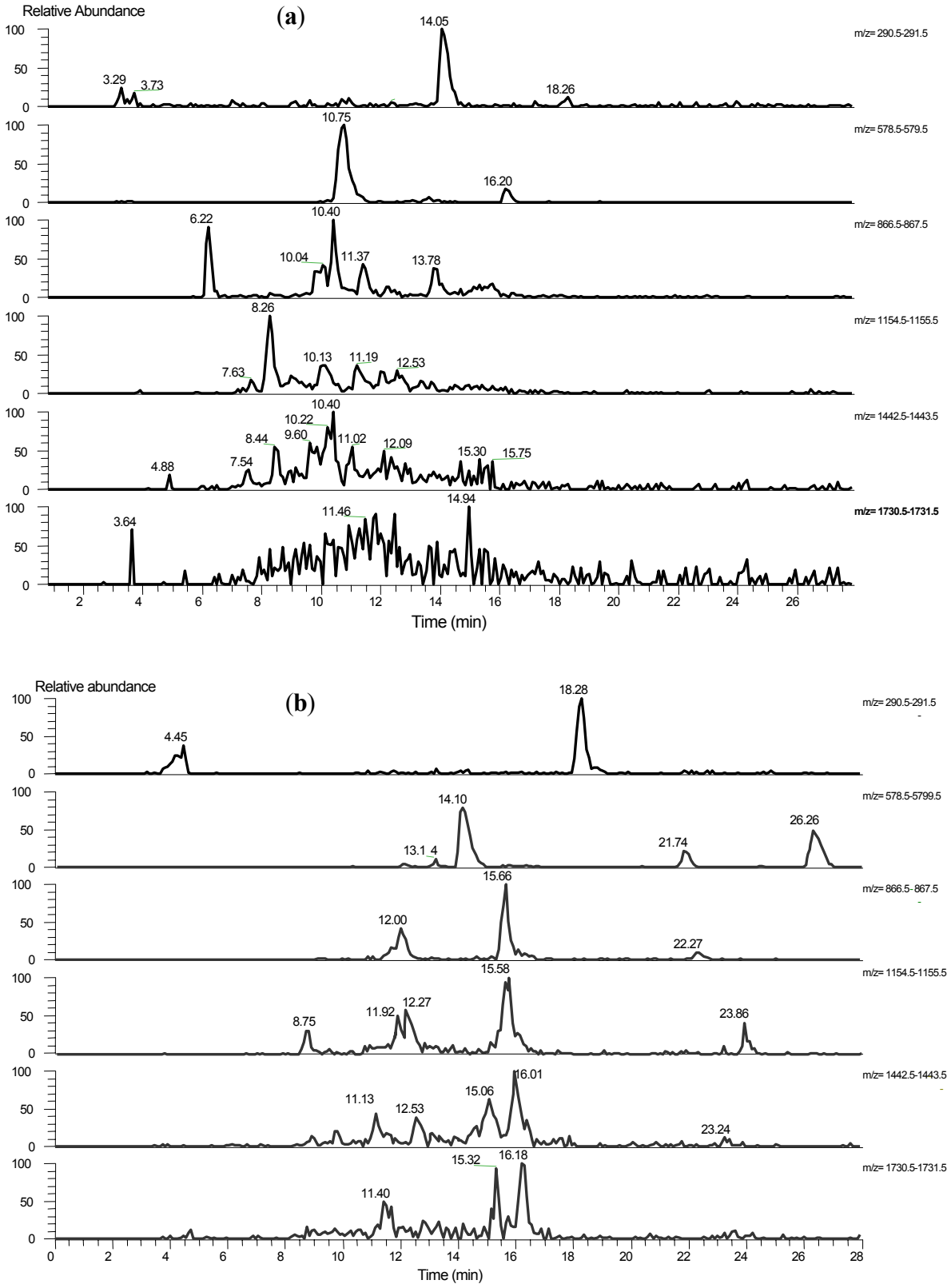

Fig. 5.13. Ion traces of proanthocyanidins in pycnogenol and star fruit. (**a**) Ion traces of monomers through heptamers in pycnogenol. (**b**) Ion traces of monomers through heptamers in star fruit.

The high content of polyphenolics (mainly proanthocyanidins) and strong antioxidant capacity of residue extract suggested its great commercial potential as a nutraceutical resource or functional food ingredient. Further research on structural identification and quantification of proanthocyanidins in star fruit is necessary in order to understand its proanthocyanidin profiles and possible dietary intake of these compounds. While pycnogenol has been reported and claimed to have health effects (11, 17-20), the potential health effects of star fruit need yet to be studied.

5.9 Chapter Summary

Based on TAC assays of SPE fractions and HPLC assays of SPE fractions, juice/extract and reaction solution with free radicals, the major antioxidants in star fruit were investigated and further identified by tandem mass spectrometry. Phenolic compounds were found to be the major antioxidants in star fruit. (-)Epicatechin and proanthocyanidins, which existed as dimers through hexamers, were reported in star fruit for the first time and preliminarily considered as major phenolic compounds in star fruit.

The residue of star fruit, which is normally discarded during juice drink processing, was found to possess a higher antioxidant activity than the corresponding juice in several modes for assessing antioxidant activity. The residue extract also showed strong antioxidant activity in delaying oxidative rancidity of soya bean oil at 110°C. High performance liquid chromatograph coupled with mass spectrometry (HPLC/MS) showed that major proanthocyanidins in star fruit were different from their isomers in pycnogenol. The high content of phenolics and strong antioxidant activity of residue extract indicate that residue

powder may impart health benefits when used in functional food products to add polyphenolics and that residual extract should also be regarded as a potential nutraceutical resource.

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Chapter 6

Antioxidants in Lady's Finger (*Hibiscus Esculentus Linn*)

6.1 Introduction

Knowledge of the potential antioxidant components present in fruits and vegetables may provide us with useful nutritional information as well as their specific health effects. Recently, several methods, which were based on online-detection of antioxidants by post-column neutralization of eluates with free radicals, have been successfully utilized to identify and quantify antioxidants in certain biological samples [1-5]. However, limitations of these methods are obvious when standards of unknown antioxidant components are not available and the reaction stoichiometry of an antioxidant and free radical is still not clear. Therefore, it is difficult to measure and identify the major antioxidant(s) in biological samples using these existing methods. This work has successfully characterized and further identified antioxidant(s) in star fruit by comparing chromatograms of sample extract with oxidized sample extract by $ABTS^{\bullet+}$, which is prepared with ABTS and potassium persulphate [6]. These background peaks might interfere characterization of some antioxidant peaks. Here, approach was improved by using $ABTS^{\bullet+}$, which is prepared by oxidation of ABTS with MnO_2, to characterize antioxidant peaks, and thus reduce interferences of peaks from $ABTS^{\bullet+}$ solution in the spiking tests. The improved approach was systematically used to analyze the major antioxidants in Lady's finger, a common vegetable in Southeast Asia. Lady's finger is a native of tropical Africa and is now widely grown in the tropics and the sub-tropics. It has been reported that

lady's finger is a good source of trace elements such as zinc, copper, magnesium, calcium and selenium [7]. Surprisingly, its antioxidant capacity and major antioxidant components appear not yet reported. This study covers characterization of major antioxidants in lady's finger, structure elucidation of characterized antioxidants using tandem mass spectrometry and their structure confirmation using nuclear magnetic resonance spectroscopy.

6.2. Characterisation of major antioxidant peaks in lady's finger

As shown previously, several background peaks were eluted from $ABTS^{\bullet+}$ solution that was prepared by oxidation of ABTS by potassium persulphate [6]. Here, it was found that no peaks were eluted from $ABTS^{\bullet+}$ solution prepared by oxidizing ABTS with MnO_2. Thus $ABTS^{\bullet+}$ solution oxidized with MnO_2 will be a better oxidant for characterizing antioxidant peaks as no interference will be introduced from the background solution (Fig. 6.1). Peak heights of antioxidant peaks were significantly reduced after reaction with $ABTS^{\bullet+}$ (Fig. 6. 2). As shown in Fig. 6. 2, several major peaks, which had retention times between 20 and 30 min, were initially identified as major antioxidant components as those peaks disappeared or were significantly reduced after reaction with free radicals. The four antioxidants, which had retention times between 20 and 30 min, were initially identified as major antioxidants and named as compounds **1-4** according to their elution order. In addition, one small peak, which was eluted at around 9.5 min and named compound **5**, was also identified as an antioxidant.

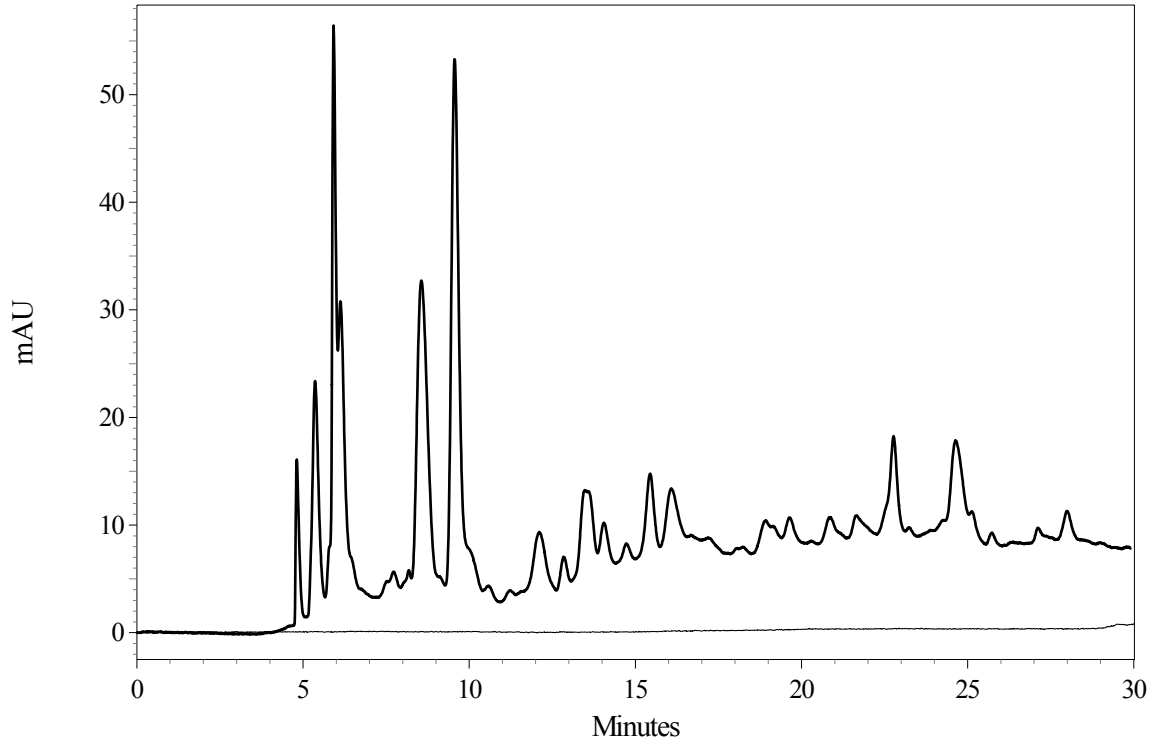


Fig. 6.1. Chromatograms of $ABTS^{+}$• solution oxidized by potassium persulphate and manganese dioxide. Solid line (—), $ABTS^{+}$• oxidized by manganese dioxide; dashed line (-·····-), $ABTS^{+}$• oxidized by potassium persulphate.

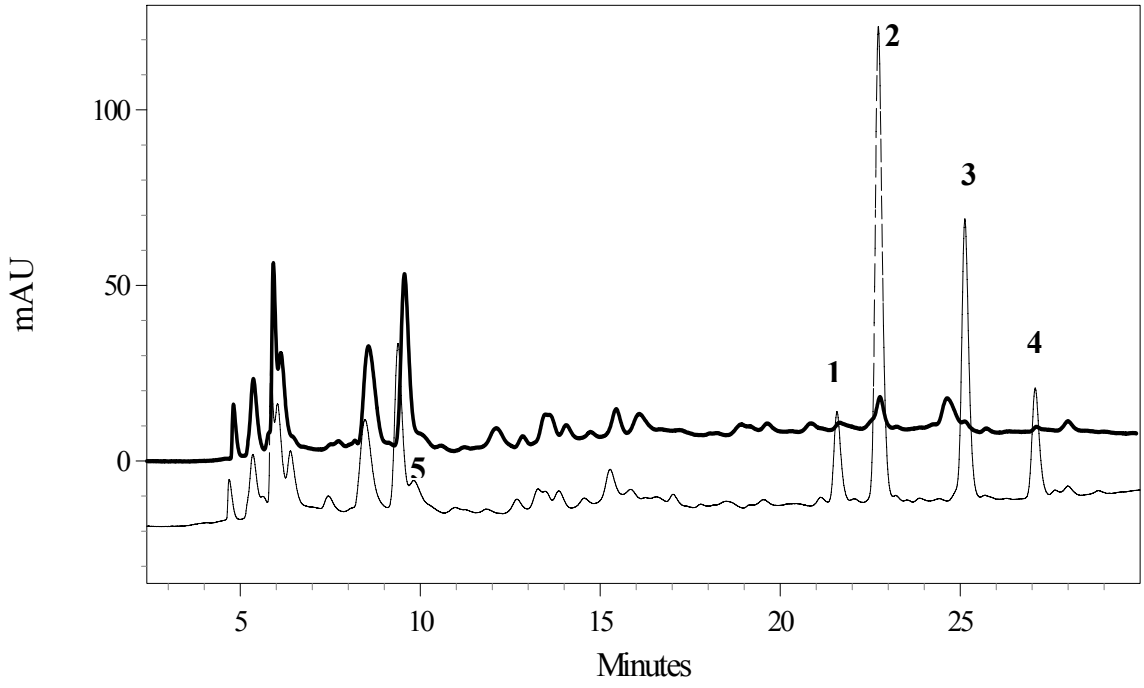


Fig. 6. 2. Chromatograms of extract and reaction solution of extract and $ABTS^{+}$•. Solid line, 1:5 of extract/ $ABTS^{+}$• (v/v); dashed line, 1:5 of extract/0.1% formic acid (v/v). Detection wavelength: 280 nm

Elution period of major antioxidant peaks was further investigated by measuring TAC of individual fractions from solid phase extraction (SPE). Table 6.1 lists the antioxidant activity performance of FRC 1 to FRC 3 and their percentage contributions to TAC. As shown in Table 6.1, FRC 2 accounted for around 70% of the TAC of the extract.

Table 6. 1. Antioxidant activity performance of fractions.

	FRC 1	FRC 2	FRC 3	Original extract
Vol. (mL)	1.20±0.07	0.91±0.08	0.98±0.04	1.0
ΔA_{414nm}	0.217±0.041	0.697±0.055	0.009±0.001	0.816±0.003
AC (Vol.× ΔA_{414nm})	0.260	0.634	0.009	0.816
TAA %	31.9(28.8 [a])	77.7(70.2 [a])	1.1(1.0 [a])	

[a] The percentage contribution to TAA in bracket was modified according to that the sum of TAA% by FRCs is 100%.

Chromatograms of FRC 1, FRC 2 and FRC 3 showed that FRC 2 mainly included peaks with retention times from 20 to 30 min (Fig. 6.3). Other non-polar compounds, which possibly existed in extract, did not show any antioxidant activity. Therefore, the major antioxidant peaks were the four major peaks, compounds **1-4,** as shown in Fig. 6.2.

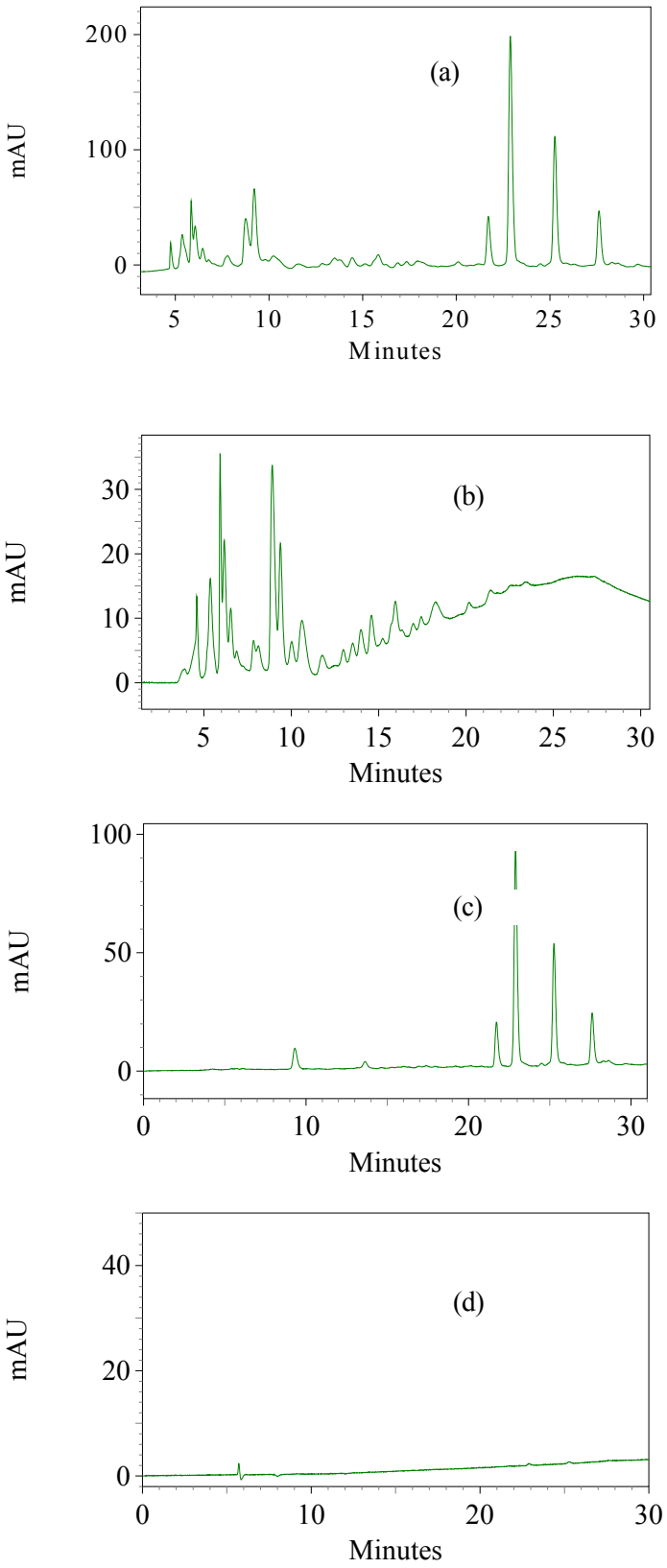


Fig. 6. 3. Chromatograms of extract and FRCs. (a), 1:3 of extract/0.1% formic acid; (b), FRC 1; (c), 1:1 of FRC 2/0.1% formic acid; (d), FRC 3. Detection wavelength: 280 nm

6.3. Identification of antioxidants using HPLC/MS^n

Results from HPLC-DAD shows that the major antioxidant components **1-4** all had spectra similar to that of quercetin (R_t = 29 min). In addition, acidic hydrolysis released quercetin aglycones. Thus they were considered as quercetin derivatives. The procedure for structure elucidation of those compounds may involve (1) confirmation of the existence of the quercetin aglycone as well as (2) identification of the substitution group and its substitution position on the aglycone.

As discussed above, FRC 2, which accounted for 70 % of TAC, mainly included compounds **1-4**. Although HPLC coupled with DAD provided good separation of compounds of interest and gave much information on the possible class of these compounds, it did not provide any confirmation of compound without authentic standards. As reviewed previously, mass spectrometry and HPLC coupled with mass spectrometry are very powerful tools for identification and structural elucidation of naturally occurring substances including flavonoids and related compounds [8]. The obvious advantage of HPLC-MS is that it provides the possibilities of separating and identifying different compounds in a complex mixture. Here, mass spectra from HPLC-MS analysis provided very useful information on possible chemical structures of antioxidants. Compound **5** was identified as (-)-epigallocatechin by spiking the sample with real standard and comparing its mass spectra and collision induced dissociation (CID) spectra with those of the authentic standard (Table. 6. 2).

Table 6. 2. ESI-MS^n spectra of compounds **1~5**

Compounds	ESI-MS [a]	ESI-MS^2 [b]	ESI-MS^3 [b]	ESI-MS^4 [b]
1	***597*** (+)	***465***, 303	***303***	257, 285, 229, etc
2	***627*** (+)	***465***,303	***303***	257, 285, 229, etc
3	***465*** (+)	***303***	257, 285, 229, etc	
4	***549*** (-)	***505***	***463***, 301, 300	301, 300
	551 (+)	***303***	257, 229, 285, etc.	
5	***307*** (+)	139,151,169, 181,289		

[a.] Only $[M+H]^+$ (100%) were listed, other ion peaks were $[M+Na]^+$, $[M+M+H]^+$, $[M+M+Na]^+$.

[b.] CID spectra were obtained with the previously italicised bold ions

Table 6.2 also gave the HPLC-DAD-ESI^n (n=1-4) mass spectra of compounds **1-4**. Other than major ion peaks $[M+H]^+$ in HPLC-MS mode, other ion peaks such as $[M+Na]^+$, $[M+M+H]^+$, $[M+M+Na]^+$ and the ion at m/z 303 were also observed. The ESI mass spectra of compounds **1-4** all include a common peak ion at m/z 303, which is corresponding to the ion peak of quercetin aglycone. The structural information can be enhanced by using tandem MS with collision induced dissociation. Table 6.2 also shows that compounds **1-4** gave similar CID spectra for ion peak m/z 303. Further CID spectra at m/z 303 were compared to the MS-MS results for quercetin. The results confirmed that they were different derivatives of quercetin. Therefore, the common aglycone of compound **1-4** was identified as quercetin. Fig.6.4 shows the MS/MS spectra of parent ions at $[M+H]^+$. For compound **1**, the daughter ions at m/z 465 and 303 by ESI-MS^2 and ESI-MS^3, respectively, indicated sequential loss of pentose and hexose (Table 6.2). In addition, high resolution electron ionization MS (HREIMS) gave the molecular formula of compound **1** as being $C_{26}H_{28}O_{16}$. Therefore, the most likely structure of compound **1** is quercetin-hexose-pentose. For compounds **2~4**, HREIMS indicated their molecular formulae to be $C_{27}H_{30}O_{17}$, $C_{21}H_{20}O_{12}$ and $C_{24}H_{22}O_{15}$, respectively. According to the CID spectra of their parent

ions, loss of hexose in compound **3** and sequential loss of hexose in compound **2** indicated compounds **2** and **3** to be quercetin-hexose-hexose and quercetin-hexose respectively. For compound **4**, its CID spectra from parent ions at m/z 551 (positive), which were observed at different collision energy levels, only gave the information of the existence of quercetin aglycone. However, its CID spectra from parent ions at m/z 549 (negative) and 505 (daughter ion of parent ions at m/z 549) indicated sequential loss of carboxyl group (CO_2), C_2H_2O group and hexose group (Fig. 6.5). These results indicated that the main fragmentation pattern in negative mode was related to the cleavage of the acylation bond between hexose and malonic acid. Therefore, compound **4** was tentatively identified as malonylated-hexose-quercetin.

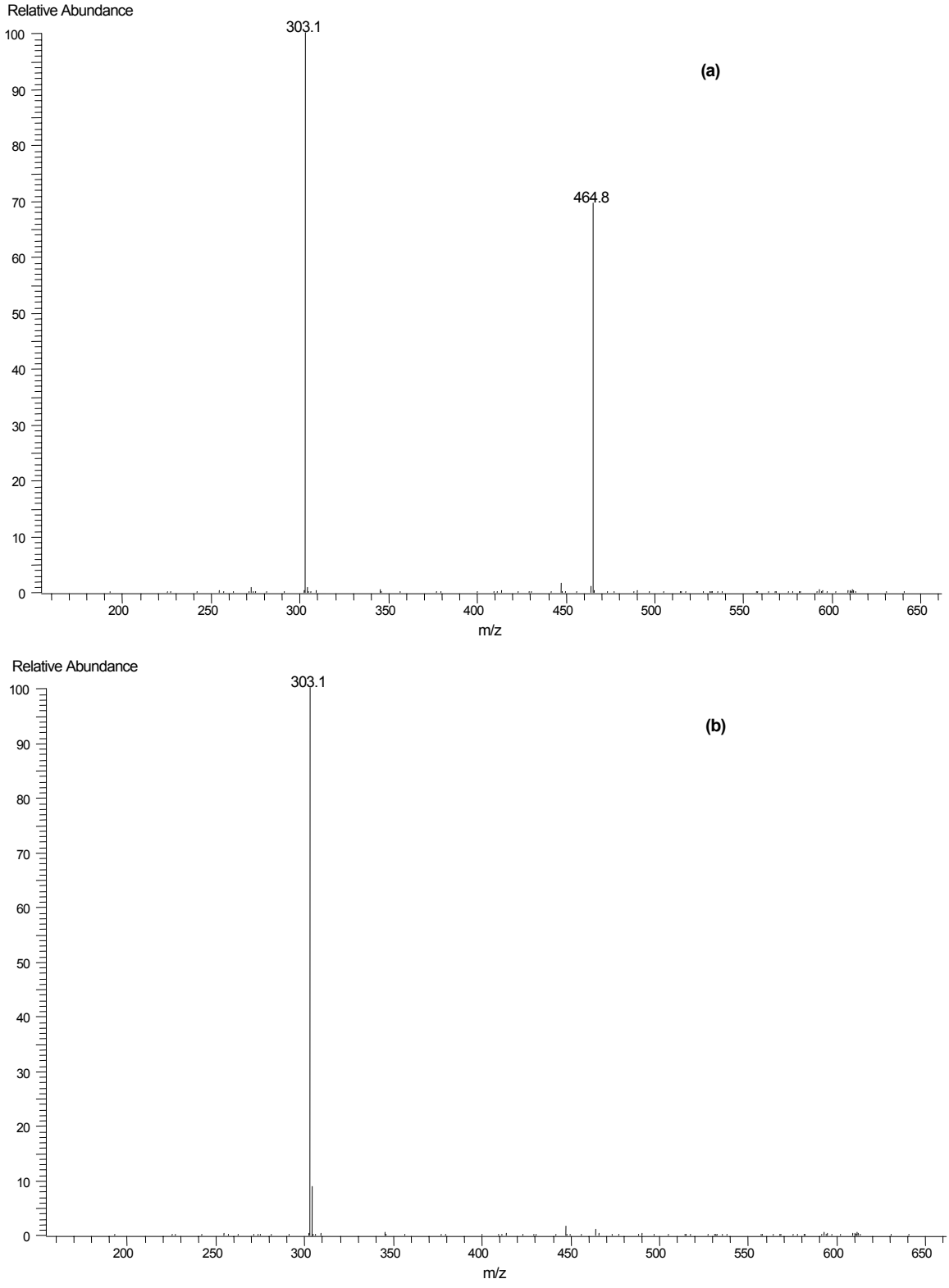


Fig. 6. 4. ESI-MS^2 mass spectra of compounds at positive mode. (a) CID spectrum corresponds to the positive parent ion(s) at m/z 597 (MS/MS Scan), collision energy: 50%; (b) CID spectrum corresponds to the positive parent ion(s) at m/z 551 (MS/MS Scan), collision energy: 50%. CID spectra at m/z 627 and 465 are similar to those at m/z 597 and 551 respectively.

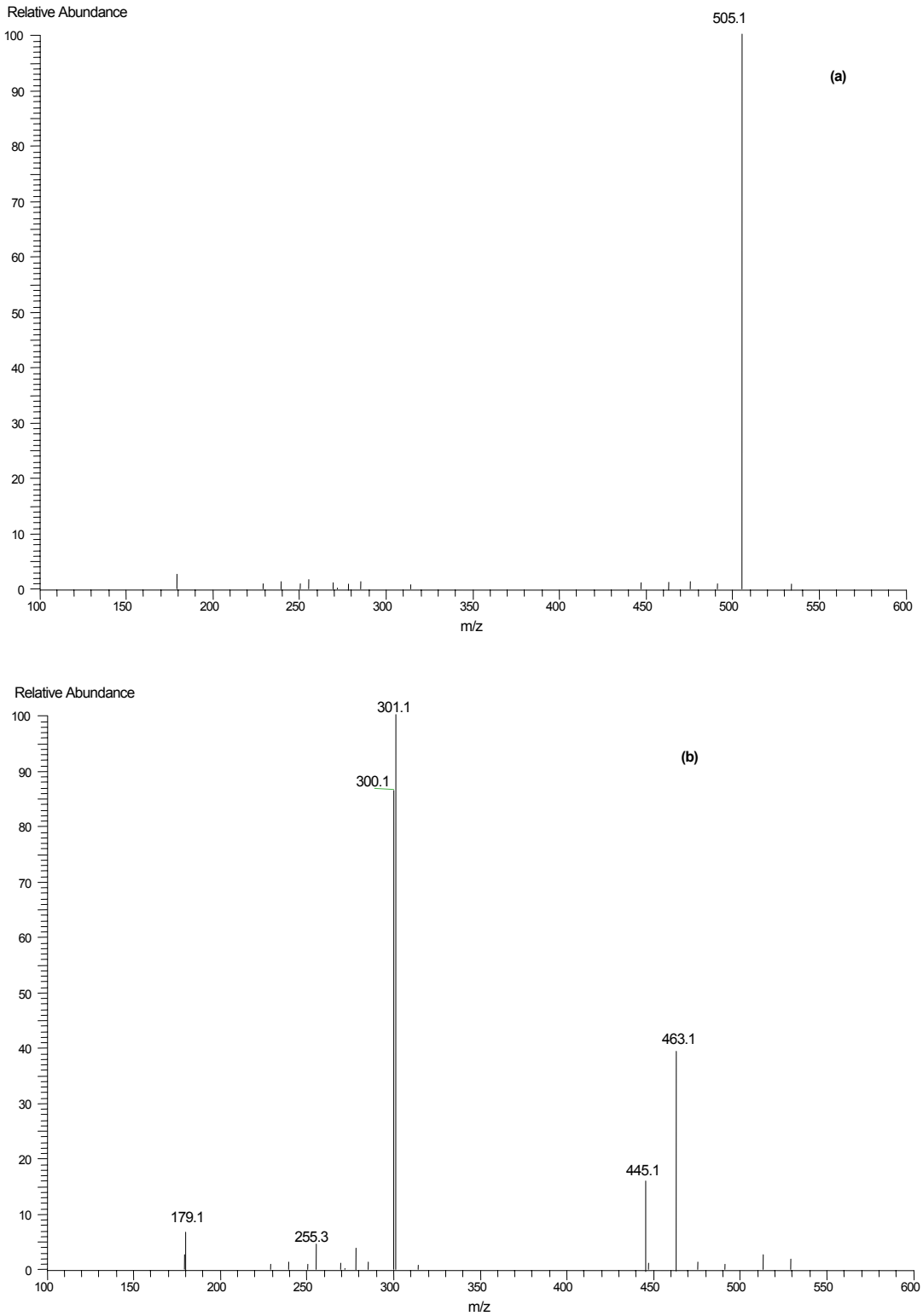


Fig. 6. 5. ESI-MS^n mass spectra of compounds **4** at negative mode. (a) CID spectrum corresponds to the negative parent ion(s) at m/z 549 (MS/MS Scan), collision energy: 80%. (b) CID spectrum corresponds to the negative parent ion(s) at m/z 505 from m/z 549 (MS^3), collision energy: 80%.

6.4. Structure confirmation using spectrometric methods

As discussed above, (-)-epigallocatechin and the molecular formulae of four quercetin derivatives were identified using HPLC combined with the ESI-MS^n techniques. However, the glycosylation position between sugar and aglycon and acylation position between sugar and acid remains unknown. Therefore, their structure identification was further investigated using spectrometric methods.

The UV-Vis spectra of compounds **1, 2, 3 and 4** in MeOH showed absorption maxima at around 256 and 357 nm. The UV spectral changes induced by various shift reagents indicated that the flavonoid had no free hydroxyl group at C-3 and free hydroxyl groups at C-5, C-7, C-3' and C-4' positions [9]. In addition, their ^1H and ^{13}C NMR spectroscopic data in DMSO-d_6 confirmed that their aglycone was quercetin and glycosylation occurred at the C-3 position. Mass spectra indicated that compound **1** was quercetin monodesmoside with one inner hexose moiety bonded at the C-3 and one terminal pentose moiety. The NMR spectrum of compound 1 (Table 6.3) was resolved by best fit matching to appropriate monosaccharide spectra or through a comparative study of the simpler mono- or diglucosides that constitute part of the structure. The assignments in the spectrum of compound **1** were deduced by reference to the ^{13}C NMR spectra of a number of flavonol monodesmosides [10], luteolin 3-xylosyl (1'''→2'') glucoside [11], quercetin 3-O-glucoside (**3**) and kampferol 3-O-xylosyl (1'''→2'') glucoside [12]. Glucose was assigned to be the inner sugar while the terminal sugar was identified as xylose. The other possible combination of hexose and pentose will give greater differences on 13C NMR spectrum [10]. Compared with the NMR spectrum of quercetin 3-O-glucoside (**3**), a downfield shift at C-2'' (glc) to 81.9

ppm was observed due to 1→**2** intersugar linkage. The assignments of xylosyl carbons are almost identical to those of the counterparts of apigenin 7-O- xylosyl (1'''→2'') glucoside and luteolin 3-xylosyl (1'''→2'') glucoside [11].

Other than carbons from quercetin aglycone, the ^{13}C NMR spectral data of compound **2** (Table 6.3) also revealed the presence of two sugar molecules with a total of 12 carbon signals. The chemical shifts of 12 sugar carbons are almost identical to those of the counterparts of two flavonol 3-O-glucosyl (1'''→6'') glucoside [10, 13]. A downfield shift of C-6'' to 68.0 was due to a 1→6 linkage between the two sugars. The compound **3** was identified as quercetin-hexose by ESI-MS^n. Its ^{13}C NMR spectrum (Table 6.3) in DMSO-d_6 was consistent with those of quercetin 3-O-glucoside [14]. Similarly, the ^{13}C NMR spectroscopic data of compound **4** (Table 3) was consistent with quercetin-3-O-(6-malonylglucoside) [15, 16].

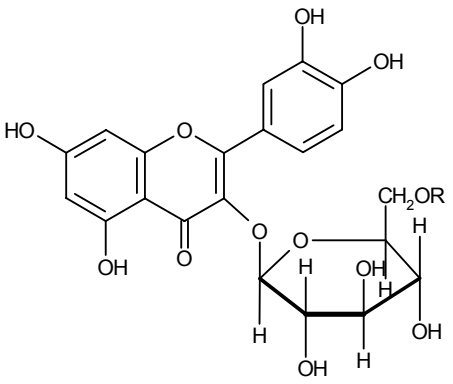
Compound **1.** Quercetin 3-O-xylosyl (1'''→2'') glucoside; UV λ_{max}MeOH: 357, 256; +NaOMe 404, 325, 273; +$AlCl_3$ 433, 273; +$AlCl_3$+HCl 398, 269; +NaOAc 382, 270; +NaOAc+H_3BO_3 378, 262 nm. ^{1}H NMR spectral data (300 MHz, DMSO-d_6): δ 12.65 (OH-5), δ 7.67 (H-6'), δ 7.57 (H-2'), δ 6.85 (H-5'), δ 6.40 (H-8), δ 6.18 (H-2), δ 5.75(H-1''), δ 4.60 (H-1'''). ^{13}C NMR spectral data (300 MHz, DMSO-d_6) are shown in Table 6.3.

Compound **2.** Quercetin 3-O-glucosyl (1'''→ 6'') glucoside; UV λ_{max}MeOH: 358, 256; +NaOMe 412, 324, 272; +$AlCl_3$ 437, 274; +$AlCl_3$+HCl 399, 269; +NaOAc 380, 271; +NaOAc+H_3BO_3 381, 261 nm. ^{1}H NMR spectral data (500 MHz, DMSO-d_6) :): δ 12.62 (OH-5), δ 7.59 (H-6'), δ 7.57 (H-2'), δ 6.85 (H-5'), δ 6.39 (H-8), δ 6.18 (H-6), δ 5.41 (H-1''). ^{13}C NMR spectral data (500 MHz, DMSO-d_6) are shown in Table 6.3.

Compound **3.** Quercetin 3-O-glucoside; UV λ_{max}^{MeOH}: 358, 257; +NaOMe 408, 320, 271; +$AlCl_3$ 436, 274; +$AlCl_3$+HCl 399, 269; +NaOAc 384, 270; +NaOAc+H_3BO_3 381, 261 nm. ^{1}H NMR spectral data (300 MHz, DMSO-d_6): δ 12.65 (OH-5), δ 7.59 (H-6'), δ 7.57 (H-2'), δ 6.84 (H-5'), δ 6.40 (H-8), δ 6.19 (H-6), δ 5.47 (H-1"). ^{13}C NMR spectral data (300 MHz, DMSO-d_6) are shown in Table 6.3.

Compound **4.** Quercetin 3-O-(6"-O-malonyl)-β-glucoside; UV λ_{max}^{MeOH}: 357, 257; +NaOMe 410, 324, 271; +$AlCl_3$ 431, 274; +$AlCl_3$+HCl 399, 269; +NaOAc 401, 271; +NaOAc+H_3BO_3 384, 263 nm. ^{1}H NMR spectral data (300 MHz, DMSO-d_6): δ 12.62 (OH-5), δ 7.54 (H-6'), δ 7.51 (H-2'), δ 6.84 (H-5'), δ 6.41 (H-8), δ 6.20 (H-6), δ 5.37 (H-1"). ^{13}C NMR spectral data (300 MHz, DMSO-d_6) are shown in Table 6.3.

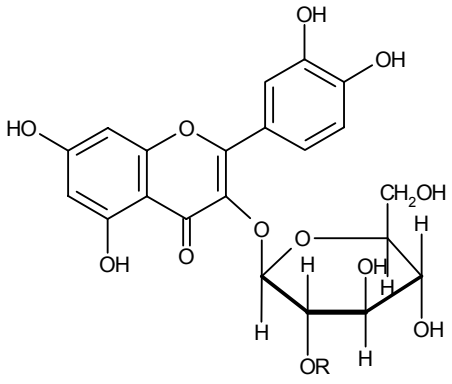
Chemical structures of compounds are shown in Fig. 6.6.



2: R=Glucosyl

3: R=H

4: R=$COCH_2CO_2H$



1: R=Xylosyl

Fig. 6. 6. Chemical structures of compounds **1-4**

Table 6.3. ^{13}C NMR spectral data of compounds **1–4** in DMSO-d_6 (δ: ppm)

Carbon number	**1**	**2**	**3**	**4**
Flavonoid moiety				
C-2	155.2	156.2	156	156.4
C-3	132.9	133.2	133.2	133.2
C-4	177.3	177.2	177.3	177.4
C-5	161.2	161.1	161.2	161.2
C-6	98.7	98.8	98.7	98.8
C-7	164.2	164.6	164.6	164.4
C-8	93.4	93.6	93.5	93.7
C-9	156.2	156.4	156.3	156.7
C-10	103.7	103.8	103.7	103.9
C-1′	121.0	121.0	121.1	121.1
C-2′	115.2	115.2	115.2	115.2
C-3′	144.9	144.8	144.8	145.0
C-4′	148.6	148.5	148.5	148.6
C-5′	115.9	116.1	116.1	116.3
C-6′	121.8	121.6	121.5	121.5
Sugar moiety				
C-1″	97.9	100.8	100.9	101.1
C-2″	81.9	73.3	74.05	74.0
C-3″	76.9	76.5	76.5	76.8
C-4″	69.6	69.6	69.9	69.6
C-5″	77.6	76.3	77.5	76.8
C-6″	61.3	68.0	60.9	69.6
C-1‴	104.6	103.1		
C-2‴	73.9	74.0		
C-3‴	76.1	76.3		
C-4‴	69.4	69.7		
C-5‴	65.7	76.4		
C-6‴		60.7		
Malonyl moiety				
C-7″				166.9
C-8″				41.8
C-9″				167.9

Compound **1**: quercetin 3-O-xylosyl (1'''→2'') glucoside
Compound **2**: quercetin 3-O-glucosyl (1'''→6'') glucoside
Compound **3**: quercetin 3-O-glucoside
Compound **4**: quercetin 3-O-(6''-O-malonyl)-β-glucoside

6.5 Chapter Summary

The improved approach, which mainly involved (1) Identification of major antioxidants using HPLC analysis of biological samples, free radical, reaction solution of both and fractions from SPE and TAC assay of related solution and (2) Structure elucidation of major antioxidants using modern techniques, was successfully applied for identification of major antioxidants in lady's finger. This approach could be used for identification of major antioxidants in a variety of biological samples.

To our knowledge, the four quercetin derivatives and (-)epigallocatechin were identified for the first time in lady's ginger. As flavonoids have attracted increasing attention due to their potential health benefits, the next step of the research is to quantify them for the initial understanding of the possible dietary intake of these compounds. Further research on stoichiometry and kinetic study of isolated compounds are still necessary.

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Chapter 7

Antioxidants in Salak (*Salacca Edulis Reinw*)

7.1. Introduction

As described in the last two chapters, HPLC with a UV-VIS or diode array detector can be used as a powerful tool to separate and identify antioxidants in a sample after reaction with free radicals. However, the complexity of biological samples has been a major obstacle to identification of their antioxidant compositions. Even though there have been numerous reports on this subject, only a small number of compounds have been reported at one time in each individual study. While HPLC was predominately used to separate compounds such as phenolics, different detection systems have been used for determination of the separated compounds, e.g., UV, electrochemical and DAD [1]. UV detection is likely to be the most common. However, specificity for proanthocyanidins over other polyphenolic compounds is low due to the narrow range of UV absorption of these compounds [2]. Postcolumn reaction of proanthocyanidins with dimethylaminocinnamaldehyde to produce a product with maximum absorption at around 640 nm was applied to increase sensitivity [3]. Electrochemical detection of proanthocyanidins has also become more widespread due to the smaller number of electroactive substances compared to UV-absorbing compounds [4-6]. In recent years, HPLC coupled with mass detector has widely been used to identify unknown polyphenolics [7] and proanthocyanidins [8-10] in biological samples. As Carri *et al* reviewed, HPLC coupled with mass spectrometry has been widely used to identify phenolic compounds, and ESI mass spectrometric liquid interface was thought to provide advantages in terms of sensitivity and capability to analyze

large, thermally labile and highly polar compounds [11, 12]. The advantages of mass detector make it a potential tool not only for screening but also for identifying natural antioxidants in biological samples.

It is believed that the reaction between an antioxidant and a free radical results in the oxidation of the antioxidant. This may involve the transfer of protons to the free radical, especially when the antioxidant is a phenolic compound. Once a reaction occurs, the molecular weight of products will most likely be different from their antioxidant reactants. Based on this, free radical active compounds could be monitored by observing the difference of peak intensity of individual ions between reactants and reaction products using mass spectrometry detectors. Even if the reactants and products are of the same molecular weights, by combining mass spectrometry with HPLC, they could still be characterized as they should be eluted at different retention times due to their structure difference. Therefore, HPLC coupled with a mass detector will be an ideal tool for characterizing free radical active compounds.

Another advantage of this approach is that it is possible to characterize and identify those antioxidants, which may not be sensitive to common detection systems such as UV or electrochemical detection. Previously developed methods used to identify antioxidant peaks by spiking with free radicals followed by separation with HPLC-DAD were unable to identify some antioxidants [13, 14] in salak. These methods use methanol with aqueous sulphuric acid or formic acid as mobile phase, respectively. The failure to detect some antioxidants is most likely due to poor molar extinction coefficient of proanthocyanidins under the conditions specified. A mass detector will not only be able to provide

information of free radical active components but also their possible chemical structures in a biological sample.

Salak palm fruit belongs to Arecaceae family and is indigenous throughout the Indo-Malaysian region. The fruit can be consumed fresh, candied and canned when fully ripe. As reported in Chapter 3, salak extract is an excellent free radical scavenger. However, it is still not clear which compounds are responsible for its free radical scavenging ability. The newly-developed approach was applied for systematic characterisation and identification of antioxidants in salak extract.

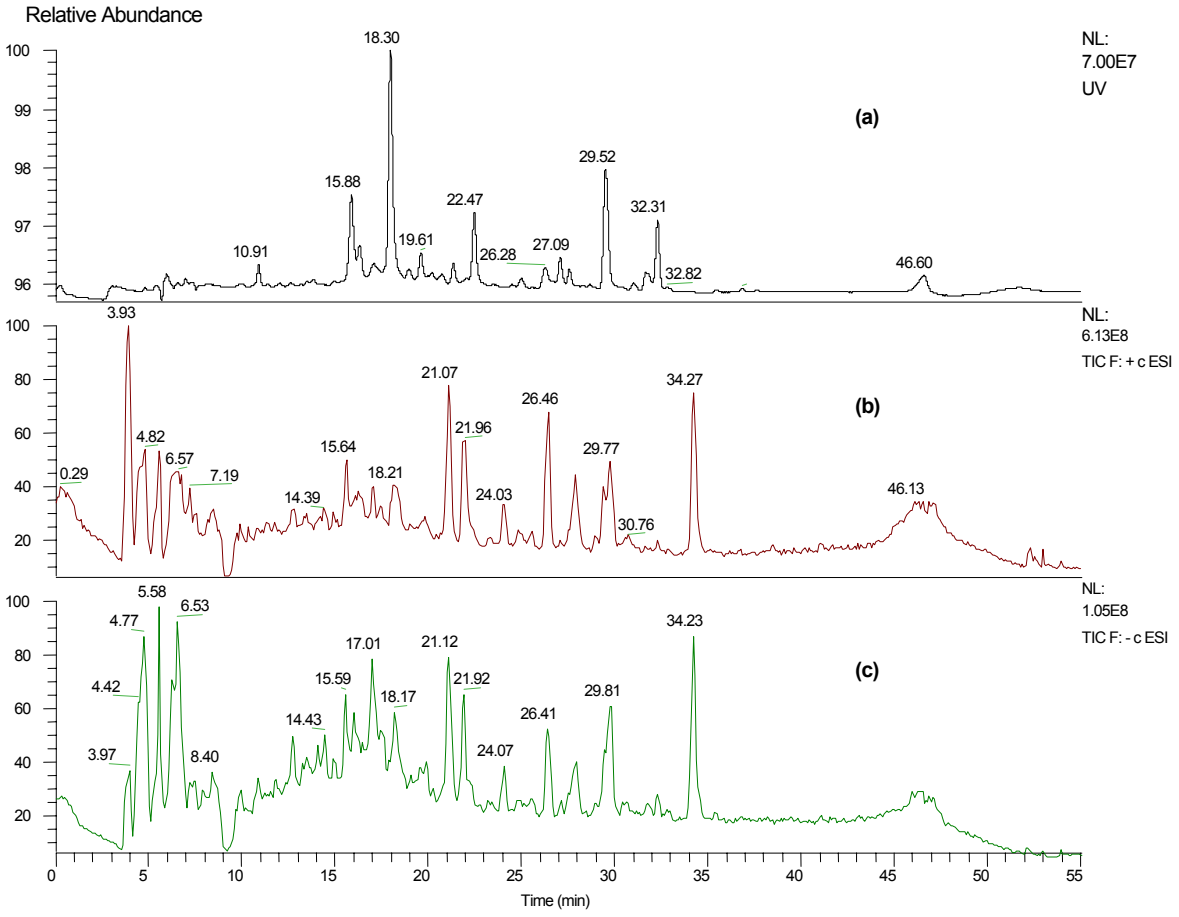

Fig. 7.1 HPLC Chromatograms of salak extract. (a) Detection wavelength: 280 nm; (b) TIC at positive mode; (c) TIC at negative mode.

7.2 Free radical active components in salak extract

Fig. 7.1 shows UV chromatogram at 280 nm, total ion chromatogram (TIC) at positive mode and TIC at negative mode of salak extract. Interestingly, both total ion chromatograms at positive ion mode and at negative ion mode by mass detector provided more peaks than those by UV detection at 280 nm, and the UV data do not clearly indicate the presence of monomer through hexamers in the salak extract (Fig. 7.1(a)). In addition, extracted ion chromatograms of any interested ions will provide absolute advantages over any other common detection system.

To investigate which peaks in salak extract belongs to antioxidants, the intensities of individual ion peaks were compared between control and reaction products of free radicals with salak extract. As so many ion peaks existed in total ion chromatograms (TICs) of salak extract, rough comparisons at different elution periods on ion peaks and their intensities between control and reaction solution were carried out. It was found that most compounds in salak extract were eluted before 40 min, therefore, ion peaks of both reaction solution and control were divided into four elution stages with an interval of 10 min. Mass spectrometry profile of each stage at both positive and negative model was investigated.

Of the four stages investigated, intensities of many ion peaks at stage 2 (from 10 to 20 min) were found to disappear or be significantly reduced after reacting with free radicals. Fig. 7.2 shows the mass spectrum profiles of control and reaction solution at stage 2 at negative mode.

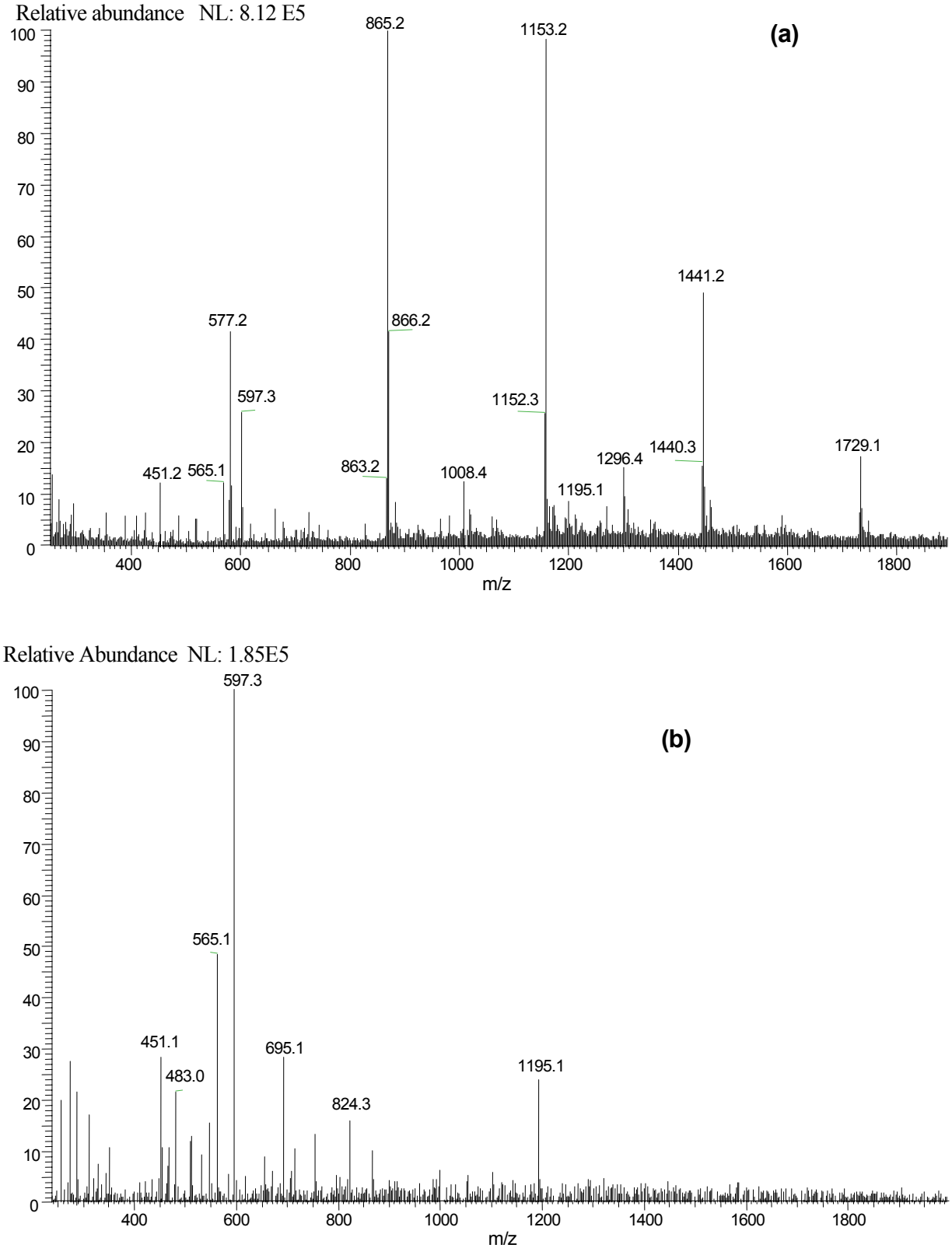

Fig. 7.2. ESI-MS profiles of extract and reaction solution at stage 2 (10-20 min) under negative modes. (a) Extract:water=1:2; (b) Extract: $ABTS^{\bullet+}$ =1:2.

Peaks eluted are summarised in Table 7.1, where their retention times, m/z of ions and intensities before and after reaction with $ABTS^{\bullet+}$ are given. Antioxidants that reacted with $ABTS^{\bullet+}$ showed lower intensities and indicated in the last column to be reactive towards $ABTS^{\bullet+}$. There were no significant changes for intensities of those ion peaks, which were eluted before 10 min. Similarly, no significant decrease of intensities of ion peaks eluted after 20 min, except for (-)epicatechin with a retention time of 20.4 min (Table 7.1). Although intensities of some peaks at stage 3 (from 20 to 30 min) and 4 (from 30 to 40 min) were slightly reduced when mixed with high concentration of $ABTS^{\bullet+}$ (Table 7.1), they are tentatively not considered as antioxidants because their intensities are not reduced when mixed with $ABTS^{\bullet+}$ at lower concentrations. This will be further discussed below.

Those peaks, which had retention times between 10 and 20 min, were identified as antioxidant-related peaks as their intensities were significantly reduced after reacting with $ABTS^{\bullet+}$. HPLC/MS/MS was further applied for elucidating the possible structures of corresponding antioxidants.

The extracted ion chromatograms of antioxidants are shown in Fig. 3. While Table 7.2 shows profiles of antioxidant peaks in salak extract, other ions e.g. ions at m/z 289 with a retention time of 3.98 min and m/z 1153 with a retention time of 30.08 min, which had the same signal of m/z as some of antioxidant peaks, were not listed in the Table as their intensities were not reduced and therefore were not characterized as antioxidants.

Table 7. 1. Ion peak intensities of extract and its reaction solution with ABTS•$^{+}$

	Rt (min)	Intensities of ion peaks		Free radical reactivity
m/z *		Control (Extract:water=1:2)	Reaction product 1 (Extract:ABTS=1:2)	ABTS$^{\cdot+}$
577(-)	14.83	7.4 ± 0.3E6	<1E5	Yes
	15.99	4.5 ± 0.2E6	<1E5	Yes
865(-)	13.15	8.3 ± 0.4E6	<1E5	Yes
	14.52	2.5 ± 0.2E6	<1E5	Yes
	15.37	2.5 ± 0.2E6	<1E5	Yes
	17.42	8.4 ± 0.3E6	<1E5	Yes
	18.03	4.2 ± 0.2E6	<1E5	Yes
1153(-)	11.28	3.7 ± 0.3E6	<1E5	Yes
	13.17	1.4 ± 0.1E6	<1E5	Yes
	13.60	2.6 ± 0.2E6	<1E5	Yes
	14.80	2.5 ± 0.1E6	<1E5	Yes
	17.50	4.9 ± 0.3E6	<1E5	Yes
	30.12	1.6 ± 0.2E6	1.3 ± 0.2E6	No
1441(-)	12.71	1.0 ± 0.1E6	<1E5	Yes
	16.53	2.2 ± 0.2E6	<1E5	Yes
	17.77	1.4 ± 0.1E6	<1E5	Yes
1729(-)	17.42	0.5 ± 0.1E6	<1E5	Yes
453(+)	16.84	5.5 ± 0.2E6	5.1 ± 0.5E6	No
355(+)	18.41	3.8 ± 0.1E6	1.3 ± 0.1E6	Yes
289(-)	20.4	1.3 ± 0.3E6	<1E5	Yes
464 (+)	21.2	5.4 ± 0.3E7	6.1 ± 0.7E6	No
482(-)	22.3	6.5 ± 0.3E6	5.3 ± 0.6E6	No
393	24.4	8.5 ± 0.3E6	7.3 ± 0.4E6	No
	30.1	1.5 ± 0.3E7	1.2 ± 0.2E6	No
419	22.5	0.9 ± 0.3E6	0.9 ± 0.3E6	No
	28.0	3.0 ± 0.3E6	2.7 ± 0.3E6	No
	29.6	1.2 ± 0.3E6	1.1 ± 0.3E6	No
444	26.7	4.0 ± 0.3E6	4.2 ± 0.3E6	No
875	26.7	3.4 ± 0.3E7	3.1 ± 0.3E7	No
471	26.7	8.1 ± 0.3E6	7.7 ± 0.3E6	No
739	34.5	6.9 ± 0.3E6	5.2 ± 0.3E6	No
723	34.4	1.3 ± 0.3E7	1.1 ± 0.3E6	No
721	34.4	4.0 ± 0.3E6	3.9 ± 0.3E6	No
745	34.5	5.1 ± 0.3E6	5.2 ± 0.3E6	No
699	34.5	7.2 ± 0.3E6	6.3 ± 0.3E6	No

n+SD=3, each performed in triplicate.

7.3. Identification of antioxidants using HPLC/MS^n

HPLC coupled with mass spectrometry has been widely used to identify phenolic compounds, and electronspray ionization (ESI) mass spectrometric liquid interface was thought to provide advantages in terms of sensitivity and capability to analyze large, thermally labile and highly polar compounds [11, 12]. In ESI-MS, deprotonated molecular ions represented the base peak in the negative ion spectra. In contrast, protonated molecular ions, sodium adducts and potassium adduct were the base peaks in the positive ion mode. In addition, tandem mass spectrometry can be further applied to obtain the structural information using collision induced dissociation (CID) of positive and/or negative parental ions.

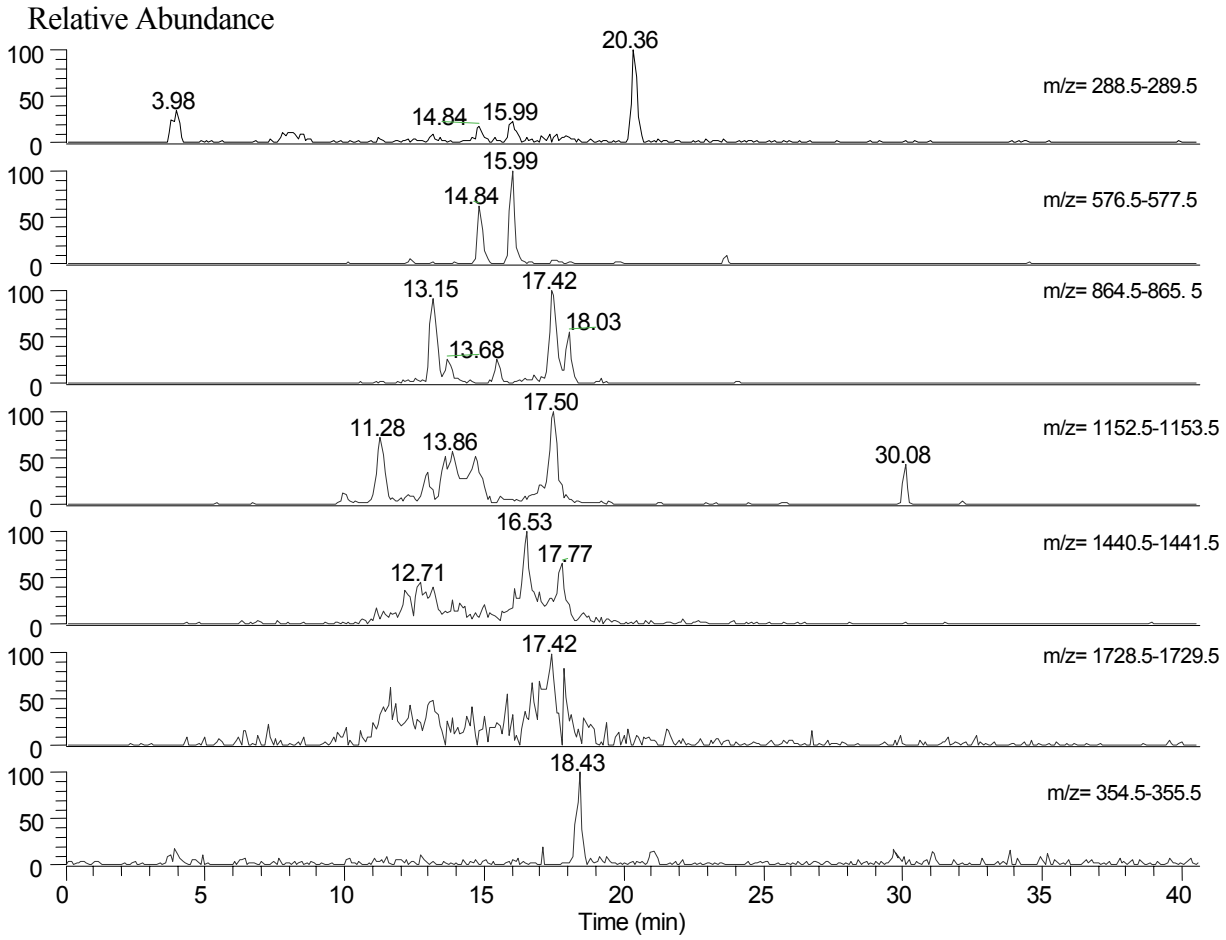

Fig.7.3. Extracted ion chromatograms for possible antioxidant ion peaks

Initial total ion chromatograms (TICs) of salak juice/extract, which is obtained by HPLC-ESI-MS at both positive and negative modes, indicated that many antioxidant peaks have molecular weights of 578, 866, 1154, 1442, 1731, respectively. The extracted ion chromatograms of these ions are shown in Fig. 7.3. They had molecular weights of [290×n - (n-1) ×2], which indicated they were proanthocyanidins that were formed by catechin and epicatechin units and had been proven to be excellent free radical scavengers [15, 16]. The interflavanic linkage usually occurs between C4 and C6 or between C4 and C8 (Fig. 7.4).

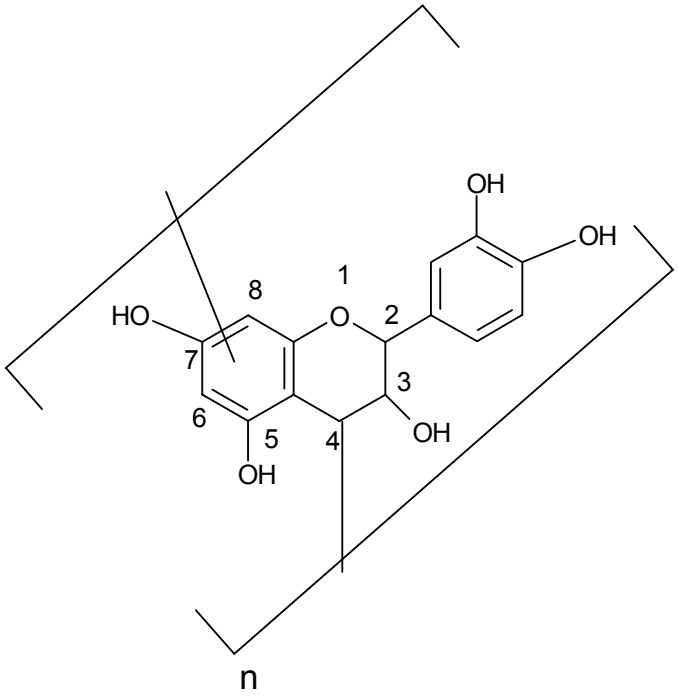

Fig. 7.4. Chemical structures of proanthocyanidins in salak extract (n=1-6)

To confirm the existence of proanthocyanidins, tandem mass spectrometry of those antioxidant peaks were further investigated. The CID spectra of these compounds are given in Table 7.2. The main fragment ions of parent ions at m/z 579 were m/z 427 $[M+H-152]^+$

from RDA-F of the heterocyclic rings, 409 $[M+H-170]^+$ from RDA-F of the heterocyclic rings and loss of water, m/z 291 $[M+H-288]^+$ from interflavanic bond cleavage. Similarly, fragment ions of m/z 867 included those at m/z 579 $[M+H-288]^+$ from interflavanic bond cleavage, m/z 715 $[M+H-152]^+$ from retro-Diels-Alder fission (RDA-F) of the heterocyclic, etc. For compounds with molecular weights of 1154, 1442 and 1730, their CID spectra underwent similar fragmentation pattern to those with molecular weights of 578 and 866 were observed. Their fragment patterns were the same as proanthocyanidins in star fruit and pycnogenol [14]. Therefore, these compounds were assigned as singly-linked proanthocyanidins existing as dimers through hexamers respectively. As shown in Fig. 7.2(a), another two peaks with ion m/z 1008.4 and 1296.2, respectively, were also significantly reduced after reaction with $ABTS^{\bullet+}$. They might arise from a doubly charged heptamer and nanomer, respectively. No significant peaks were observed in their extracted chromatograms probably due to the low concentrations in the extract. Because of the limitation of instrumentation on mass range i.e. 50-2000 Da, their parent ion could not be detected. Their charged states were also not observed by further zoom scan probably due to too low contents contained in sample.

Other than proanthocyanidins identified by tandem mass spectrometry, the intensities of the two other peaks were also significantly reduced after reaction with $ABTS^{\bullet+}$ for one hour. One peak had a retention time of 18.42 min and ions at m/z 355 $[M+H]^+$ and 353 $[M-H]^-$ respectively. Fragment ions m/z 179 and 191 from $[M-H]^-$ at m/z 353 indicated that the peak arose from the ester of caffeic acid with quinic acid. The peak was confirmed to be chlorogenic acid by spiking the extract with a standard sample. Another peak was found to be (-)epicatechin by spiking test and comparing its CID spectra with a standard.

Table 7. 2. Positive and negative ions and their corresponding CID ions of antioxidants

Rt (min)	ESI MS m/z			Major CID ion peaks of $[M\pm H]^{+/-}$
	Mode	$[M\pm H]^{+/-}$	$[M+Na]^{+}$	
14.83, 15.99	+	579	601	427, 409, 291, 289, 453,
	-	577		not investigated
13.15, 14.52, 15.37, 17.42,18.03	+	867	889	715, 697, 579, 577, 427, 409, etc
	-	865		not investigated
11.28, 13.17, 13.60, 14.80, 17.50	+	1155	1177	1003, 985, 867, 865, 715, 697, 579, 577, 427, 409, etc
	-	1153		not investigated
12.71, 16.53, 17.77	+	1443	1465	1291, 1273, 1155, 1153, 867, 865, 579, 577, etc.
	-	1441		not investigated
17.42	+	1731		1579, 1561, 1443, 1441, 1155, 1153, 867, 865, etc.
	-	1729		not investigated
18.42	+	355		163
	_	353		191, 179
20.4	+	291		123, 139, 165, etc
	_	289		245, 205, 179, etc

While using these ionisation techniques in HPLC/MS, some precautions were taken. Although $[M+H]^{+}$ or $[M-H]^{-}$ predominated in most compounds, mass spectrometry from ESI technique may include adduct ion peaks such as $[M+Na]^{+}$, $[M+K]^{+}$, $[M+M+Na]^{+}$, $[M+M+H]^{+}$, $[M+M-H]^{-}$ and multiple charged ions. Therefore, much attention was given when any of those additional peaks appeared in high relative abundance together with protonated or deprotonated ions. In such case, predominant ion peak(s) should be used for characterisation of free radical active components.

7.4. Reactivity of antioxidants with free radicals

Different antioxidants may exhibit different reaction types with free radicals because of existing differences in their chemical structures. Antioxidants, which react with free radicals rapidly, are considered to be more efficient. For example, after reacting with high

concentration of ABTS•$^{+}$ for 1 hour, around 30% of chlorogenic acid in extract remained unused. This indicated that the reaction between chlorogenic acid and ABTS•$^{+}$ is slow. While for (-)epicatechin, it was almost fully consumed after reaction with ABTS•$^{+}$ for one hour. Similarly, proanthocyanidins also reacted with ABTS•$^{+}$ rapidly. The results are consistent with kinetics of ABTS•$^{+}$ with (-)epicatechin and chlorogenic acid (Fig.7.5).

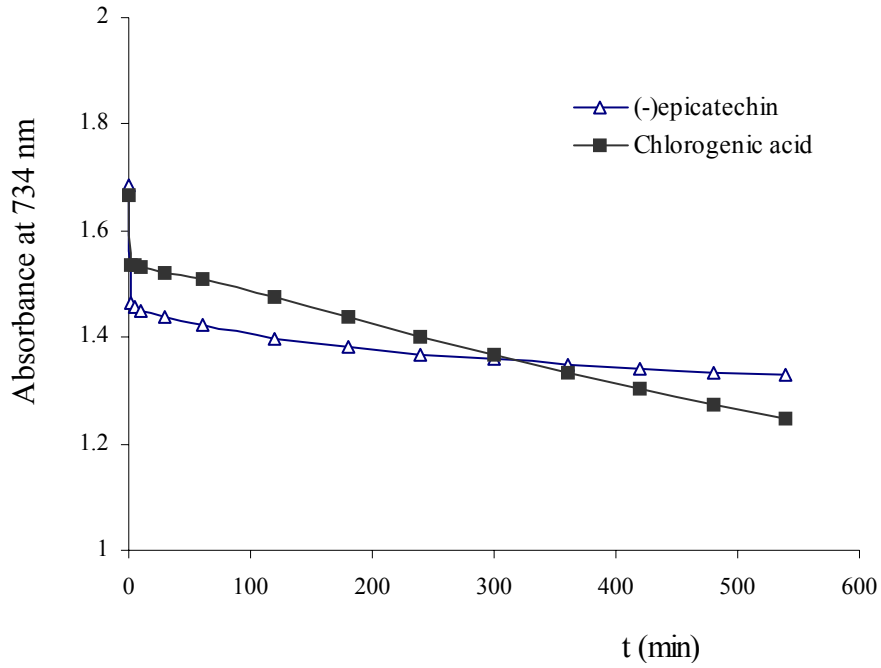

Fig. 7.5. Kinetics of ABTS·$^{+}$ with (-)epicatechin and chlorogenic acid. Reaction conditions: room temperature (~28ºC); 30 µL addition of 3.1 mM of (-)epicatechin and 30 µL addition of 3.3 mM of chlorogenic acid into 3 mL of 1.0 mM ABTS•$^{+}$ solution.

Although some peaks, eluted at stage 3 and 4 were slightly reduced when mixing with a high concentration of ABTS•$^{+}$, they were tentatively not considered as antioxidants because they were not efficient free radical scavengers even when compared with chlorogenic acid which reacted with free radical at a much slower rate than both (-)epicatechin and proanthocyanidins. Similar results were obtained when the solution was spiked with DPPH free radicals.

7.5 Chapter Summary

A new approach, which is based on HPLC/MS screening of free radical active components in biological samples and HPLC/MS^n structural elucidation of active components, was successfully applied to rapidly identify the major antioxidants in salak. Chlorogenic acid, (-)epicatechin, proanthocyanidins existed as dimers through hexamers were reported for the first time in salak and preliminarily considered as major antioxidants in salak. The new approach was successfully applied to identify antioxidants in ciku king and ulam raja, and also could be widely used for identification of antioxidants in other biological samples.

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Chapter 8

Antioxidants in Ciku King (*Manilkara Zapota*)

8.1. Introduction

The ciku king (Manilkara zapota), also known as sapodilla, is a small evergreen tree native to Mexico's tropical America and now is well spread throughout the tropics. The fruit ripens within 9 days at ambient temperature and spoils within 13 days after harvest [1]. In Singapore, fruits are normally eaten fresh, but the pulp has been reported to be incorporated into sherbets, milkshakes and ice-cream. As described in chapter 3, both unripe ciku and ciku king are found to possess extremely high antioxidant capacity that is not attributed by L-ascorbic acid, a constituent that is normally responsible for the antioxidant capacity in many fruits.

The unripe fruits of ciku are astringent to taste, probably due to their high tannin and polyphenolic content it contains [2]. In a recent study by Ma and co-workers, 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid were reported in ciku fruit [3]. The same author researchers also reported that ciku possesses high antioxidant capacity using the 1, 1'-diphenyl-2-picryl hydrazyl (DPPH•) scavenging assay and cytotoxicity in the HCT-116 and SW-480 human colon cell lines. 5-Caffeoyl-quinic acid was also detected in the peel and pulp of ripe ciku fruits [4]. Although unripe fruits possess high antioxidant capacities, they are not edible as they are sappy and extremely astringent. Leaving the unripe fruits to ripen is often necessary, so that the amount of reducing sugars increases, thereby increasing the fruits' sweetness while

allowing the astringency due to tannins and acidity caused by organic acids to decrease with time [5]. However, with the advancement of ripening of the fruit, texture of the fruit also changes to become softer due to pectin breakdown [1]. Ciku fruits are best eaten while the flesh is still firm and not mushy but sweet to taste, without too much astringency.

Ciku king, as one of the ciku family member, is more popular in the Singapore market probably due to its better taste and bigger size. As described in Chapter 3, unripe ciku king also possessed extremely high antioxidant capacity. But how much antioxidants are usually consumed from the fruit, and which components are responsible for its antioxidant activity remains unclear. In this study, the variation of total antioxidant capacity (TAC) and total phenolic contents (TPC) in ciku king fruit were determined with storage. An attempt is undertaken to estimate the best storage time to consume ciku king fruit with reasonable sensory attributes while maintaining a high antioxidant capacity of the fruit. Antioxidant components were further investigated using the approach described in Chapter 7.

8.2 Changes of TAC & TPC during storage

For all replicates, the total antioxidant capacity of ciku king fruits was found to follow a general decreasing trend with storage time, as fruits in the mature, ripened stage was reached (Fig. 8.1). The variation in the antioxidant capacity of fruits for different replicates can be understood due to natural variation, especially when cultivation conditions and stages at harvests are hard to ascertain as the fruits were obtained from

a supermarket. Hence it is not unusual for consumers to encounter fruits of a large variation of antioxidant capacity although the fruits were of the same size and a similar appearance. Also, a sudden fall in antioxidant capacity occurred around day 3 to 4, where the fruits started to ripen (Fig. 8.1). A trend similar to that of variation of TAC was also observed for the total phenolic content of ciku king fruits with storage time (Fig.8.2). This trend is also similar to the variation of tannin content in ciku fruits during the ripening process. Both Mohamed *et al.* [1] and de Brito & Narain [5] reported the decline of tannin content during the ripening process.

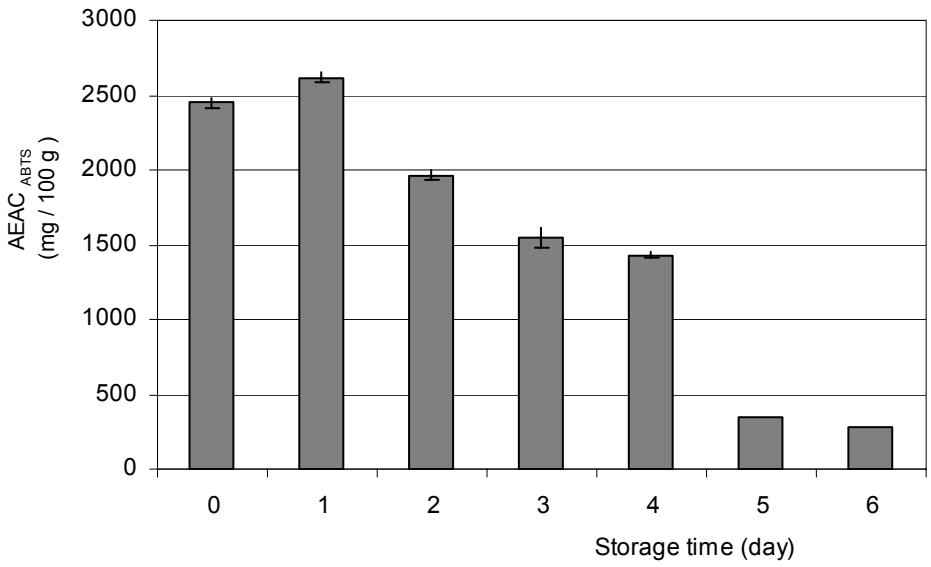


Fig. 8.1. Variation of total antioxidant capacity of ciku king fruits with storage time (days after purchase)

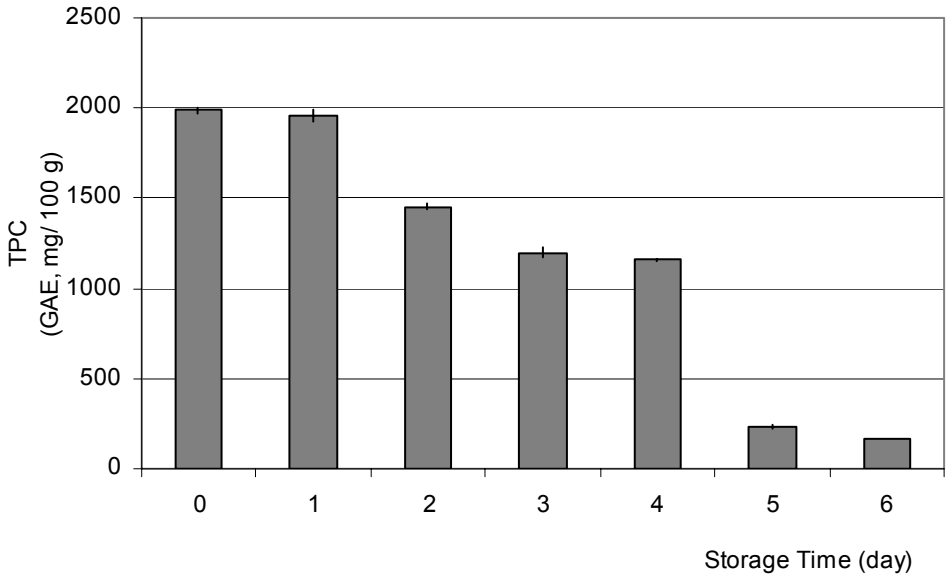


Fig. 8.2. Variation of total phenolics content of ciku king fruits with storage time

An excellent correlation between total antioxidant capacity and total phenolics content was obtained (Fig. 8.3). The good correlation indicated that phenolic compounds are the major antioxidants in ciku king fruits.

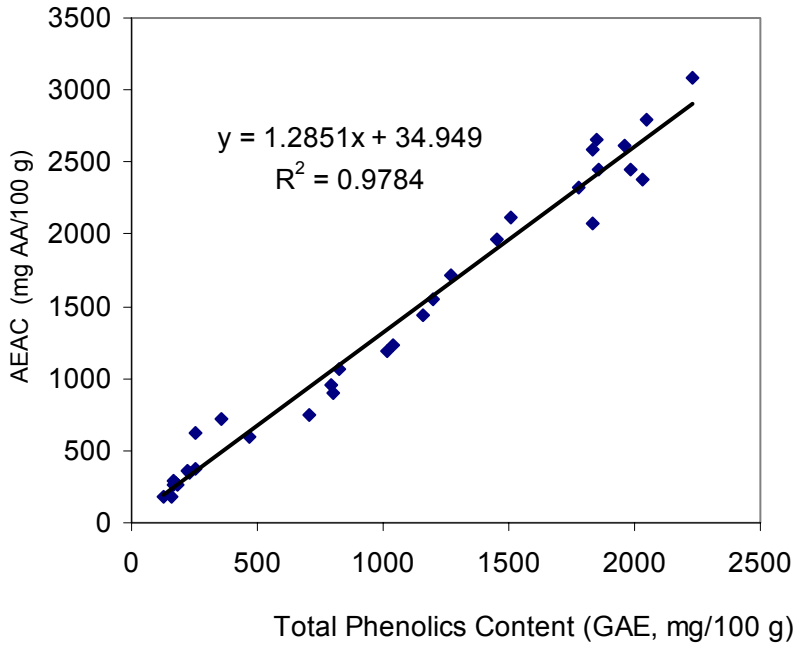


Fig. 8.3. Correlations between TAC and TPC

As both TAC and TPC of ciku king fruit decreased significantly during naturally ripening, consumption of fruits at different days will lead to the intake of different amounts of antioxidants. In this study it was found that the best time for consumption of ciku king fruits at a flavourful stage and still contain high amounts of antioxidants and total phenolics was 3rd or 4th -days of storage at room temperature. However, this storage time might not be exactly the same for all ciku king fruits because of the difference of cold storage time, harvest time, etc. For example, the best time for one batch of fruits was found to be 2 or 3 days of storage while 3 or 4 days storage was the best for other four batches. It was most likely that the best time for one to consume ciku king fruit was just-ripe-to-eat, and fruits at this stage still had extremely high antioxidant capacity ranging from 600 to 1200 mg AA_{eq} per 100 g flesh. Over-ripe ciku king fruits only had antioxidant capacity level with AEAC around 200 mg AA_{eq} per 100 g flesh.

8.3 Identification of antioxidants in ciku king using HPLC/MS^n

The previously-developed approach, which is described in Chapter 7, was applied for characterisation and identification of antioxidants in the extract of ciku king fruit.

By comparing mass spectral profile of the extract and that of the reaction solution of the extract with $ABTS^{\bullet+}$, a number of ion peaks were identified as antioxidant-related peaks. Those major antioxidant-related ion peaks were found to be eluted with retention times varying from around 6 to 19 min (Fig. 8.4) except that a very small amount of (-)epicatechin was eluted around 20.3 min. Fig. 8.5 shows extracted ion chromatograms for

those possible antioxidant ions in the extract. The extracted peaks of each antioxidant-related ion from both the extract and reaction solution of the extract with free radical was further compared to characterize antioxidant peaks.

It was found that not all the extracted peaks of antioxidant ions in Fig 8.5 were reduced after reacting with free radicals. Only those peaks which showed lowered intensities after mixing with $ABTS^{\bullet+}$ compared to control were considered as antioxidants and listed in Table 8.1. Table 8.1 summarises the retention times of antioxidants, m/z of their parent ions and their corresponding fragment ions from collision induced dissociation (CID).

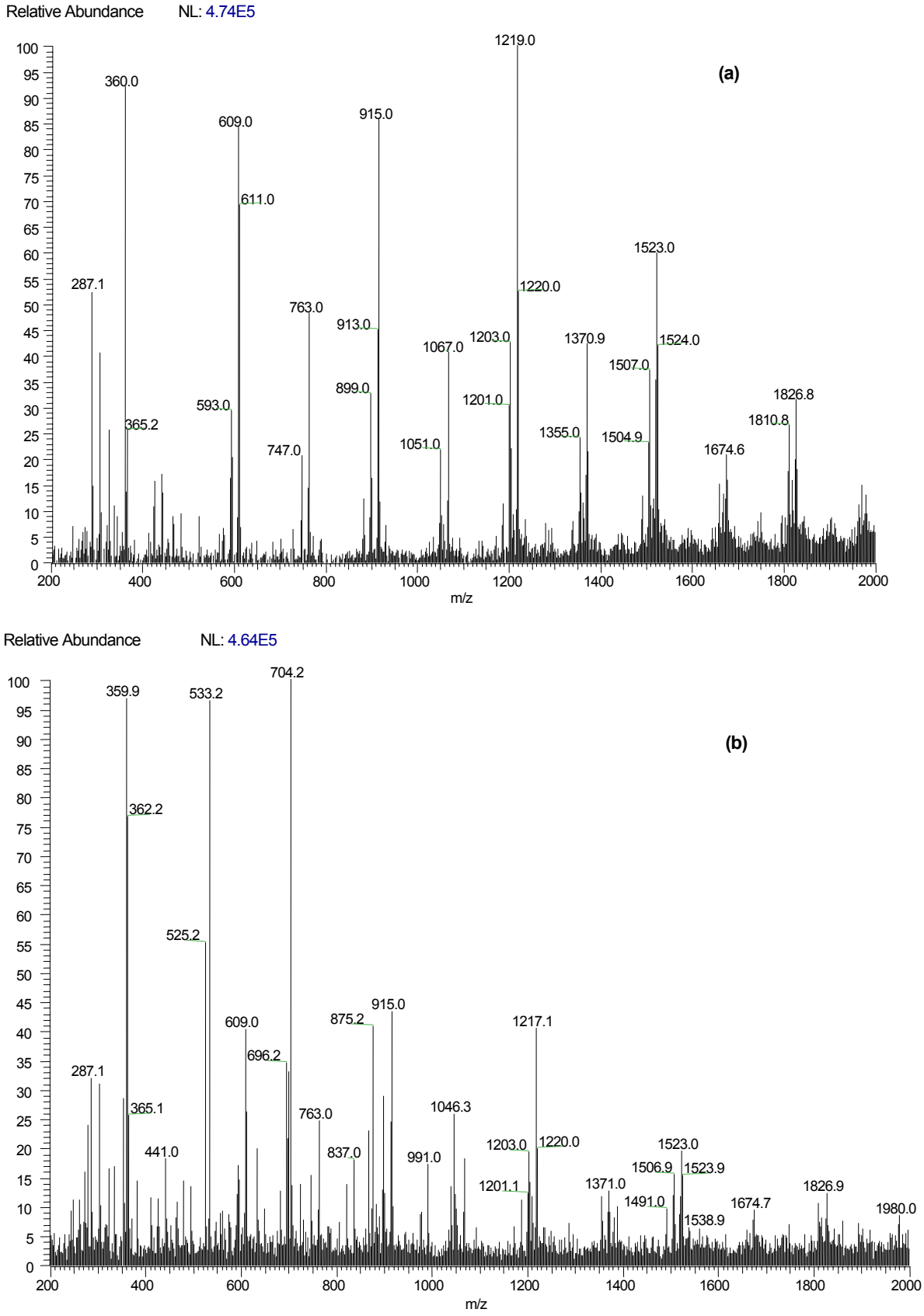
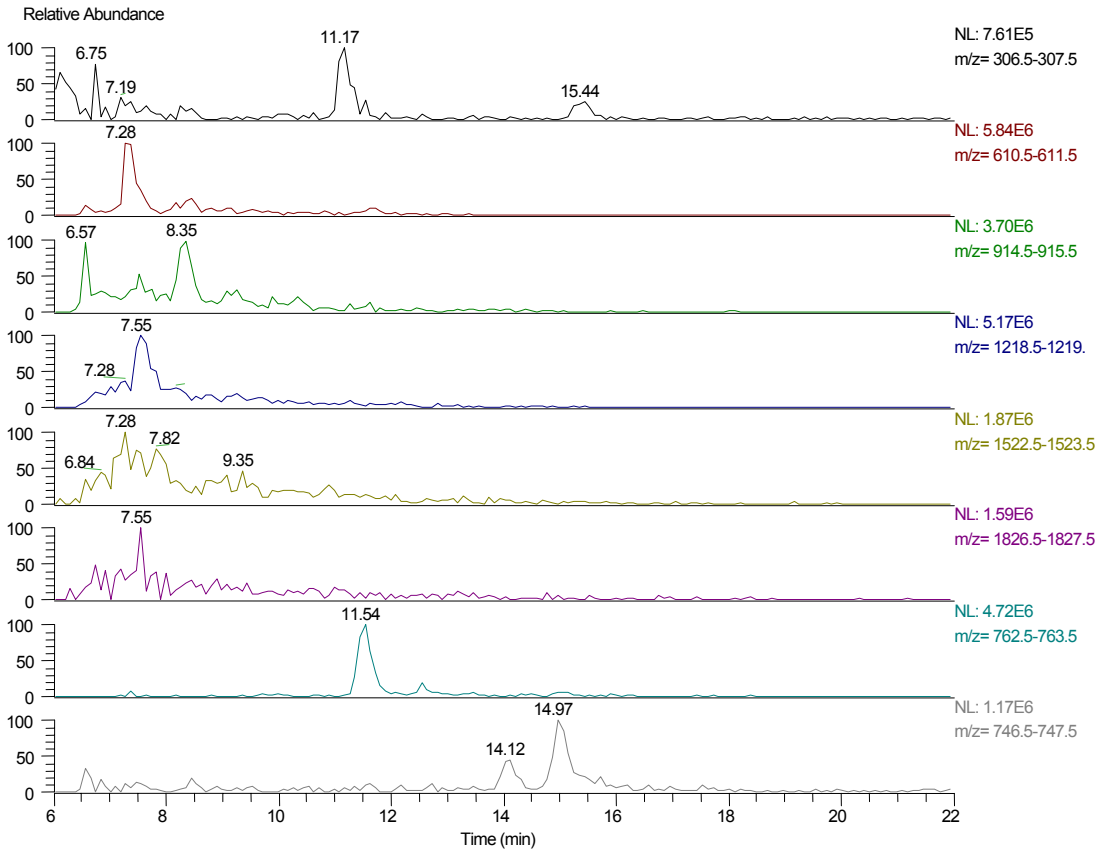
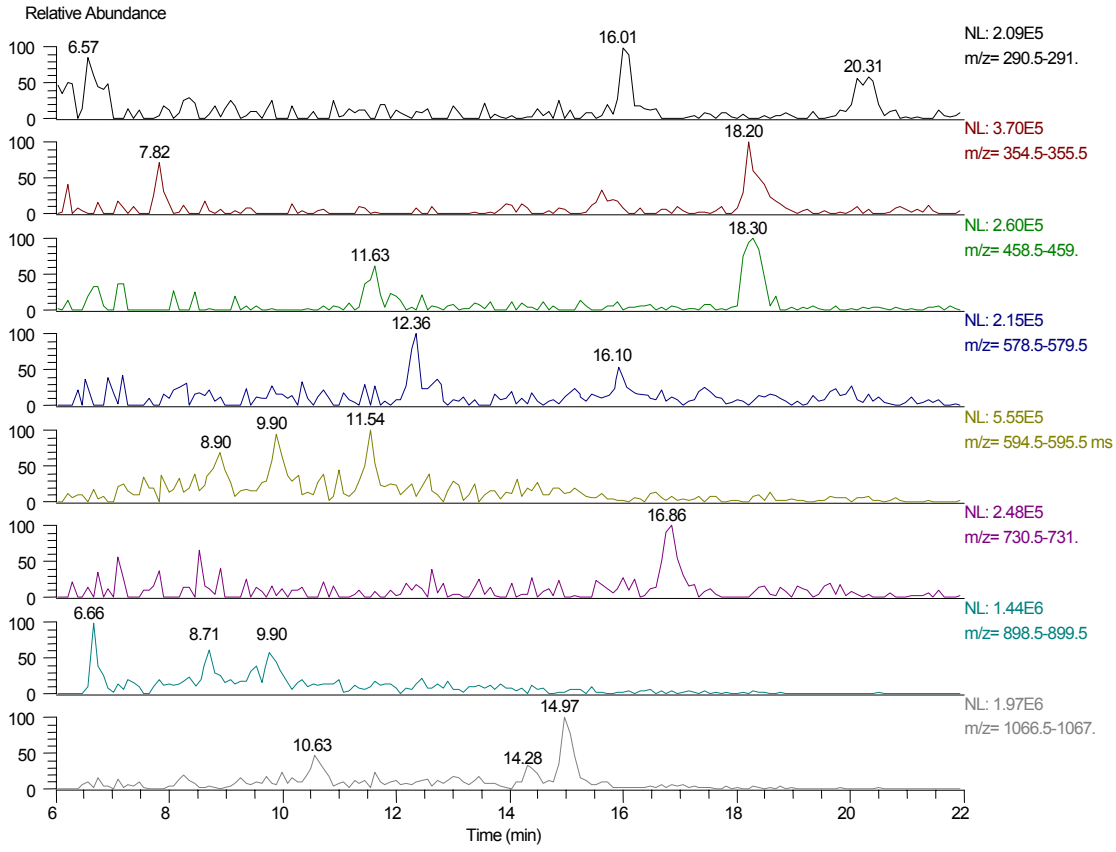


Fig. 8.4. ESI/MS profiles of antioxidant elution period (6-19 min). (a) Sample with water; (b) Sample with ABTS•$^{+}$.


Relative Abundance
NL: 7.61E5
m/z= 306.5-307.5
NL: 5.84E6
m/z= 610.5-611.5
NL: 3.70E6
m/z= 914.5-915.5
NL: 5.17E6
m/z= 1218.5-1219.
NL: 1.87E6
m/z= 1522.5-1523.5
NL: 1.59E6
m/z= 1826.5-1827.5
NL: 4.72E6
m/z= 762.5-763.5
NL: 1.17E6
m/z= 746.5-747.5
Time (min)


Relative Abundance
NL: 2.09E5
m/z= 290.5-291.
NL: 3.70E5
m/z= 354.5-355.5
NL: 2.60E5
m/z= 458.5-459.
NL: 2.15E5
m/z= 578.5-579.5
NL: 5.55E5
m/z= 594.5-595.5 ms
NL: 2.48E5
m/z= 730.5-731.
NL: 1.44E6
m/z= 898.5-899.5
NL: 1.97E6
m/z= 1066.5-1067.
Time (min)

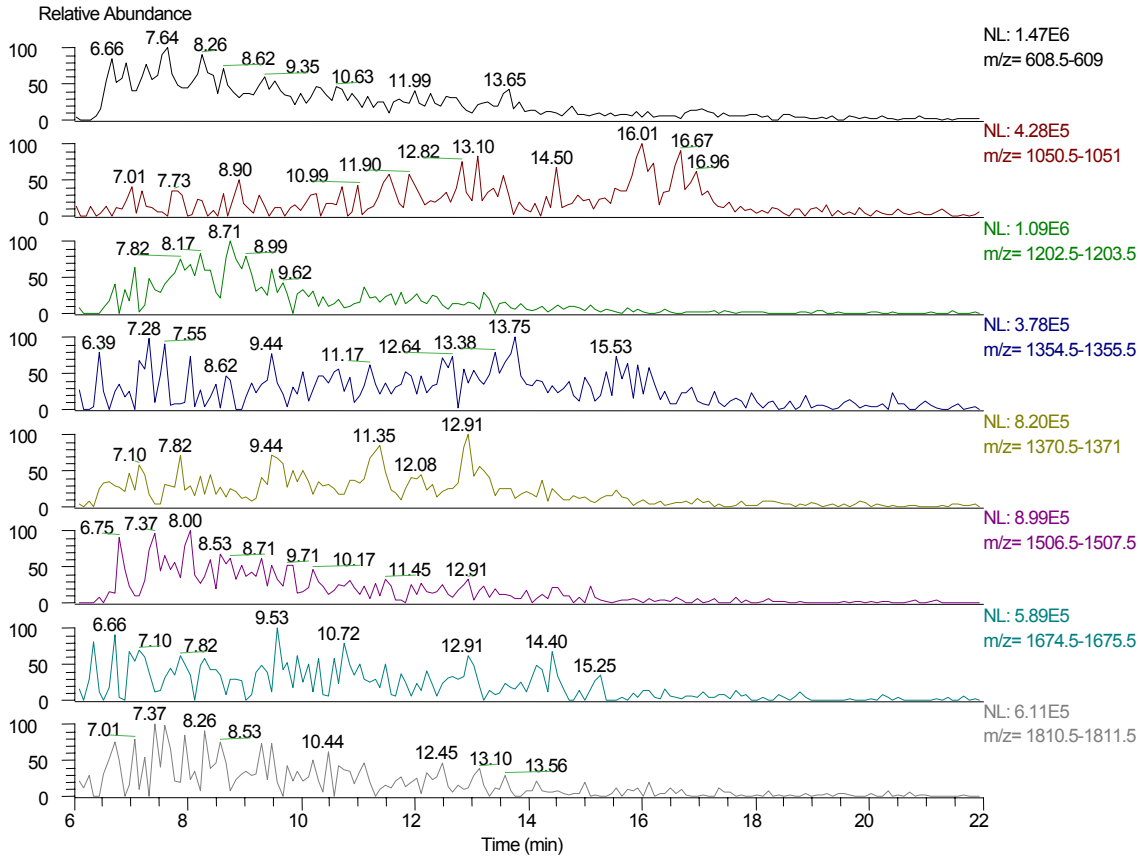


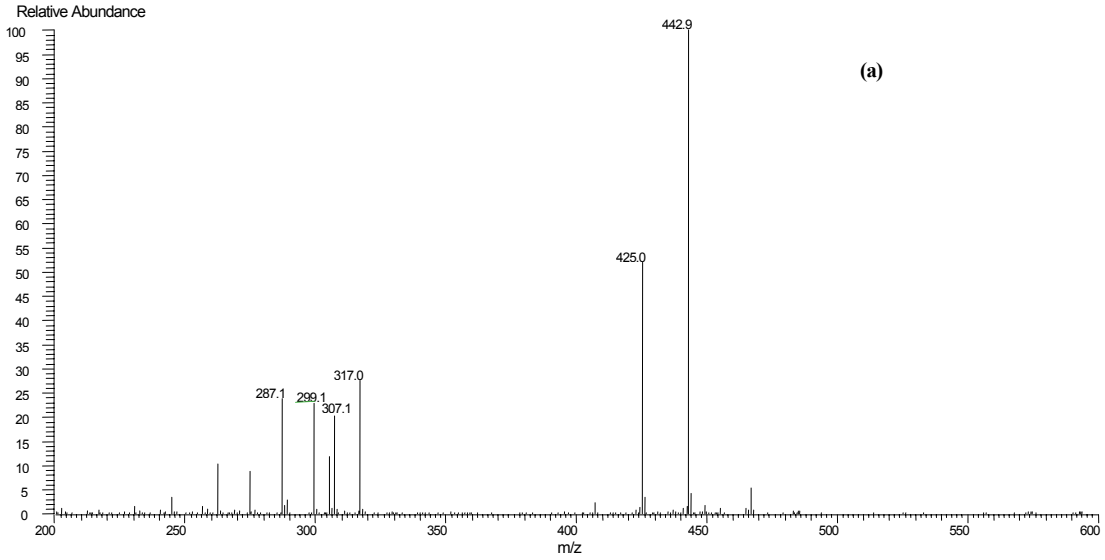
Fig. 8.5. Extracted chromatograms of antioxidant ion peaks in ciku king extract.

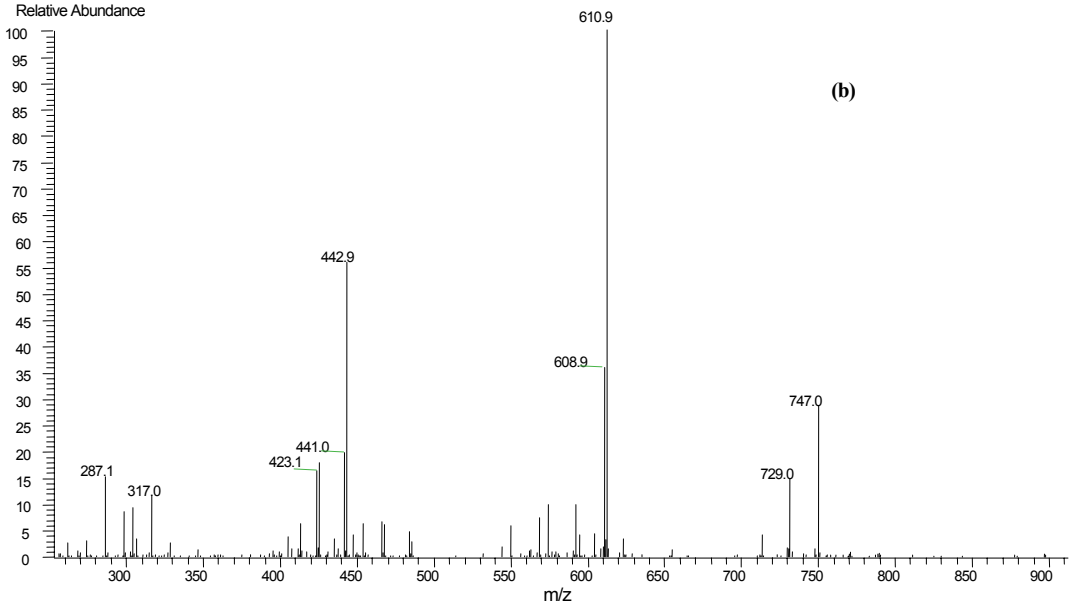
Table 8.1. Positive and negative ions and corresponding CID ions of some antioxidants

Rt (min)	ESI MS m/z		Major CID ion peaks of $[M\pm H]^{+/-}$
	Mode	$[M\pm H]^{+/-}$	
7.22 (**C1**)	+	611	443, 425, 317, 307, 299, etc
	-	609	/
7.46 (**C2**)	+	1219	611, 443, 915, 1051, 747, 745, 609, etc
	-	1217	/
6.57(**C3**), 8.33 (**C4**)	+	915	611, 443, 747, etc
	-	913	/
7.28 (**C5**)	+	1523	609, 611, 913, 915, 1217, 1219, 1353, 1355, etc.
	_	1521	/
7.55 (**C6**)	+	1827	911, 913, 1219, 1523, 1521 1657, 1659, 611, etc
	_	1825	/
6.66 (**C7**), 8.71 (**C8**), 9.90 (**C9**)	+	899	595, 593, 731, 611, 443, 427, 425, etc
	_	897	/
11.59 (**C10**)	+	763	425, 443, 595, 459, 317, 299, etc
	-	761	/
14.20 (**C11**)	+	747	595, 427, 409, 579, 459, 301, 247, etc
	_	745	/
15.08 (**C12**)	+	747	443, 425, 579, 299, 287, 317, etc
	_	745	/
10.63(**C13**), 14.28(**C14**), 14.97 (**C15**)	+	1067	763, 609, 899, 609, 443, 459, 425, etc
	_	1065	/
16.80 (**C16**)	+	731	409, 579, 427, 443, etc.
	_	729	/
18.35 (**C17**)	+	459	289, 151, 307, 271
	_	457	169, 331, 305, 287,269
16.17 (**C18**), 20.33 (**C19**)	+	291	139, 165, 123, 151, 273, etc
	_	289	/
11.17 (**C20**), 15.42 (**C21**)	+	307	139, 169, 181,289, 151
	_	305	/
12.34 (**C22**), 16.10 (**C23**)	+	579	427, 409, 453, etc
	_	577	/
18.20 **(C24)**	+	355	163
	_	353	191,179, 135, etc

Initial total ion chromatograms (TICs) of ciku king extract, which were obtained by HPLC-ESI-MS at both positive and negative modes, indicated that **C1** had a molecular weight of 610 [306×n - (n-1) ×2] Da. As shown in Table 8.1 and Fig. 8.6(a), the parent ion $[M+H]^+$ at m/z 611 gave main daughter ion peaks at m/z 443, 425, 307, etc. Further MS^3 results showed that daughter ions from ions at m/z 307 gave the same fragmentation pattern as gallocatechin. Main fragment ion at m/z 443 $[M+H-168]^+$ from RDA-F of the heterocyclic

rings (Fig. 8.7). Ion at m/z 425 arose from RDA-F of the heterocyclic rings and loss of water. As mass spectrometry cannot distinguish between stereoisomers, for the convenience of explanation, all ions which gave similar CID spectra to (+)gallocatechin will be addressed as gallocatechin. Thus, compound **C1** was assigned as a gallocatechin dimer. Its chemical structure is shown in Fig. 8.8 with n equal to 2.





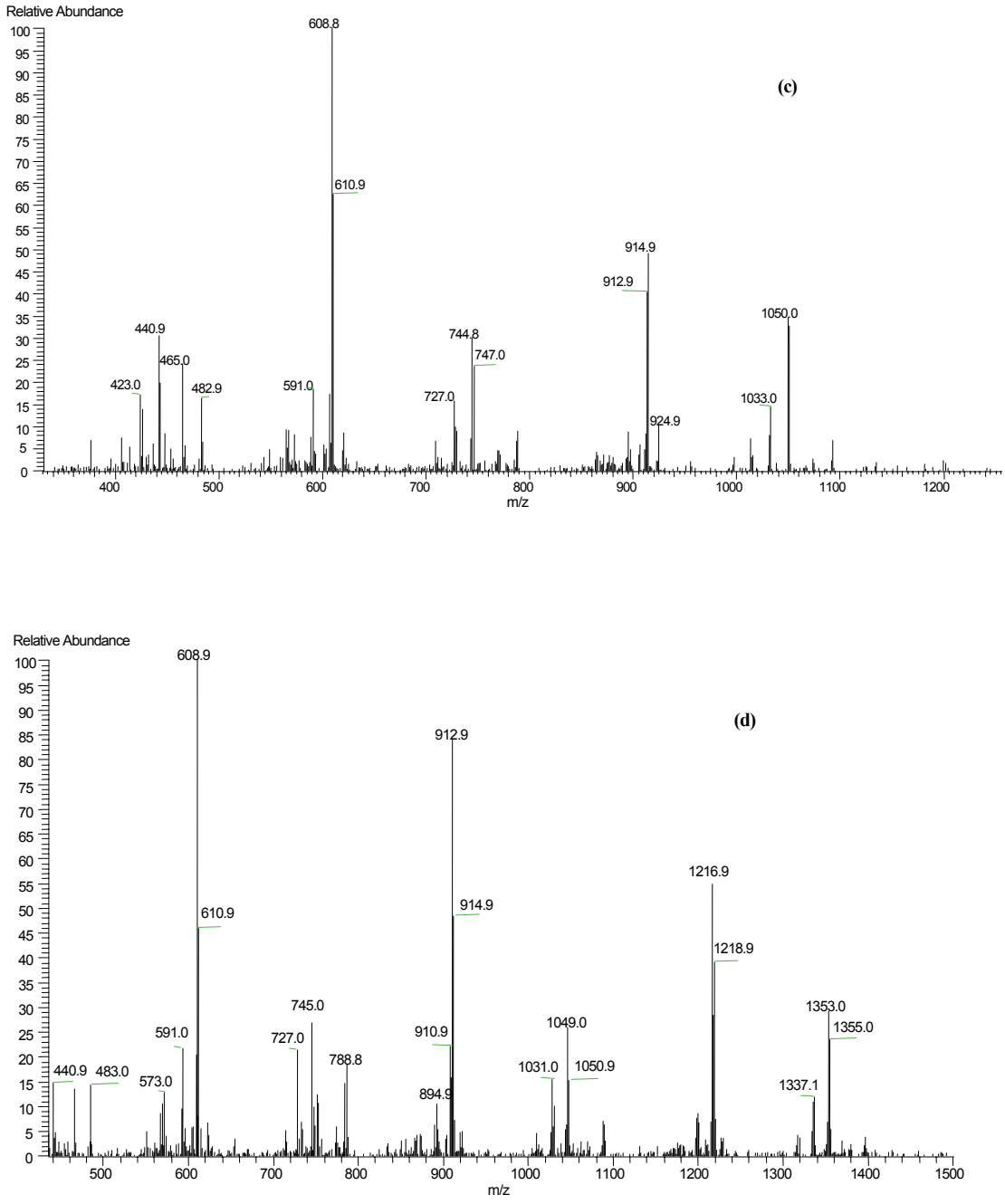


Fig. 8. 6. ESI-MS/MS mass spectra of gallocatechin dimers through pentamers at positive mode. (a) CID spectrum corresponds to the positive parent ion(s) at m/z 611 (MS/MS Scan); (b) CID spectrum corresponds to the positive parent ion(s) at m/z 915 (MS/MS Scan); (c) CID spectrum corresponds to the positive parent ion(s) at m/z 1219 (MS/MS Scan); (d) CID spectrum corresponds to the positive parent ion(s) at m/z 1523 (MS/MS Scan). Collision energy, 70%.

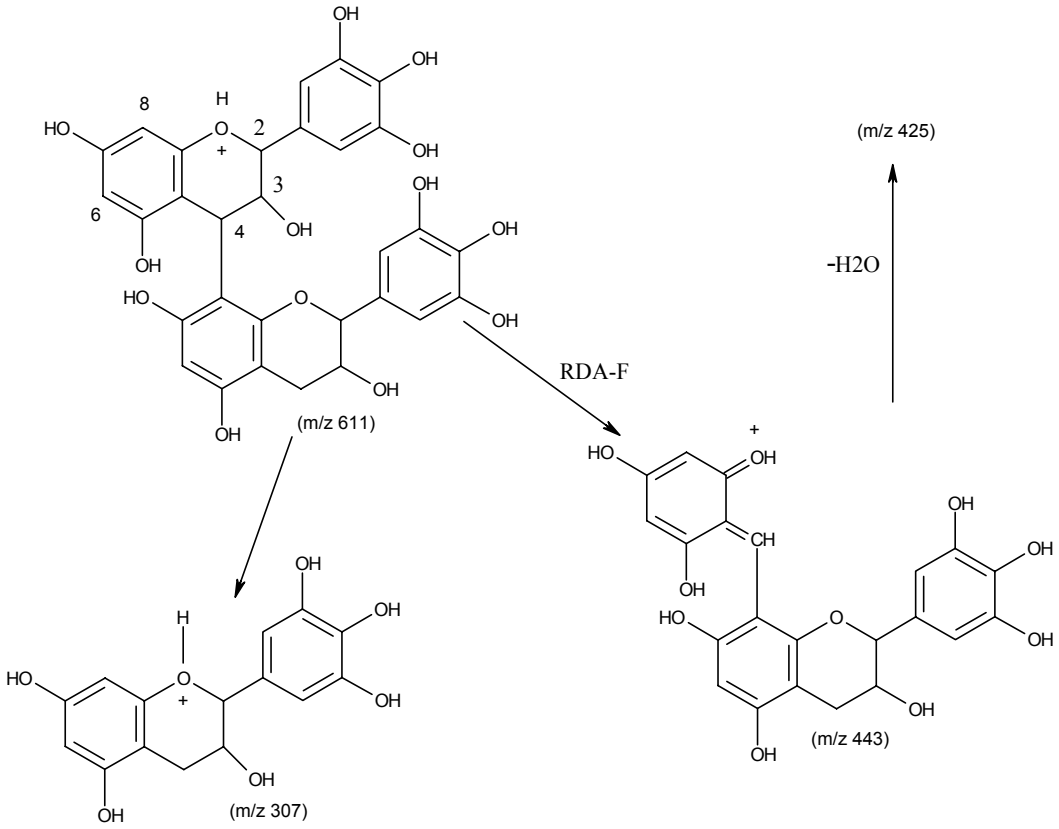


Fig. 8.7. Fragmentation patterns of gallocatechin dimer

Similarly, **C2** had a molecular weight of 1218 [306×n - (n-1)×2] Da. Fig. 8.6(c) and Table 8.2 showed the fragment ions of its parent ion at m/z 1219. The main fragment ions of parent ions at m/z 611 $[M+H-608]^+$ and 915 $[M+H-304]^+$ were from interflavanic bond cleavage, 1051 $[M+H-168]^+$ from RDA-F of the heterocyclic rings, 443 and 747 from interflavanic bond cleavage and followed by RDA-F of the heterocyclic rings, 609 $[M+H-610]^+$ from interflavanic bond cleavage following the quinone-methide mechanism. Therefore, compound **C2** was assigned to be a tetramer of gallocatechin. Its chemical structure is shown in Fig. 8.8 with n equal to 4.

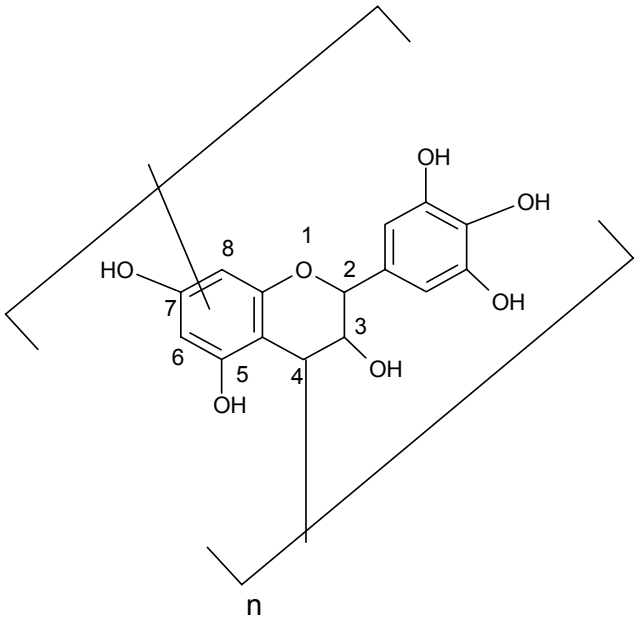


Fig. 8.8. Chemical structures of prodelphinidins with gallocatechin as basic units in ciku king extract (n=1-6)

Compounds **C3** and **C4** had a molecular weight of 914 [306×n - (n-1)×2] Da. Fig. 8.6(b) and Table 8.2 show the fragment ions of its parent ion at m/z 915. The main fragment ions of parent ions at m/z 611 $[M+H-304]^+$ were from interflavanic bond cleavage, 747 $[M+H-168]^+$ from RDA-F of the heterocyclic rings, 443 $[M+H-304-168]^+$ from interflavanic bond cleavage and followed by RDA-F of the heterocyclic rings, etc. Therefore, compounds **C3** and **C4** were assigned to be two trimers of gallocatechin. Their chemical structures are shown in Fig. 8.8 with n equal to 3

Compound **C5** and a few of its isomers with a molecular weight of 1522 [306×n-(n-1)×2] Da might exist in the extract of ciku king fruit although no significant extracted ion peaks were observed. Fig. 8.6(d) and Table 8.2 show the fragment ions of its parent ion at m/z 1523. The main fragment ions of parent ions at m/z 611 $[M+H-304\times3]^+$, 915$[M+H-304\times2]^+$, and 1219 $[M+H-304]^+$ were from

interflavanic bond cleavage, 609, 913 and 1217 due to interflavanic bond cleavage following the quinone-methide mechanism, etc. Therefore, compounds **C5** and a few of its isomers were assigned to pentamers of gallocatechin. Their chemical structures are shown in Fig. 8.8 with n equal to 5.

Compound **C6** had a molecular weight of 1826 [306×n - (n-1)×2] Da. Table 8.2 shows the fragment ions of its parent ion at m/z 1827. The main fragment ions of parent ions at m/z 915 $[M+H-304\times3]^+$, 1219 $[M+H-304\times2]^+$, 1523 $[M+H-304]^+$ were from interflavanic bond cleavages, 913, 1217 and 1521 due to interflavanic bond cleavage following the quinone-methide mechanism, etc. Therefore, compounds **C6** and a few of its isomers were assigned to be a haxamer of gallocatechin. Its chemical structure is shown in Fig. 8.8 with n equal to 6.

Compounds **C7, C8** and **C9** had the same molecular weight, and their CID spectra were similar to parent ions at m/z 899. The main fragment ions of parent ions at m/z 611 $[M+H-288]^+$ and 595 $[M+H-304]^+$ from interflavanic bond cleavages, 593 $[M+H-306]^+$ due to interflavanic bond cleavage following the quinone-methide mechanism, 731 $[M+H-168]^+$ from RDA-F of the heterocyclic rings, 443 $[M+H-288-168]^+$ and 427$[M+H-304-168]^+$ from interflavanic bond cleavages and RDA-F of the heterocyclic rings, etc. were noted. Therefore, compounds **C7, C8** and **C9** were assigned to be three trimers constituted of two gallocatechins and one catechin.

Compound **C10** had a molecular weight of 762 Da. Its CID spectra from parent ions at m/z 763 are shown in Table 8.2. The main fragment ions of parent ions at

m/z 443[M+H-168-152]$^+$ RDA-F of the heterocyclic rings and loss of gallyol part, 425[M+H-168-152-18]$^+$ from RDA-F of the heterocyclic rings and losses of gallyol part and water, 459 [M+H-304]$^+$ from interflavanic bond cleavage, etc. Therefore, compounds **C10** was assigned to be a dimer constituted of one gallocatechin and one gallocatechin gallate (Fig. 8.9).

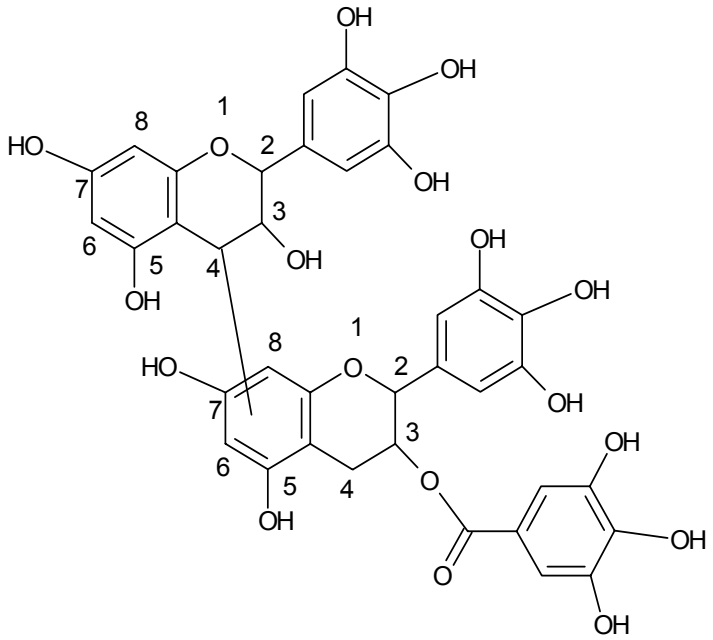


Fig. 8.9. Chemical structures of a dimer constituted of a gallocatechin and a gallocatechin gallate (Compound **C10**)

Compound **C11** had a molecular weight of 746 Da. Its main fragment ions were at m/z 595 $[M+H-152]^+$ from RDA-F of the heterocyclic ring of catechin moiety, 459$[M+H-288]^+$ from interflavanic bond cleavage, 579 $[M+H-168]^+$ possibly from RDA-F of the heterocyclic ring of gallocatechin gallate moiety or from RDA-F of the heterocyclic ring of catechin moiety and loss of water, 427 might arise from RDA-F of the heterocyclic ring of catechin moiety from parent ions m/z 579, etc. Therefore, compound **C11** was assigned to be a dimer constituted of one catechin and one gallocatechin gallate.

Compound **C12** had a molecular weight of 746 Da. Its main fragment ions at m/z 443 $[M+H-304]^+$ from interflavanic bond cleavage, 579 $[M+H-168]^+$ from RDA-F of the heterocyclic ring of gallocatechin moiety, 425 from RDA-F of the heterocyclic ring of gallocatechin moiety plus loss of H_2O, etc. Therefore, compound **C12** was tentatively assigned to be a dimer constituted of one gallocatechin and one catechin gallate.

Compounds **C13, C14** and **C15** had molecular weight of 1066 Da. Their main fragment ions at m/z 763 $[M+H-304]^+$ from interflavanic bond cleavage, 899 $[M+H-168]^+$ from RDA-F of the heterocyclic rings, 609 $[M+H-306-152]^+$ from interflavanic bond cleavage following the quinone-methide mechanism, etc Therefore, compounds **C13, C14** and **15** were assigned to be three trimers constituted of two gallocatechins and one gallocatechin gallate.

Compound **C16** had a molecular weight of 730 Da. Its main fragment ions at m/z 579 $[M+H-152]^+$ from loss of gallyol moiety or RDA-F of the heterocyclic ring of

catechin moiety, 427 $[M+H-152-152]^+$ from RDA-F of the heterocyclic ring of catechin gallate moiety, 409 from RDA-F of the heterocyclic ring of gallocatechin moiety plus loss of H_2O, etc. Therefore, compound **C16** was tentatively assigned to be a dimer constituted of one catechin and one catechin gallate.

Compound **C17** had a molecular weight of 458 Da. Its main fragment ions at m/z 289 $[M+H-152-18]^+$ from losses of gallyol moiety and water, 307 $[M+H-152]^+$ from losses of gallyol moiety, etc. It was assigned to be (-) epigallocatechin gallate by further spiking and comparing its CID spectra with authentic standard.

Compounds **C18** and **C19** were assigned to be (+)catechin and (-)epicatechin respectively by spiking and comparing its CID spectra with standards. Compounds **C20** and **C21** had a molecular weight of 306 Da. By spiking and comparing their CID spectra with authentic standard, compounds **C20** and **C21** were assigned to be (-)gallocatechin and (-)epigallocatechin, respectively.

Compounds **C22** and **C23** had molecular weight of 578 Da. Their main fragment ions at m/z 427 $[M+H-152]^+$ from RDA-F of the heterocyclic rings, 409 $[M+H-170]^+$ from RDA-F of the heterocyclic rings and loss of water, etc. Their fragmentation patterns indicated that they were two catechin dimers.

Compound **C24** had a molecular weight of 354 Da. Its parent ion at m/z 353 gave fragment ions at m/z 191 $[\text{quinic acid-H}]^-$ and 179 $[\text{caffeic acid-H}]^-$, which were generated by cleavage of the ester bond and consistent with the structures of caffeoylquinic acid derivatives. Compound **C24** was assigned to be chlorogenic

acid (5-O-caffeoyl quinic acid) by spiking and comparing its CID spectra with authentic standard.

Other than the above compounds, which had at least one distinct ion peak, the intensities of several other ion peaks e.g. ions at m/z 609, 913 and 593 were also significantly reduced after reacting with $ABTS^{\bullet+}$, however, as shown in Fig 8.5, no obvious corresponding ion peaks were observed in ion extracted chromatograms. They might not come from a compound itself but from fragment ions of other bigger molecular ions such as ions at m/z 915, 1067, 1219, etc. Similarly, no obvious peaks were observed for antioxidant ions at m/z 1051, 1203, 1355, 1371, 1507, 1675 and 1811, which shows quite relatively high peak intensities in Fig. 8.4. These ions might arise from fragmentation of other bigger molecular ions. The relatively high intensities of these ions in Fig. 8.4 might also be an overall reflection of the sum of a number of isomers of each ion. Their corresponding CID spectra from parent ions at m/z $[M+H]^+$ indicted that they were proanthocyanidins with basic units of gallocatechin or catechin or both. In addition, although three antioxidant ions at m/z 595 with low peak intensities and retention times of 8.90, 9.90 and 11.54 were observed, they were tentatively considered as fragment ions of other main antioxidant ions at m/z 899, 899 and 763 respectively. They might also arise from dimers constituted of one catechin and one gallocatechin, but this needs to be confirmed under improved chromatographic separation such as using normal phase HPLC.

Except for those compounds **C16-C20**, which had been confirmed with authentic standards, the chemical structures and stereochemistry of other compounds still need to be elucidated by isolation of individual compounds for NMR assay.

The peak intensities of major antioxidants were also found to decrease with storage time. This was consistent with results of section 8.2, which indicated that both antioxidant capacity and total phenolic content decreased with storage time.

8.4 Chapter Summary

The changes of antioxidant capacity and total phenolics of ciku king with storage time decreased significantly during the days from unripe to overripe stages. The best time for one to consume ciku fruits at a flavourful stage and yet still able to acquire high amounts of antioxidants and total phenolics was suggested to be just-ripe-to eat. The change of the content of major antioxidant peaks during storage was consistent with changes of antioxidant levels. The possible structures of 24 antioxidants were proposed using high performance liquid chromatography and mass spectrometry (HPLC/MS) and HPLC/MS/MS. Polyphenolics with basic blocks of gallocatechin, catechin or both were found to exist widely in the extract of ciku king fruit and were preliminarily considered as its major antioxidants.

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Chapter 9

Antioxidant in Ulam Raja (*Cosmos caudatus*)

9.1 Introduction

Ulam raja (*Cosmos caudatus*) is an annual, short-lived, perennial, aromatic herb. It originated from tropical Middle America and is now widespread in almost all tropical regions. Ulam raja is recommended in the traditional medicine for improving blood circulation. Its young leaves are often eaten raw with chilli or coconut paste and are used in dishes such as kerabu. They are also used as an appetiser and food flavouring due to their unique taste and aroma.

Ragasa and co-workers have reported several antimutagen and antifungal compounds from ulam raja, e.g. cotunolide, stigmasterol, lutein and 4, 4'-bipyridine[1]. In addition, Zanariah and co-workers have reported protein and amino acid compositions of ulam raja [2]. As described in Chapter 3, it was found that ulam raja had extremely high antioxidant capacity of about 2,400 mg AEAC/100 g of fresh sample. Its antioxidant capacity was much higher than that of any other vegetables tested in this study. However, it is still not clear which compounds are responsible for its antioxidant activity. The main objective of this study was to identify those antioxidants using the approach described in Chapter 7.

9.2 Free radical active components in ulam raja

Fig 9.1 shows HPLC chromatograms of UV and mass spectral data, respectively. Numbers of peaks from total ion chromatograms (TICs) was much more than those from UV detector. This indicated that mass detector were more sensitive than UV detector for those compounds only appearing in TICs. To investigate which peaks contained antioxidants, the intensities of individual ion peaks at four elution periods were compared before and after reacting with $ABTS^{\bullet+}$. Fig. 9.2 to 9.5 shows the intensities of individual peaks of both control and reaction products at elution period 1 (0-10 min), 2 (10-20 min), 3 (10-20 min) and 4 (30-40 min), respectively.

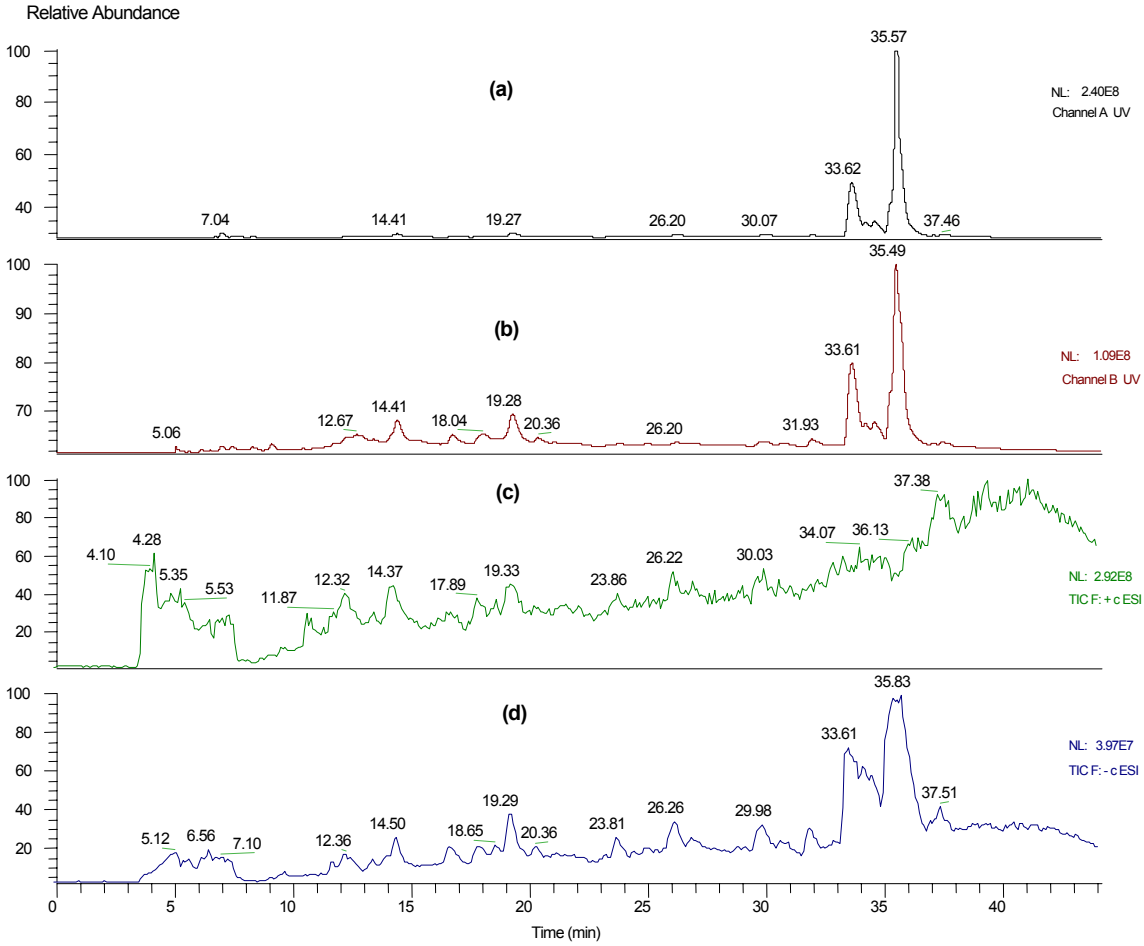

Fig. 9.1. HPLC chromatograms of ulam raja extract (a) UV at 250 nm; (b) UV at 280 nm; (c) TIC at positive mode; (d) TIC at negative mode..

Fig.9.2 shows that, at elution period 1, only the intensity of ion peaks at m/z 1155 was significantly reduced from 1.76×10^5 ($6.78\times10^6\times26\%$) in Fig 9.2(a) to less than 4.2×10^4 ($2.12\times10^6\times2\%$) in Fig 9.2(b) while no obvious decrease for other ion peaks was observed. Extract ion chromatogram of m/z 1155 in the control and reaction solution were compared to characterise antioxidant peaks.

Fig. 9.3 shows that, at elution period 2, the intensity of ion peaks at m/z 579, 867, 1155, 1443 and 1731 were significantly reduced from 1.19×10^6 ($1.75\times10^6\times68\%$),

1.75×10^6, 1.37×10^6 ($1.75\times10^6\times78\%$), 1.09×10^6 ($1.75\times10^6\times62\%$) and 0.56×10^5 ($1.75\times10^6\times32\%$) respectively to less than 2.4×10^5 ($1.20\times10^6\times20\%$) for all ion peaks. Two ion peaks at m/z 355 and 731 were found to be reduced from 7×10^5 ($1.75\times10^6\times40\%$), and 6.6×10^5 ($1.75\times10^6\times38\%$) to 5×10^5 ($1.20\times10^6\times42\%$) and 3.4×10^5 ($1.20\times10^6\times28\%$) respectively. Therefore, extract ion chromatogram at m/z 355, 731, 579, 1155, 1443, and 1721 in control and reaction solution were compared to identify antioxidant peaks.

Intensities of major ion peaks from extract at m/z 927, 949, 765, 603 and 595 were almost not reduced after reaction with free radicals (Fig. 9.4). However, intensities of ion peaks at m/z 433 and 355 were reduced. Extract ion chromatogram of ions at m/z 433 and 355 in the control and reaction solution were compared to identify antioxidant peaks.

Fig. 9.5 shows that the intensity of ion peaks at m/z 303, 465, 435, 449, 433, 611 and 891, 919, and 951 were significantly reduced after reaction with free radicals. Therefore, extract ion chromatogram at m/z 303, 465, 435, 449, 433, 611 and 891, 919, and 951 in the control and reaction solution were compared to identify antioxidant peaks.

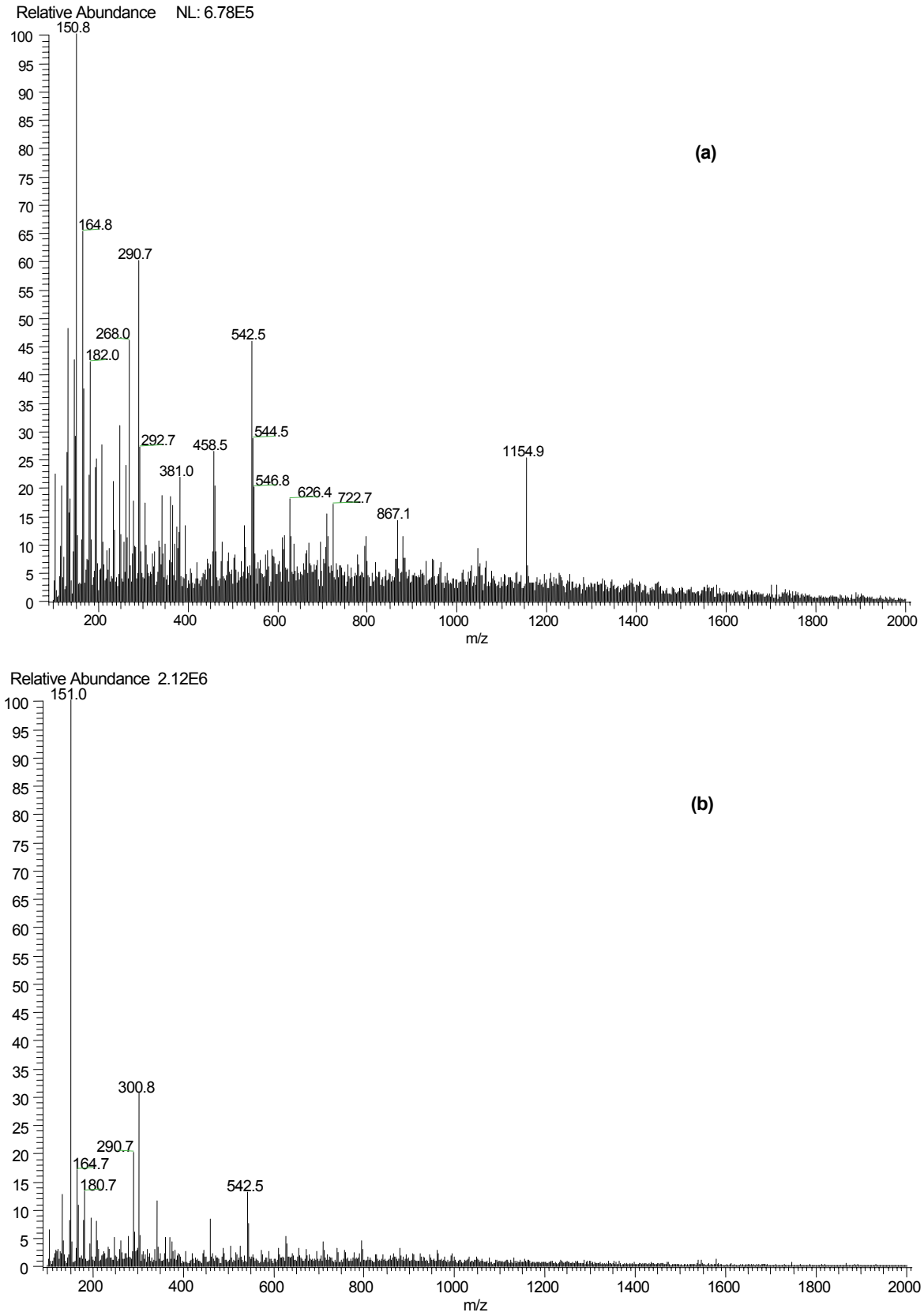

Fig. 9.2. ESI/MS profiles of elution period 1 (0-10 min). (a) Sample with water; (b) Sample with $ABTS^{\bullet+}$

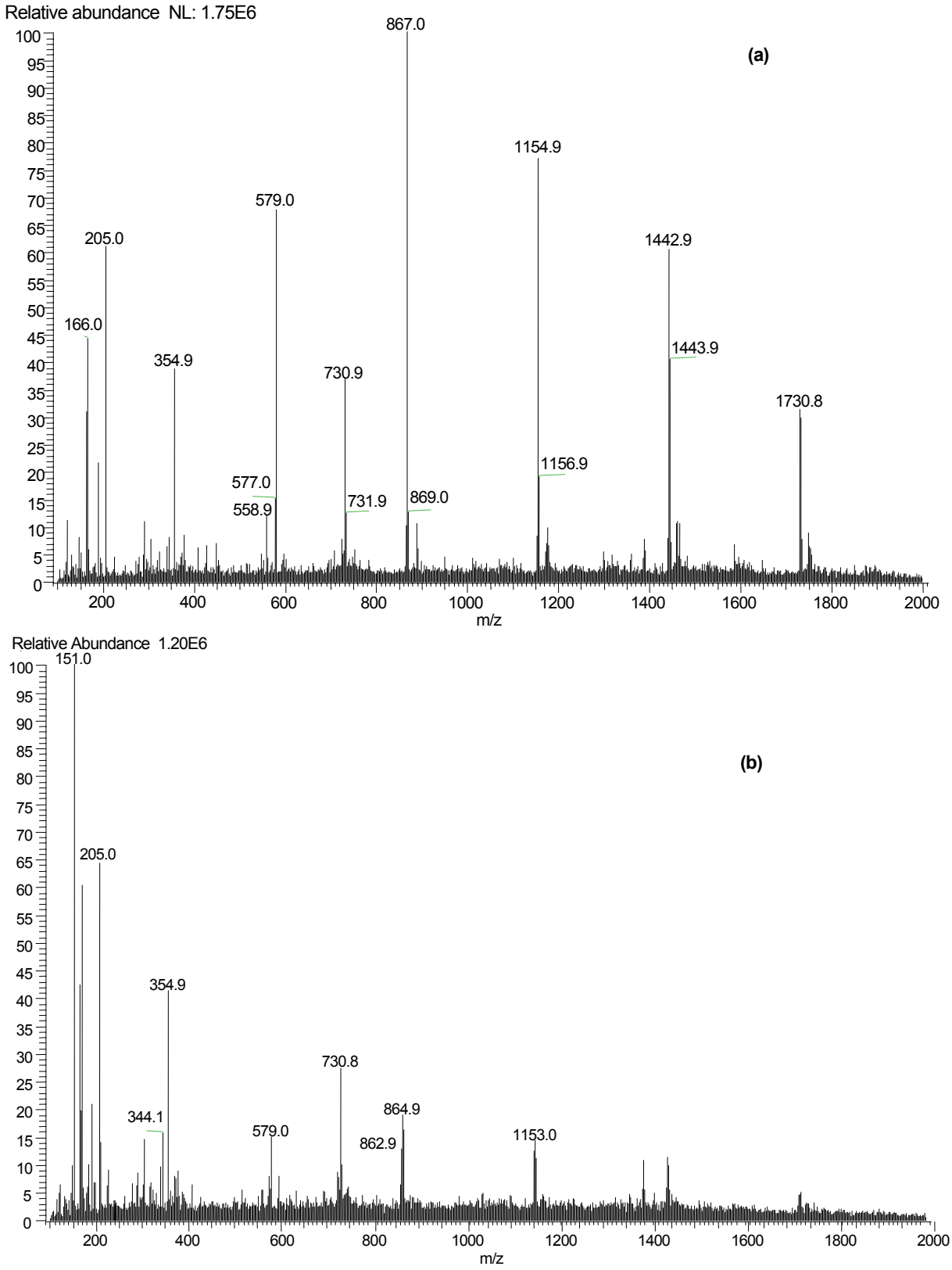

Fig. 9.3. ESI/MS profiles of elution period 2 (10-20 min). (a) Sample with water; (b) Sample with ABTS$^{\bullet+}$

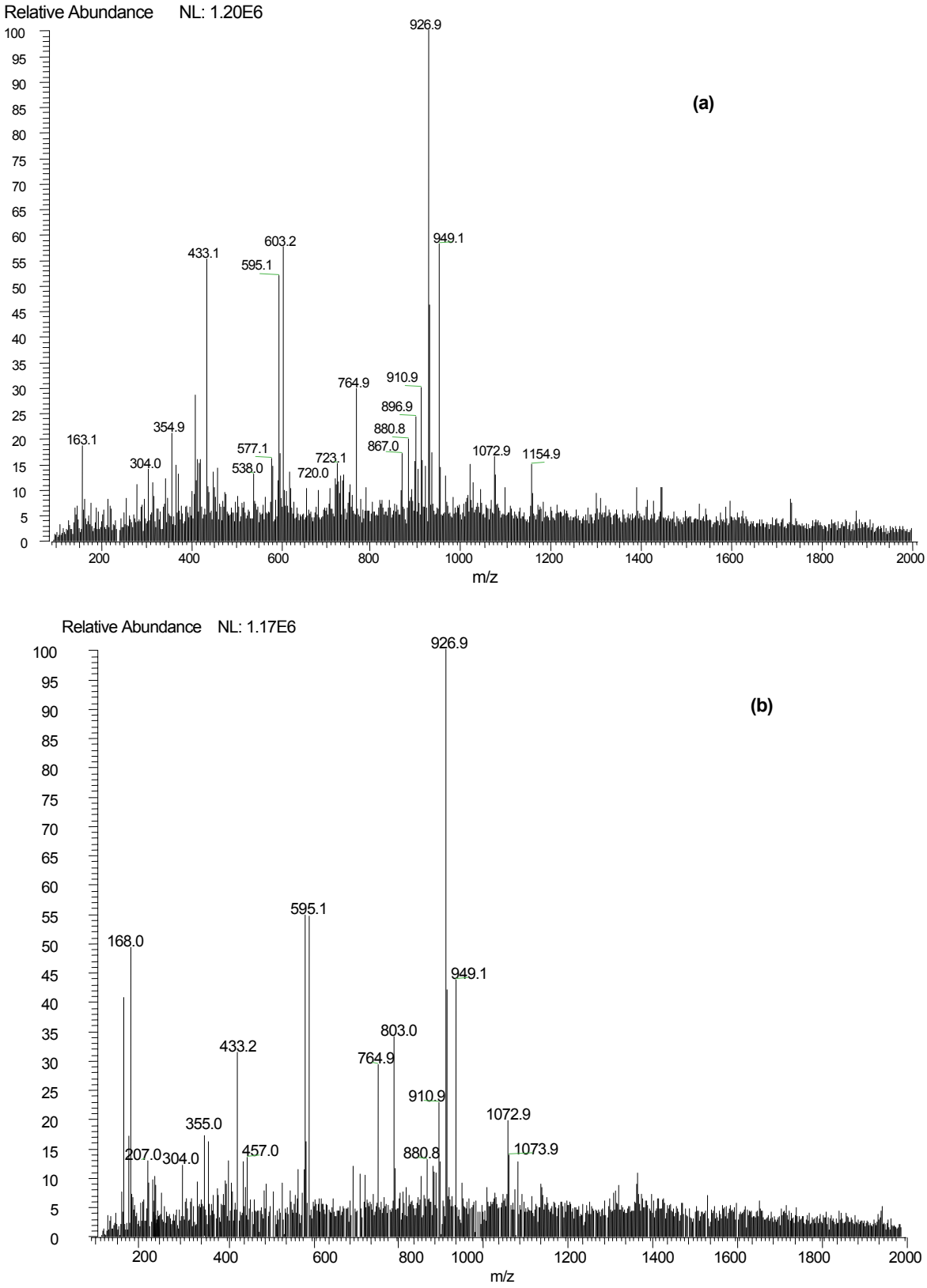

Fig. 9.4. ESI/MS profiles of elution period 3 (20-30 min). (a) Sample with water; (b) Sample with $ABTS^{\bullet+}$

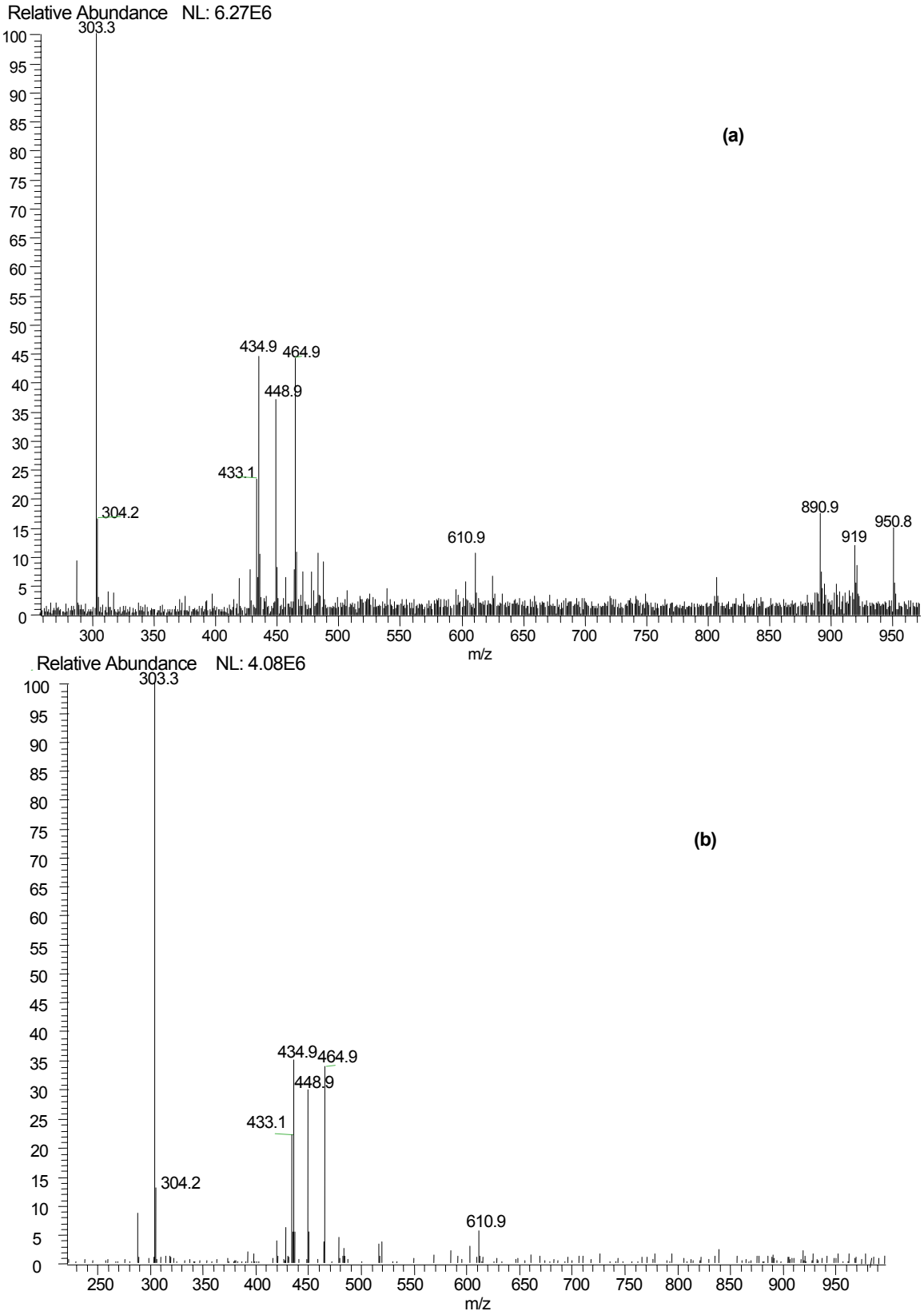

Fig. 9.5. ESI/MS profiles of elution period 4 (30-40 min). (a) Sample with water; (b) Sample with ABTS•$^{+}$

Table 9.1 shows the profiles of antioxidant peaks in ulam raja extract, which were characterised from extracted ion chromatograms of extract of ulam raja and reaction solution extract with free radicals. Other ions, which had the same signal of m/z as some of antioxidant peaks, were not listed in the table as their intensities were not reduced and therefore were not characterized as antioxidants. Other than those antioxidant ion peaks listed in Table 9.1, several other ion peaks at m/z 731 (Fig. 9.3), 891, 919 and 951(Fig. 9.5) were also identified as antioxidant peaks. However, they arose from other antioxidant peaks at m/z 355 $[M+H]^+$, 435$[M+H]^+$, 449$[M+H]^+$ and 465$[M+H]^+$, respectively. Therefore, their tandem mass spectra are not discussed in detail.

Table 9.1. Positive and negative ions and their corresponding CID ions of antioxidants

Rt (min)	ESI MS m/z		Major CID ion peaks of $[M\pm H]^{+/-}$
	Mode	$[M\pm H]^{+/-}$	
12.70, 16.85, 19.51 (**U1-3**)	+	579	427, 409, 291, 289, etc
	-	577	not investigated
12.39, 13.64, 16.87, 18.70 (**U4-7**)	+	867	715, 697, 579, 577, 427, 409, etc
	-	865	not investigated
9.88, 11.94, 18.86 (**U8-11**)	+	1155	1003, 985, 867, 865, 715, 697, 579, 577, 427, etc
	-	1153	not investigated
11.49, 12.21, 15.34, 19.31(**U12-15**)	+	1443	1291, 1273, 1155, 1153, 867, 865, 579, 577, etc.
	-	1441	not investigated
12.21, 13.10 (**U16, U17**)	+	1731	1579, 1561, 1443, 1441, 1155, 1153, 867, etc.
	-	1729	not investigated
16.96 (**U18**)	+	291	139, 123, 165, etc
	_	289	not investigated
14.56 (**U19**)	+	355	163, etc
	_	353	191, 179, 135
19.48 (**U20**)	+	355	163, etc
	_	353	191,179, 135, etc
20.73 (**U21**)	+	355	163, etc
	_	353	191, 179, 173, 135, etc
30.02 (**U22**)	+	433	367, 397, 415, 379, 337, 313, 283, 271
	_	431	311, etc
32.17 (**U23**)	+	433	367, 397, 415, 379, 337, 313, 283, 271
	_	431	311, etc
37.66 (**U24**)	+	433	367, 397, 415, 379, 337, 313, 283, 271
	_	431	311, etc
33.68 (**U25**)	+	611	465, 303
	_	609	463, 301
35.36 (**U26**)	+	465	303
	_	463	301
35.82 (**U27**)	+	435	303
	_	433	301
33.56 (**U28**)	+	449	303
	_	447	301

9.3 Identification of antioxidants in Ulam Raja using HPLC/MS^n

Initial total ion chromatograms (TICs) of ulam raja extract, which were obtained by HPLC-ESI-MS at both positive and negative modes, indicated that compounds **U1-3**

have a molecular weight of 578 Da. As their parent ion $[M+H]^+$ at m/z 579 gave similar fragment ions as that by proanthocyanidin dimers of star fruit, they were assigned to be three isomers of proanthocyanidin dimers.

Similarly, compounds **U4-7** had a molecular weight of 866 Da, and their parent ions $[M+H]^+$ at m/z 867 gave similar fragment ions as that by proanthocyanidin trimers of star fruit. Therefore, Compounds **U4-7** were assigned to be four proanthocyanidin trimers. Similarly, compounds **U8-11** were assigned to be four tetramers. Compounds **U12-15** had a molecular weight of 1443 Da and were identified to be four pentamers. Compounds **U16** and **U17** had a molecular weight of 1730 Da and were identified as two hexamers of catechin. Compound **U18** had a molecular weight of 290 Da. Compound **U18** was assigned to be (+)catechin by spiking and comparing its CID spectra with standard. Fig. 9.6 shows possible chemical structure of compounds **U1-18** with basic unit of (+/-)catechin/epicatechin through C4-C6 or C4-C8 interflavanic linkages.

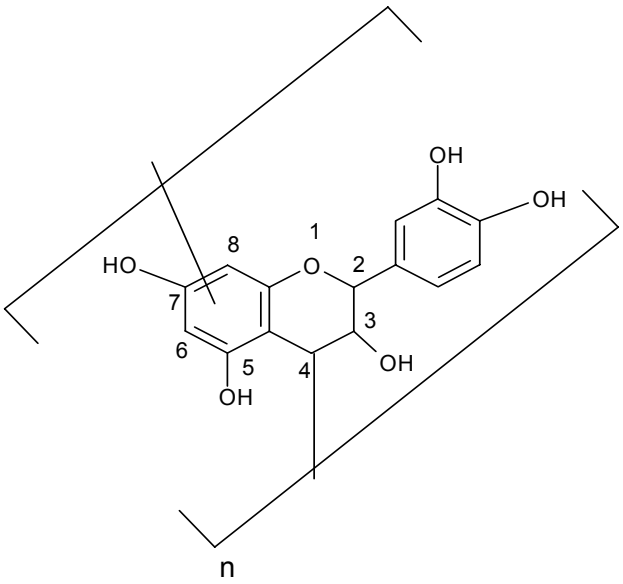

Fig. 9.6. Chemical structure of compounds **U1-U18** (n=1-6) with basic unit of (+/-)catechin/epicatechin

Compounds **U19-21** had the same molecular weight of 354 Da. They also showed identical $[M-H]^-$ ions at similar fragmentation patterns with ions at m/z 191 [quinic acid-H]$^-$ and 179 [caffeic acid-H]$^-$, which were generated by cleavage of the ester bond and found to be consistent with the structures of caffeoylquinic acid derivatives (Fig. 9.7). Compound **U20** was assigned to be chlorogenic acid (5-O-caffeoyl quinic acid) by spiking and comparing its CID spectra with authentic standard. Compounds **U19** and **U21** were most likely naturally occurring isomers of chlorogenic acid, i.e. neochlorogenic acid and cryptochlorogenic acid. According their elution order, neochlorogenic acid was eluted prior to cryptochlorogenic acid [3]. Therefore, compounds **U19** and **U21** were identified as neochlorogenic acid and cryptochlorogenic acid, respectively. As shown in fig 9.7, the intensity patterns of these characteristic fragments for compound **U21** were substantially different, e.g. the base peak for compounds **U19** and **U20** was the ion at m/z 191, and for compound **U21** that at m/z 179 or 173. These results are consistent with those obtained by Carini *et al* [3].

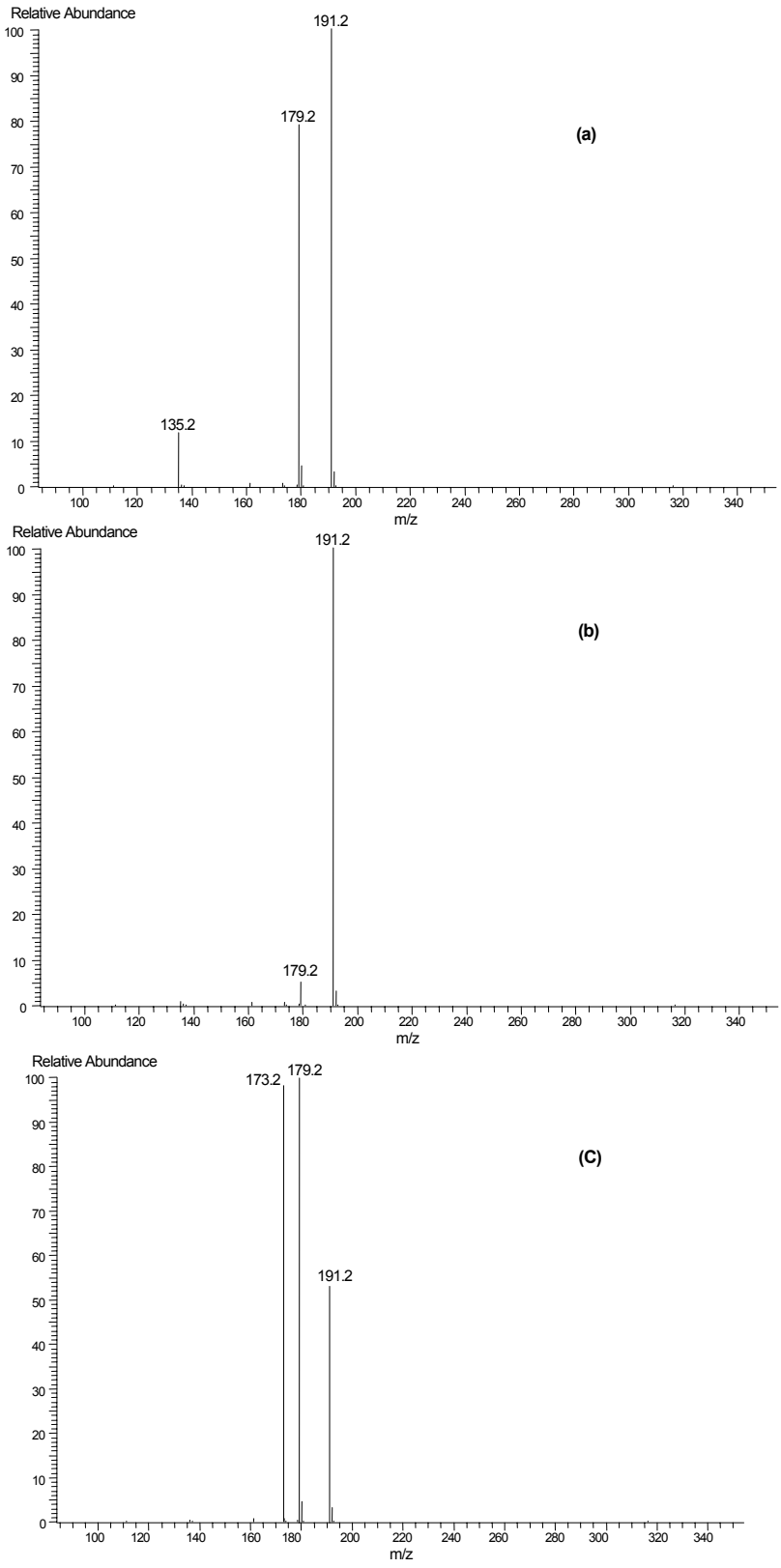

Fig. 9.7 CID spectra compounds **19-21** from parent ions at m/z 355. (**a**), Compound **19.** (**b**), Compound **20**. (**c**), Compound **21**.

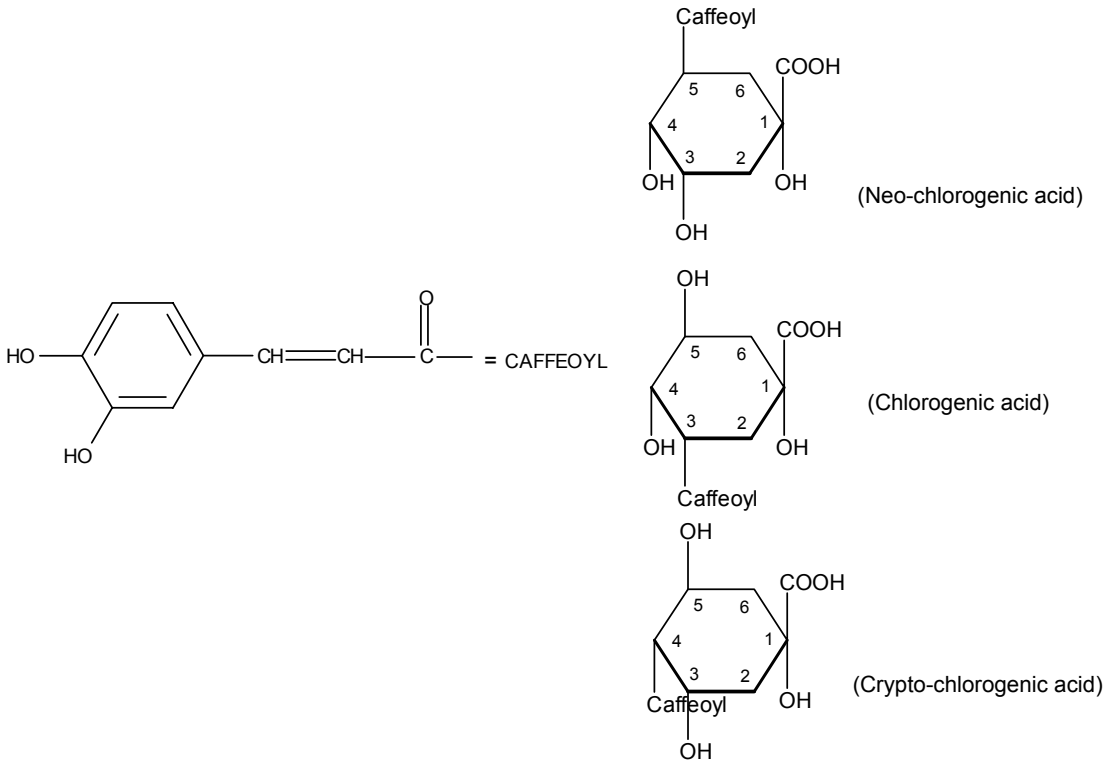

Fig. 9.8. Chemical structures of compounds **19-21**

Both ion peaks at m/z 731 and 355 at positive mode and ion peaks at 707 and 353 at negative mode were found to be eluted simultaneously. Ion peak at m/z 731 and 707 arose from $[M+M+Na]^+$ and $[M+M-H]^-$, respectively. Therefore, ion peaks at m/z 731 actually arose from chlorogenic acids and its isomers.

Compounds **U22-24** had a molecular weight of 432 Da. Their parent ions $[M+H]^+$ at m/z 433 and $[M-H]^-$ at m/z 431 gave daughter ions at m/z 313 and 311, respectively. Their chemical structures need to be further investigated.

Compound **U25** had a molecular weight of 610 Da. The parent ion [M+H]+ at m/z 611 gave daughter ion at m/z 465 and 303. The fragmentation pattern was the same as that of quercetin rutinoside[4, 5]. Therefore, Compound **U25** was tentatively assigned

to be rutin. Compound **U26** had a molecular weight of 464 Da. Its CID spectrum indicated it was a quercetin hexose. Compound **U26** was assigned to be quercetin 3-O-glucoside by spiking and comparing its CID spectra with pure compounds isolated from lady's finger.

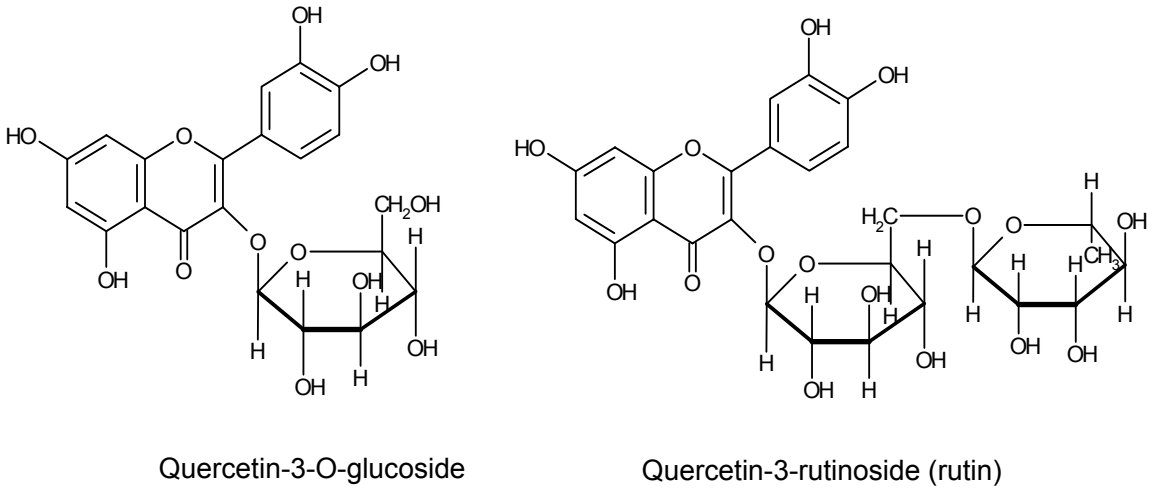

Fig. 9.9. Chemical structures of some quercetin derivatives

Compound **U27** had a molecular weight of 434 Da. Its parent ions $[M+H]^+$ at m/z 435 and $[M-H]^-$ at m/z 433 gave daughter ions at m/z 303 and 301, respectively. Its CID spectra indicated that compound **U27** were a quercetin pentose. Compound **U28** had a molecular weight of 448 Da. Its parent ions $[M+H]^+$ at m/z 449 and $[M-H]^-$ at m/z 447 gave daughter ions at m/z 303 and 301 respectively. Its CID spectra indicated that compound **U28** was a quercetin deoxyl-hexose.

9.4 Chapter Summary

Antioxidants in ulam raja were firstly characterized using high performance liquid chromatography and mass spectrometry (HPLC/MS) and HPLC/MS/MS. A number of antioxidants were identified for the first time in ulam raja, and their chemical structures were proposed. The major antioxidants in ulam raja were attributed to a number of proanthocyanidins that existed as dimers through hexamers, quercetin glycosides, chlorogenic, neochlorogenic, cryptochlorogenic acid acid and (+) catechin.

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PART IV

CONCLUSIONS & FUTURE WORK

Chapter 10

Conclusions

Investigations on antioxidant capacity of a variety of fruits and vegetables confirmed that most fruits and vegetables are good sources of natural antioxidants. According to their antioxidant capacity, unripe ciku fruit, ciku king, ulam raja and blueberry had extremely high antioxidant capacity with AEAC over 600 mg AAeq/100 g fresh sample; strawberry, plum, star fruit, guava, seedless grape, salak, red chilly and beetroot had high antioxidant capacity with AEAC over 200 AAeq/100 g fresh sample; red onion, Fuji apple, red cabbage, long bean, kiwi fruit, lady's finger, mangosteen, solo papaya, cempedak, avocado, oval orange, mango, kiwi fruit, cempedak, pomelo, lemon, pineapple, lettuce, foot long papaya, rambutan and rambutan king had medium high antioxidant capacity with AEAC over 70 mg AAeq/100 g fresh sample; banana, bittergourd, kang kong, tomato, eggplant, coconut pulp, tomato, rockmelon, honeydew, watermelon, French bean and coconut milk had low antioxidant capacity with AEAC lower than 70 mg AAeq/100 g fresh sample.

The L-ascorbic acid contribution to AEAC of fruits varied greatly among species, from 0.06% in ciku to 70.2% in rambutan. L-Ascorbic acid accounted for a high percentage contribution to $ABTS^{\cdot+}$ scavenging activity in rambutan and rambutan king (70%), pineapple (63%), guava (48.3%), lemon (53.2%) and solo papaya (48%), foot long papaya (62.3%), kiwi fruit ((38.7%), pomelo (34.7%), watermelon (31.7%), tomato (29.1%) and oval orange (25.5%). The contribution of L-ascorbic acid to AEAC among other fruits and vegetables was low, especially for ciku, plum, starfruit, salak, seedless grape, mangosteen, apple and cempedak. It seems that fruits with high

AEAC value are more likely to have a lower percentage contribution from L-ascorbic acid to AEAC in the whole extract. Antioxidants in a whole extract might work synergistically. L-Ascorbic acid is normally a good antioxidant for regeneration of other antioxidants. Total antioxidant capacity of selected fruits and vegetables obtained from ABTS$^{\bullet+}$, DPPH$^{\bullet}$ and FRAP methods correlated well with their total phenolic contents. This strongly implicates that phenolic compounds are the major antioxidants of water/ethanol extracts of many fruits and vegetables.

A new HPLC method was developed for preliminary screening of antioxidants varying from a very polar compound e.g L-ascorbic acid to middle polar compounds e.g. flavonoid aglycones. Twenty-nine organic acids and phenolic compounds were well separated using the developed method. The method was successfully applied for determining organic acid and phenolic profiles in apple juices. More importantly, the newly-developed method could be used to monitor antioxidant profiles from very polar to middle polar compounds in fruit extracts, e.g. L-ascorbic acid contents and phenolic profiles in star fruit and lady's finger.

A new approach was developed for identification of antioxidants in biological samples and applied to star fruit extract. Firstly, high performance liquid chromatography (HPLC) was employed to identify antioxidant peaks in a sample by spiking the sample extract with ABTS$^{\bullet+}$, which was prepared from potassium persulphate and ABTS. Secondly, in order to identify the elution period of major antioxidant peaks, the antioxidant capacities of different fractions from solid phase extraction (SPE) were measured, and the chromatograms of fractions were also

recorded. Lastly, tandem mass spectrometry (MS^n) was used to elucidate the possible chemical structures of antioxidants. Based on TAC assays of SPE fractions and HPLC assays of SPE fractions, juice/extract and reaction solution with $ABTS^{\bullet+}$, and tandem mass spectrometry of antioxidant peaks, L-ascorbic acid, (-)epicatechin and proanthocyanidins, which existed as dimers through hexamers, were identified as antioxidants in star fruit. (-)Epicatechin and proanthocyanidins were reported in star fruit for the first time, and proanthocyanidins were preliminarily considered as major phenolic compounds in star fruit.

Major antioxidants of aqueous ethanolic extract from Lady's Finger (*Hibiscus esculentus Linn*) were systematically investigated using an improved approach from that given above. $ABTS^{\bullet+}$ prepared from ABTS and manganese dioxide instead of potassium persulphate was used to avoid the interference from the background peaks. The major antioxidant compounds in Lady's Finger were identified to be (-)-epigallocatechin and quercetin derivatives using HPLC-MS and HPLC-MS^n (n=2~4) techniques. It was found that about 70% of total antioxidant activity was contributed by four quercetin derivatives. The structures of major antioxidants, which were isolated by semi-preparative RP-HPLC with two tandem C18 columns, were further confirmed using UV-visible absorption spectroscopy and ^{13}C NMR spectroscopy. Quercetin 3-O-xylosyl (1'''→2'') glucoside, quercetin 3-O-glucosyl (1'''→6'') glucoside, quercetin 3-O-glucoside and quercetin 3-O-(6''-O-malonyl)-glucoside were first identified and characterized as major antioxidants in lady's finger.

Antioxidants in salak were systematically investigated using another newly-developed approach. The approach was based on significant decrease in the intensity of antioxidant ion peaks obtained from high performance liquid chromatograph coupled with mass spectrometry (MS) after reaction with $ABTS^{\bullet+}$. HPLC/MS/MS was further applied to elucidate the structure of antioxidant peaks characterized in spiking tests. The antioxidants in salak were identified as chlorogenic acid, (-)epicatechin, singly-linked proanthocyanidins that mainly existed as dimers through hexamers of catechin or epicatechin. The new approach proved to be useful for rapid characterisation and identification of antioxidants in biological samples e.g ciku king fruit and ulam raja, especially for screening those antioxidants, which might not be sensitive to common detectors such as UV-Vis or diode array detector (DAD).

The best time for one to consume ciku king fruits at a flavourful stage and yet still be able to acquire high amounts of antioxidants and total phenolics was suggested. The change of the content of major antioxidant peaks was consistent with changes of antioxidant levels during storage. The antioxidant compositions of ciku king fruit were identified as oligomers of gallocatechins dimer through hexamer, a trimer constituted of two gallocatechins and one gallocatechin gallate, a dimer constituted of one gallocatechin and one gallocatechin gallate, catechin dimers, a dimer constituted of one catechin and one gallocatechin gallate, a dimer constituted of one gallocatechin and one catechin gallate, among others.

Antioxidants in ulam raja were firstly characterized using high performance liquid chromatography and mass spectrometry (HPLC/MS) and HPLC/MS/MS. A number of antioxidants were firstly identified in ulam raja, and their chemical structures were

proposed. The major antioxidants in unripe ulam raja were due to a number of proanthocyanidins that existed mainly as dimers through hexamers of catechin or epicatechin, quercetin glycosides, chlorogenic acid and its isomers.

10.1 Future works

While this study has investigated antioxidant capacity of selected fruits and vegetables in the Singapore market and studied the antioxidant compositions of selected fruits and vegetables that have shown strong free radical scavenging activity, more studies need to unravel other relevant information.

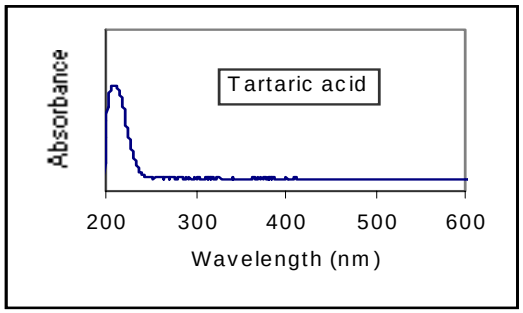
Firstly, to understand the stereochemistry of most antioxidants in this study, further separation and isolation of those compounds such as tannins by large-scale column separation or preparative HPLC need to be carried out. As tannins are very polar, normal phase column such as LH-20 will give a better separation than reversed-phase column separation. While the mass detection range for the instrument used for this study is 50-2,000 Da, hence it could not provide a parent ion for oligomers with n>7, a time-of-flight mass detector will provide more information on compounds with a molecular weight above 2,000 Da. In addition, quantification of those antioxidant components is also very important in order to understand possible dietary intake of these compounds.

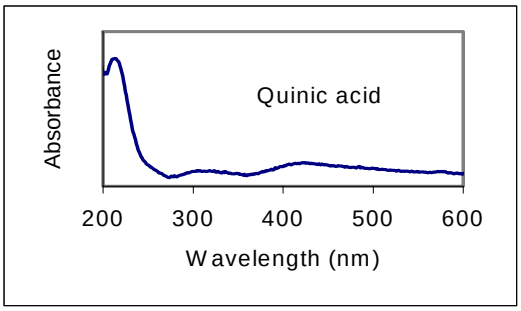
Secondly, it is worth looking into the detailed study on the capacity and efficiency of individual antioxidants and how they each can contribute to the TAC of the whole extract, and possible synergistic effects among antioxidants are also worth studying.

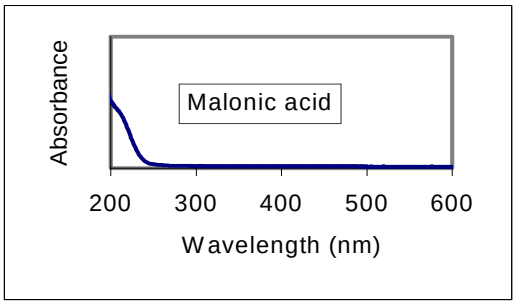
Thirdly, it is also worth characterising antioxoidant profiles of other tropical fruits using those approaches developed in this study.

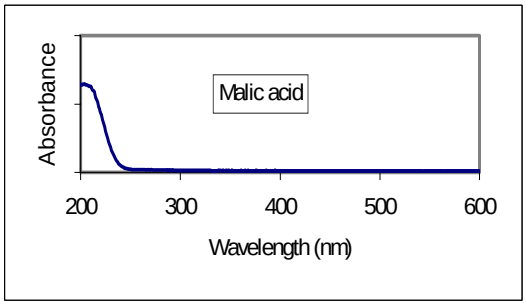
Lastly, potential health effects of tropical fruits and vegetables also need to be investigated systematically through animal or human studies. As this study revealed that tropical fruits and vegetables are good sources of antioxidants, it is possible to make use of them or related wastes from the processing industry in the development of functional foods.

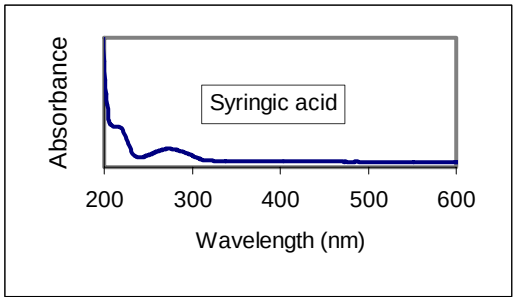
Appendix I UV-visible spectra of individual standards

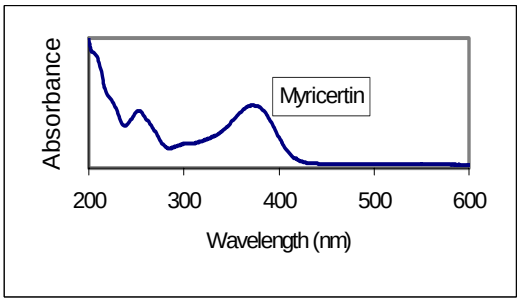


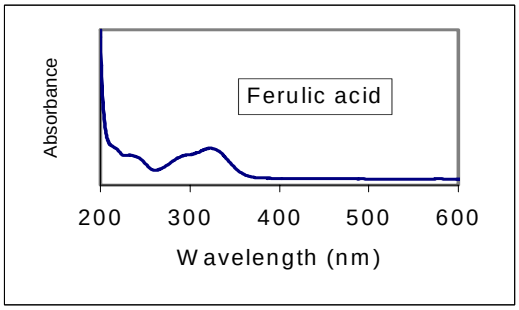


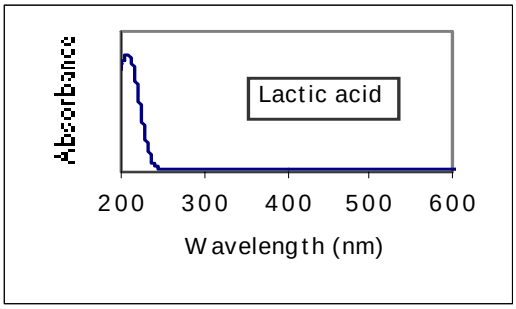


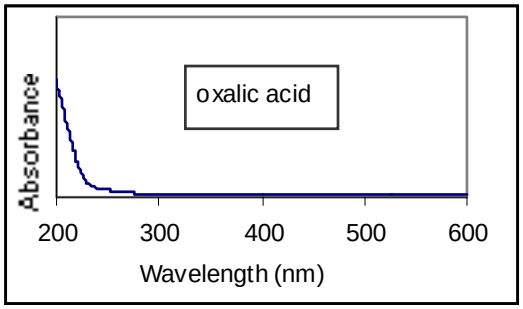


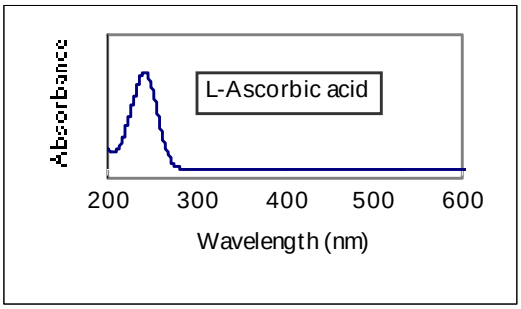


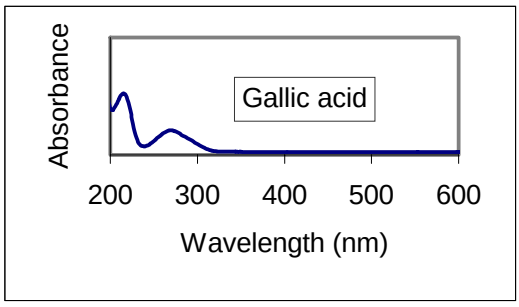


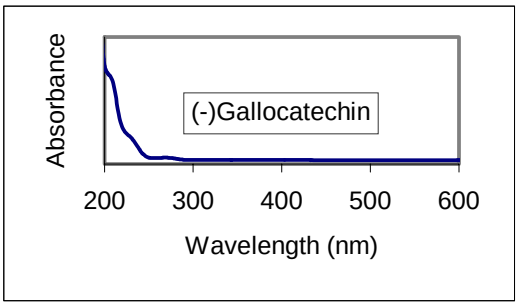


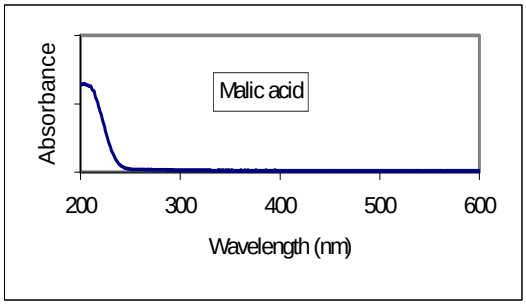


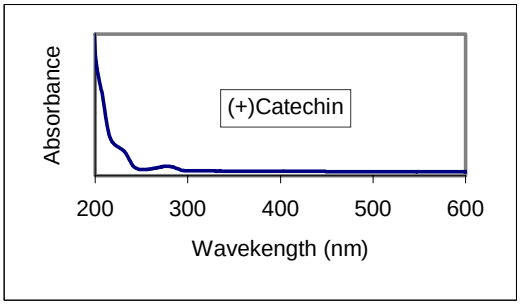


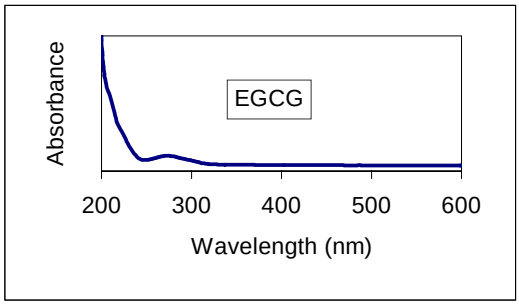


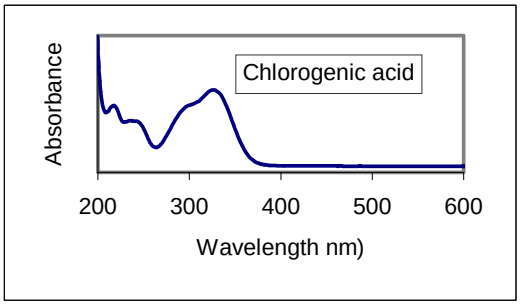

Absorbance
Gallic acid
200 300 400 500 600
Wavelength (nm)

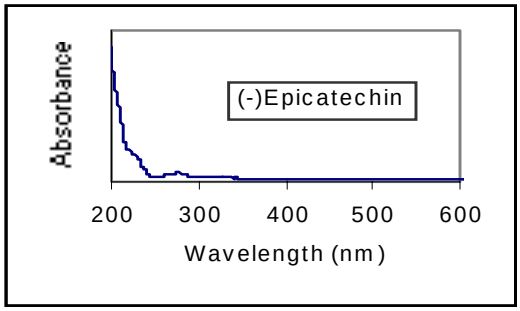

Absorbance
(-)Gallocatechin
200 300 400 500 600
Wavelength (nm)

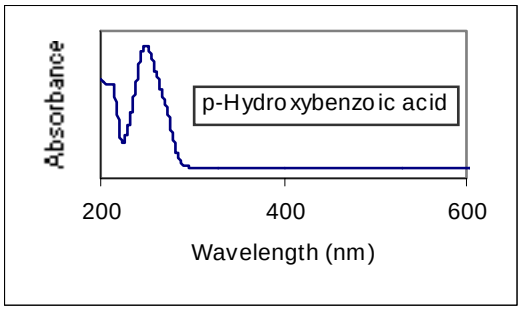

Absorbance
Malic acid
200 300 400 500 600
Wavelength (nm)

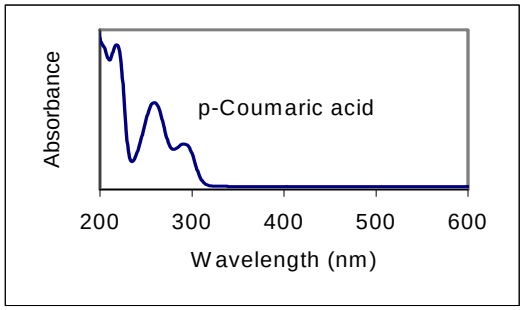

Absorbance
(+)Catechin
200 300 400 500 600
Wavekength (nm)


Absorbance
EGCG
200 300 400 500 600
Wavelength (nm)


Absorbance
Chlorogenic acid
200 300 400 500 600
Wavelength nm)


Absorbance
(-)Epicatechin
200 300 400 500 600
Wavelength (nm)


Absorbance
p-Hydroxybenzoic acid
200 400 600
Wavelength (nm)


Absorbance
p-Coumaric acid
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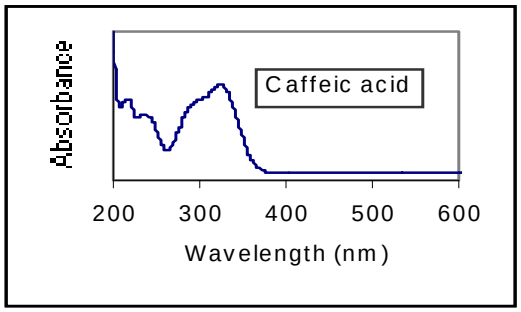
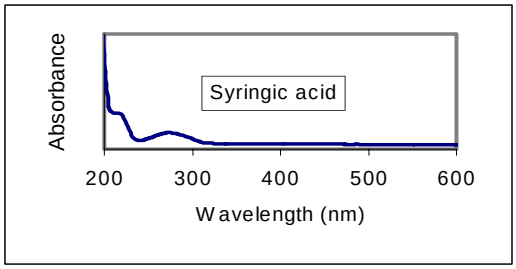
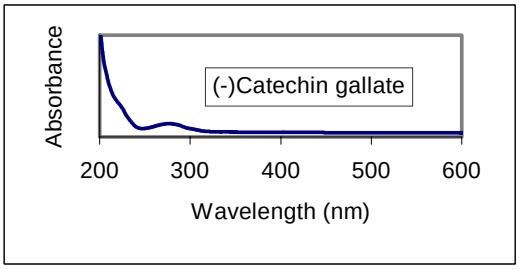
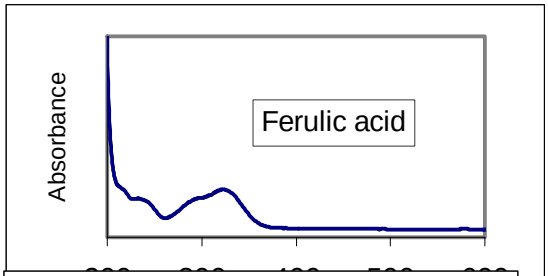
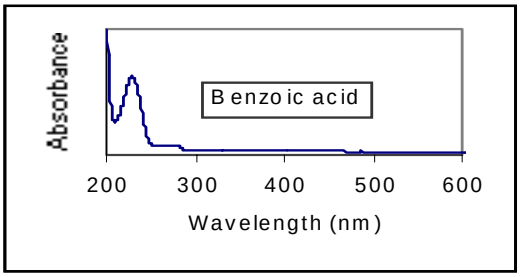
Wavelength (nm)

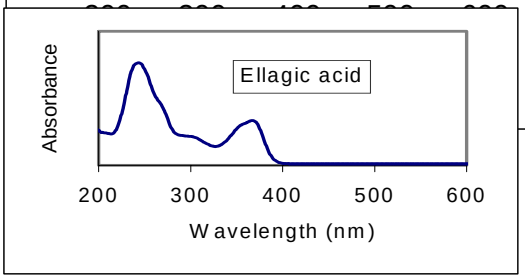

Absorbance
Caffeic acid
200 300 400 500 600
Wavelength (nm)

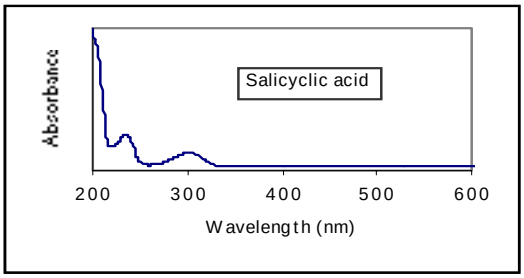

Absorbance
Syringic acid
200
300
400
500
600
Wavelength (nm)

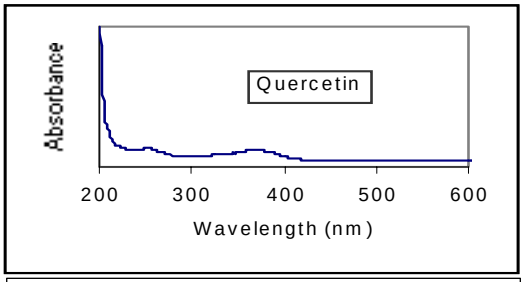

Absorbance
(-)Catechin gallate
200
300
400
500
600
Wavelength (nm)


Absorbance
Ferulic acid


Absorbance
Benzoic acid
200
300
400
500
600
Wavelength (nm)


Absorbance
Ellagic acid
200
300
400
500
600
Wavelength (nm)


Absorbance
Salicyclic acid
200
300
400
500
600
Wavelength (nm)


Absorbance
Quercetin
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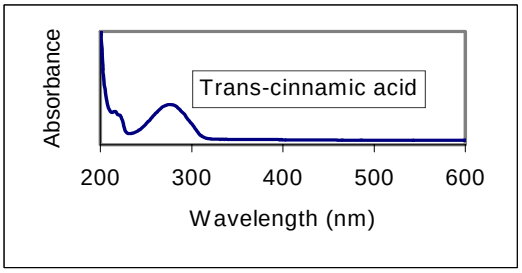
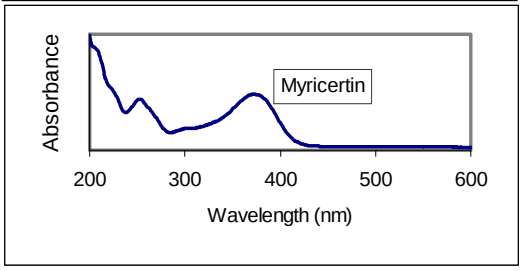
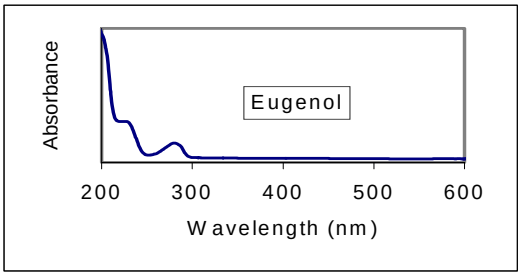
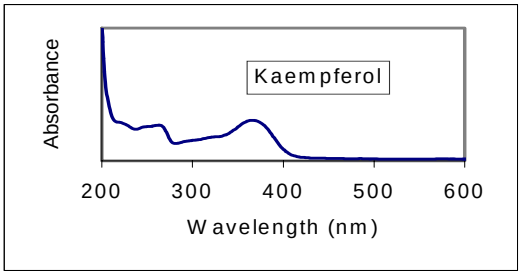
300
400
500
600
Wavelength (nm)


Absorbance
Trans-cinnamic acid
200
300
400
500
600
Wavelength (nm)


Absorbance
Myricertin
200
300
400
500
600
Wavelength (nm)


Absorbance
Eugenol
200
300
400
500
600
Wavelength (nm)


Absorbance
Kaempferol
200
300
400
500
600
Wavelength (nm)